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PEROXISOME PROLIFERATOR- ACTIVATED RECEPTOR δ

REGULATION OF SKELETAL MUSCLE METABOLISM

David Kitz Krämer



**Karolinska
Institutet**

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Für meine Familie

Life is all about being focused
without holding onto it
being detached
and yet, deeply in touch.

John Argent

ABSTRACT

Peroxisome Proliferator-Activated Receptor (PPAR) δ is a nuclear transcription factor which has been implicated in the regulation of lipid metabolism in skeletal muscle. In addition to the postural and locomotive functions of skeletal muscle, this organ has a major impact role on whole body metabolism. Reduced insulin sensitivity is a characteristic feature in subjects with type 2 diabetes mellitus. Physical exercise/muscle contraction alters the metabolic properties of skeletal muscle, and renders the muscle more sensitive to insulin. The underlying molecular mechanisms mediating this effect remain largely elusive. This thesis has investigated the role of PPAR δ in skeletal muscle glucose- and lipid metabolism, exercise responses and fibre-type composition in human skeletal muscle.

The effect of low-intensity exercise on clinical characteristics and skeletal muscle gene expression was evaluated in subjects with Type 2 diabetes mellitus. Skeletal muscle protein and mRNA expression of PPAR δ increased in an exercise-dependent manner. The increase skeletal muscle PPAR δ was coincident with improvements in the clinical profile of the study participants. Furthermore, despite performing the exercise protocol, some individuals did not show improvements in insulin-sensitivity or increases in skeletal muscle PPAR δ expression. This finding suggests that the exercise-training induced activation of PPAR δ is critical for mediating the beneficial effects of exercise. We hypothesised that this is dependent on PPAR δ -responsive genes altering skeletal muscle metabolism, which subsequently has an impact on whole body metabolism and the clinical profile.

To test the hypothesis that PPAR δ expression may be a regulator of oxidative metabolism and an insulin sensitive phenotype; we determined the expression of PPAR δ in elite endurance cyclists, normally active individuals and spinal cord injured individuals. These groups have profound differences in skeletal muscle fibre-type composition, with elite cyclists displaying the highest proportion of type I oxidative muscle fibres, and spinal cord injured subjects an almost total loss. We found that PPAR δ expression was positively correlated with the amount of oxidative skeletal muscle fibres in these groups.

To specifically address the role of PPAR δ in human skeletal muscle metabolism we utilised primary cultures of human skeletal muscle, and pharmacological activators of PPAR δ . Activation of PPAR δ in primary cultured human skeletal muscle increases fatty acid uptake and oxidation. This effect is linked to an increased expression of key genes involved in the intracellular transport of fatty acids, fatty acid uptake into the mitochondria and subsequent metabolism. These changes were concomitant with improved insulin sensitivity and glucose uptake. In addition to PPAR δ -dependent changes, we noted that the pharmacological PPAR δ activator induced a PPAR δ independent alteration in the cellular ATP:AMP ratio. This resulted in an increase in AMP-activated protein kinase phosphorylation, and an AMP-activated protein kinase dependent increase in glucose uptake.

In conclusion, PPAR δ plays a central role in the adaptive metabolic response of human skeletal muscle to exercise. Furthermore, PPAR δ orchestrates changes in skeletal muscle metabolism. Thus, PPAR δ is an interesting drug target for the treatment of metabolic diseases, such as type 2 diabetes mellitus.

Keywords: Peroxisome Proliferator-Activated Receptor (PPAR) δ , insulin sensitivity, insulin action, human skeletal muscle, primary cultured muscle cells, lipid-metabolism, glucose metabolism, AMP activated protein kinase (AMPK), Type 2 Diabetes Mellitus

SAMMANFATTNING

Peroxisom Proliferator-Aktiverad Receptor (PPAR) δ är en nukleär receptor och transkriptionsfaktor som har visat sig spela en nyckelroll i reglering av skelettmuskelns fettmetabolism. Skelettmuskulaturen är inte bara viktig för kroppshållning och rörelseförmåga, utan spelar dessutom en central roll i hela kroppens metabolism och insulinkänslighet. Minskad insulinkänslighet i muskel är ett karakteristiskt fynd hos personer med typ 2 diabetes mellitus. Å andra sidan leder motion och muskelkontraktion till förändringar i musklernas metabola egenskaper vilket ger en ökad insulinkänslighet. Vilka molekylära mekanismer som reglerar dessa effekter är i dagsläget inte kartlagt. Denna avhandling har undersökt vilken roll PPAR δ har när det gäller skelettmuskelns glukos- och fettmetabolism, svaret på fysisk aktivitet samt libertyppssammansättning i mänskliga. Effekten av lågintensiv fysisk aktivitet på kliniska parameterar och på genuttruck i skelettmuskeln undersöktes hos personer med typ 2 diabetes. Protein och mRNA uttryck av PPAR δ ökade i skelettmuskeln som svar på fysisk aktivitet. Dessutom sammanföll kliniska förbättringar i insulinkänslighet med det ökade uttrycket av PPAR δ i muskeln. Förutom ökat uttryck av PPAR δ ökade uttrycket av uncoupling protein (UCP) 3, vilket kan tyda på förbättrad och/eller ökad mängd mitokondrier. En grupp personer svarade inte på den fysiska aktiviteten med förbättrad insulinkänslighet, trots att de utfört samma mängd fysisk aktivitet. Hos dessa personer ökade heller inte uttrycket av PPAR δ eller UCP3 i muskeln. Detta tyder på att en ökning av PPAR δ i muskeln, vilket sannolikt leder till förändringar i PPAR δ -reglerade gener och ändringar muskelns metabola profil, påverkar helkroppsmetabolismen och styr hur motion påverkar kliniskt relevanta parameterar. För att testa denna hypotes undersökte vi om PPAR δ uttrycket i skelettmuskulaturen är korrelerat med muskelfibertyppssammansättningen. Typ 1 muskelfibrer är mycket oxidativa, och mer insulinkänsliga än typ 2 fibrer. Uttrycket av PPAR δ bestämdes i muskel från elittränaade cyklister, matchade normaltränaade kontroller, och personer med ryggmärgsskada. Dessa tre grupper skiljer sig markant i muskelfibertyppssammansättning, med den högsta andelen typ 1 fibrer i muskel från cyklister, och en nästan total avsaknad av typ 1 fibrer hos ryggmärgsskadade. Uttrycket av PPAR δ visade sig vara positivt korrelerat med mängden oxidativa typ1 muskelfibrer. Ett ökat uttryck at PPAR δ sammanföll i typ 1 muskel med ökat uttryck av PPAR α , PPAR γ coaktivator (PGC)1 α och PGC1 β . Vi aktiverade PPAR δ farmakologiskt i humana muskelcellodlingar för att mer direkt studera effekten av PPAR δ i muskelmetabolismen. Aktivering av PPAR δ ökade muskelns upptag och förbränning av fett. Detta var kopplat till en PPAR δ -medierad ökning i uttrycket av gener för nyckelsteg inom fettupptag och fettmetabolism. Samtidigt ökade muskelcellens insulinkänslighet. Förutom PPAR δ -medierade förändringar i cellen, resulterade farmakologisk aktivering i en förändring av cellens ATP:AMP kvot. Detta ledde i sin tur till aktivering av AMP kinas, och en AMP kinas-beroende ökning av glukosupptaget.

Sammanfattningsvis har PPAR δ en central roll i skelettmuskulaturens svar på fysisk aktivitet och reglering av fettmetabolism. Specifik aktivering av PPAR δ visar att den är en potentiellt lovande angreppspunkt för behandling av metabola sjukdomar så som typ 2 diabetes mellitus.

Nyckelord: Peroxisom Proliferator-Aktiverad Receptor (PPAR) δ , insulinkänslighet, skelettmuskel, humana skelettmuskelcellsodlingar, fettmetabolism, glukosmetabolism, AMP aktiverad proteinkinas (AMPK), typ 2 diabetes mellitus

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- I. **David Kitz Krämer**, Maria Ahlsén, Jessica Norrbom, Eva Jansson, Nils Hjeltnes, Thomas Gustafsson and Anna Krook.
mRNA expression of PPAR α , PPAR δ , PGC-1 α and PGC-1 β is altered following pathologically and physiologically induced variations in skeletal muscle fibre type.
ACTA Physiol 2006; 188, 207–216
- II. Tomas Fritz*, **David Kitz. Krämer***, Håkan KR Karlsson*, Dana Galuska, Peter Engfeldt, Juleen R. Zierath and Anna Krook.
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Diabet Metab Res Reviews 2006; 22: 492–498.
- III. **David Kitz Krämer**, Lubna Al-Khalili, Sebastio Perrini, Josefin Skogsberg, Per Wretenberg, Katja Kannisto, Harriet Wallberg-Henriksson, Ewa Ehrenborg, Juleen R. Zierath and Anna Krook.
Direct activation of glucose transport in primary human myotubes after activation of peroxisome proliferator-activated receptor δ .
Diabetes 2005, 54:1157–1163
- IV. **David Kitz Krämer**, Bruno Guigas, Ying Leng and Anna Krook.
Role of AMP kinase in PPAR δ regulation of lipid and glucose metabolism in skeletal muscle.
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- V. Lubna Al-Khalili, **David Krämer**, Per Wretenberg and Anna Krook.
Human skeletal muscle cell differentiation is associated with changes in myogenic markers and enhanced insulin-mediated MAPK and PKB phosphorylation.
Acta Physiol Scand 2004, 180, 395–403
- VI. **David Kitz Krämer**, Karim Bouzakri, Olle Holmqvist, Lubna Al-Khalili and Anna Krook.
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Cytotechnology 2005 48:89–95; DOI 10.1007/s10616-005-4074-7
- VII. **David Kitz Krämer**, Anna Krook.
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ABBREVIATIONS

18s	Ribosomal protein 18s
ACC	Acetyl-CoA carboxylase
AF	Activation function
AICAR	5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
AMPKK	AMPK upstream kinase(s)
AS160	Akt substrate of 160 kD
Beta-2MG	Beta 2-microglobulin
BMI	Body mass index
CAP	Cbl-associated protein
CPT	Carnitine palmitoyl transporter
DAG	Diacylglycerol
DBD	DNA binding domain
DBP	Diastolic blood pressure
DEX	Dexamethasone
DGK	Diacyl Glycerol Kinase
Dimer	Dimerisation domain
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulfoxide
DPP	Diabetes prevention program
ERK	Extracellular signal regulated kinases
FABP	Fatty acid binding protein
FAT/CD36	Fatty acid transporter
FBS	Foetal bovine serum
FFA	Free fatty acid
FOXO	Forkhead box (FOX) O1A
GAPDH	Glyceraldehyde phosphate dehydrogenase
GLUT	Glucose-transporters
GPDH	Glycerol-3-phosphate dehydrogenase
GS	Glycogen synthase
GWX	Specific PPAR δ activator (alias GW0742)
GW501516	Specific PPAR δ activator
HbA1c	Glycated Haemoglobin
HDL	High-density lipoproteins
HK	Hexokinase
HOMA	Homeostasis Model assessment
HPLC	High pressure liquid chromatography
HPRT	Hypoxanthine guanine phosphoribosyltransferase
HSMC	Human skeletal muscle cell
IFG	Increased fasting glucose
IGT	Impaired glucose tolerance
IPH	Isolated post-challenge hyperglycaemia

IR	Insulin receptor
IRAP	Insulin responsive amino peptidase
IRS	Insulin receptor substrate proteins
JNK	c-jun N-terminal Kinase
KHB	Krebs Hensleit bicarbonate buffer
LBD	Ligand binding domain
LPL	Lipoprotein lipase
MAPK	Mitogen activated protein kinases
mATPase	Muscle Adenosine Tri-Phosphatase
MEF	Myogenic enhancer factor
MyHC	Myosin heavy chain
NADH	Nicotinamide adenine dinucleotide
NRF	Nuclear Respiration Factor
NS	Not statistically significant
OGTT	Oral glucose tolerance test
PA	Phosphatidic acid
PDGF	Platelet derived growth factors
PDK	Phosphatidyl inositol dependent kinase
PERC	Alias PGC-1 β
PET	Positron emission tomography
PGC1 α / β	PPAR gamma coactivator isoforms α and β
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3 kinase
PIP ₂	PI 4,5-biphosphate
PIP ₃	PI 3,4,5 triphosphate
PKB	Protein kinase B (or <i>Akt</i>)
PKC	Protein Kinase C
Plysate	Platelet lysate
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR DNA consensus sequences
PRC	PGC-1 related coactivator
RXR	Retinoid X receptors
SBP	Systolic blood pressure
SCD	Stearoyl-CoA delta-9-desaturase
SDH	Succinate dehydrogenase
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory element binding transcription factor
T2DM	Type 2 Diabetes Mellitus
T3	Thyroid hormone
TAG	Triacylglycerol
TZD	Thiazolidinediones
UCP	Uncoupling Proteins
WHO	World health organisation
VO _{2max}	Maximum oxygen uptake capacity

INTRODUCTION

EQUILIBRIUM: BIOLOGICAL ENERGY HOMEOSTASIS

The key aspect of homeostasis is energy metabolism. Energy homeostasis is the basis for an organism retrieving, storing and utilising energy and allowing continuation of biochemical reactions by reduction of entropy. Over the course of some million years of evolution organisms have developed strategies allowing them to store a surplus of energy for later periods of starvation or malnutrition. Multiple interacting backup mechanisms have evolved in order to control the balance between storage under plentiful circumstances and reduced expenditure under scarce conditions. These mechanisms have reached a high complexity due to stabilising feedback circuits. Higher organisms have developed the capacity to utilise a number of chemical molecules for energy. These include proteins, carbohydrates and lipids and their constituent chemical components, with fatty acids and glucose being the main sources of energy supply. This thesis will investigate key molecular mechanisms integrating lipid- and glucose metabolism. Furthermore the impact of physical exercise on these mechanisms and the role of exercise therapy in the treatment of metabolic diseases such as Type 2 Diabetes Mellitus (T2DM) will be considered.

DISEQUILIBRIUM: INSULIN-RESISTANCE AND T2DM

On a whole body level the peptide hormones including insulin and glucagon, are the main-regulators of storage of lipids and glycogen. Together these hormones regulate energy homeostasis (**Figure 1**).

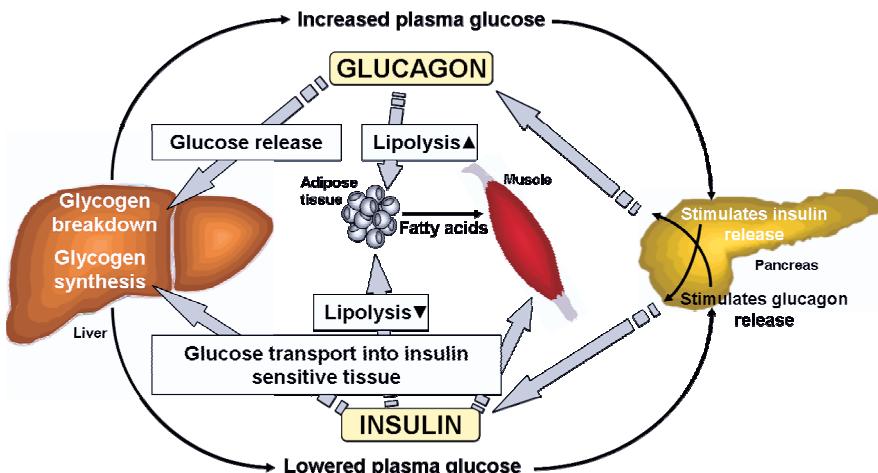


Figure 1: Regulation of plasma glucose. When glucose levels rise, insulin is released from pancreatic β -cells. This promotes lipid uptake and synthesis and inhibits the generation of energy from fat by suppressing lipid-oxidation. In response to insulin, glucose is stored in liver and muscle in the form of glycogen. When blood glucose levels fall, the pancreas reacts by releasing glucagons and reducing insulin secretion. Glucagon acts on the liver to release breakdown glycogen and release glucose. Overall glucagon has opposing effects to those of insulin and thus also increases lipolysis and fatty acid utilisation.

If the delicate regulatory mechanisms of energy-homeostasis derail, an organism glides into a state of disequilibrium. The molecular processes which have developed to support and regulate each other then loose mutual control. Such a state is the development of glucose intolerance and subsequently T2DM. T2DM is defined by the World Health Organisation (WHO) as a fasting plasma glucose concentration exceeding 6.1 mmol/L or a two hour value of 7.8 mmol/L following oral glucose tolerance test (OGTT) [2, 3] (**Figure 2**).

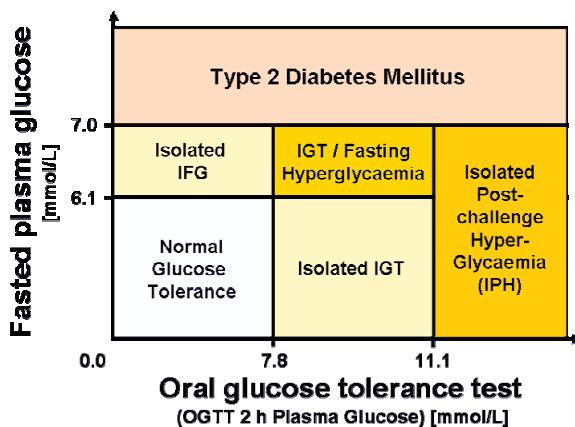


Figure 2: Definition of insulin resistance and Type 2 Diabetes Mellitus (T2DM). Fasted plasma glucose and oral glucose tolerance test (OGTT) are two measures used to identify T2DM. Early stages of impaired insulin sensitivity are characterised by isolated occurrences of impaired glucose tolerance (IGT) and increased fasting glucose (IFG), potentially manifesting as constant IGT and/or fasting hyperglycaemia.

An increased blood glucose concentration is caused by impaired insulin sensitivity and/or impaired insulin release. As glucose levels rise, this should lead to an increase in insulin secretion from the pancreatic beta-cells. Insulin resistance in peripheral tissues creates a negative spiral, initially resulting in increased insulin secretion, and eventually, when beta-cells fail to compensate sufficiently, to increased plasma glucose. In severe cases, the beta-cells decompensate completely and eventually cease to secrete insulin [4].

The prevalence of a number of metabolic disorders, including diabetes, hypertension, hyperuricaemia, lipid abnormalities, and alterations in thrombotic potential, have increased on a world-wide level. These diseases are associated with hyperinsulinaemia and insulin resistance, and have an increased risk of cardiovascular complications and stroke [5, 6]. An increased intake of high-caloric foods, with increased amounts of carbohydrates and lipids, has been suggested to contribute to the rise in the incidence of metabolic disorders. Higher amounts of fatty acids in the circulation appear to correlate and contribute to impaired insulin sensitivity in skeletal muscle [4] (**Figure 3**).

The increase in the incidence of Type 2 diabetes is well documented in the United States. In 1935, only 0.37% of Americans had diabetes [7]. By 1999, the prevalence of diabetes rose to 6.9% [8]. World-wide, India currently tops the list of people with diabetes in numbers, followed by China and the USA. Other Southeast

Asian countries, including Pakistan and Bangladesh are also within the top 10 countries with high diabetes prevalence [9].

The rapidly growing epidemic of T2DM is already, or is going to be, a challenge for industrialised societies. Hence, basic and clinical research that contributes to the understanding of the disorder and helps in the development of treatment strategies is of high importance. A corner stone in the understanding of T2DM is not just to understand glucose metabolism, but the intricate interplay of glucose- and lipid metabolism and the effect of this metabolic coordination on insulin sensitivity.

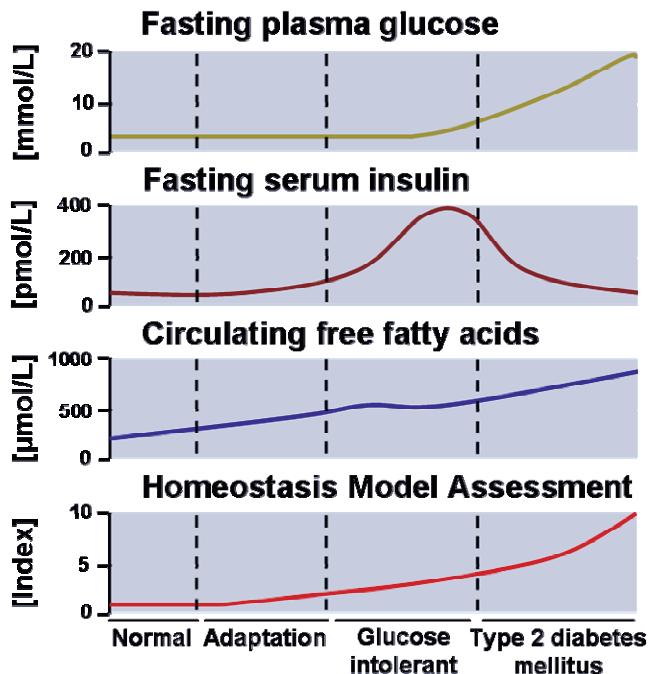


Figure 3: Hypothetical model of the pathogenesis of T2DM over time. Initially, there is a ‘normal period’ of minimal insulin resistance (defined by the Homeostasis model assessment (HOMA)) and no need for beta-cell compensation. As the degree of obesity or insulin resistance increases (coinciding with gradually increasing circulating free fatty acid (FFA) levels), there is a period of ‘adaptation’ in which fasting glucose levels are maintained by increasing fasting levels of insulin (perhaps reflective of increased number of beta-cells). A ‘glucose intolerant’ period follows, in which fasting glucose levels are maintained by even higher fasting insulin levels and beta-cell mass, in an attempt to further compensate for the insulin resistance. Although fasting glucose levels are normal in the glucose intolerant phase, there is impairment in the response to a glucose-load (glucose intolerance). This can be the beginnings of a deficit in insulin secretory capacity. Finally, in the absence of intervention to reduce insulin resistance, manifest T2DM is established. Fasting blood glucose levels markedly increase due to a decrease in circulating insulin and beta-cell mass.

BACKGROUND

The next chapters will discuss the basic molecular mechanisms of energy uptake, utilization and storage, with an emphasis on the role of the Peroxisome-Proliferator Activated Receptor (PPAR) δ , in skeletal muscle. The molecular basis for insulin resistance, obesity and T2DM are also reviewed. Treatments for T2DM, and especially the beneficial role of exercise for the pathology of T2DM and its molecular foundations, will be discussed. Furthermore, the role of PPAR δ in the molecular adaptation to exercise and its potential for future pharmacological treatment of T2DM will be discussed.

GLUCOSE METABOLISM AND INSULIN-RESISTANCE

Glucose is the most readily available energy source for most tissues. *In vivo* studies show that skeletal muscle is the principal site of glucose uptake under insulin-stimulated conditions, accounting for approximately 80-90% of glucose disposal after glucose infusion [10-12](Figure 4). In the postprandial state, insulin-mediated glucose uptake and utilisation is greatly impaired in T2DM [10, 13]. This makes skeletal muscle insulin resistance a major tissue involved in glucose-homeostasis and energy metabolism and a main target in the treatment of T2DM.

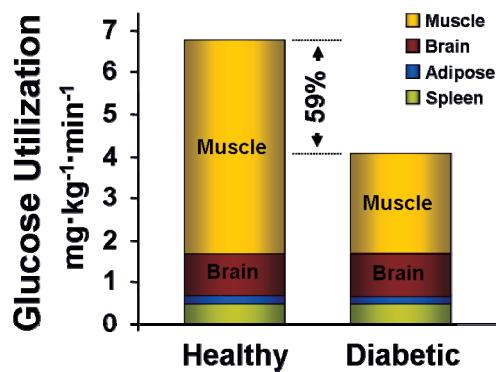


Figure 4: Glucose utilisation in healthy vs. T2DM patients. During the hyperinsulinaemic-euglycaemic clamp, glucose is given intravenously. Tissue specific analysis of glucose uptake demonstrates that skeletal muscle is the major site of glucose disposal. In T2DM patients insulin responsiveness of skeletal muscle is significantly impaired [10].

The insulin signalling network

The insulin signalling network is triggered upon insulin binding to its receptor. The insulin receptor (IR) consists of two extra-cellular alpha-subunits and two cell-membrane spanning beta-subunits. The insulin receptor possesses intrinsic tyrosine kinase activity, being responsible for autophosphorylation upon insulin binding [14]. This initial autophosphorylation results in the activation of the receptor's kinase

activity. Subsequently, this leads to a cascade of phosphorylation events of a large number of proteins, involved in transmitting signals leading to changes in glucose, lipid and protein metabolism, as well as gene-regulatory events.

Metabolic control

The Insulin Receptor Substrate (IRS) proteins are the immediate target of insulin receptor tyrosine kinase activity. To date, four different IRS isoforms have been cloned [15]. IRS proteins have multiple tyrosine and serine phosphorylation sites [15, 16]. While tyrosine phosphorylation of IRS has been associated with insulin action, phosphorylation of some serine residues rapidly suppresses insulin-stimulated glucose uptake [17]. Once phosphorylated, IRSs serve as docking proteins for multiple proteins [15, 18]. One of these proteins is phosphatidylinositol 3 Kinase (PI3K), which phosphorylates PI 4,5-biphosphate (PIP_2) to PI 3,4,5 triphosphate (PIP_3) [19]. PIP_3 leads to allosterical downstream activation of Pyruvate Dehydrogenase Kinase (PDK) 1 [20] and subsequently Protein Kinase B (PKB, also called Akt) phosphorylation [21, 22]. To date, three PKB isoforms have been cloned, $\text{PKB}\alpha$ (or $\text{Akt}1$), $\text{PKB}\beta$ (or $\text{Akt}2$) and $\text{PKB}\gamma$ (or $\text{Akt}3$) [23]. Skeletal muscle expresses $\text{PKB}\alpha$ and β . IRS1 controls aspects of glucose metabolism via $\text{PKB}\beta$ and phosphatidylinositol 3 kinase, while IRS2 regulates lipid-metabolism via $\text{PKB}\alpha$ [24] (Figure 5).

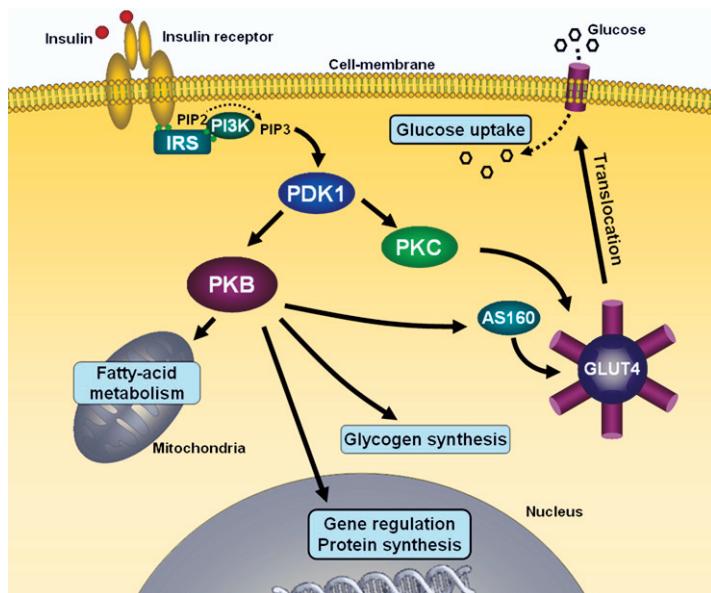


Figure 5: Insulin signalling to metabolic end points. After insulin binding to the insulin-receptor, insulin receptor substrates (IRS) are phosphorylated which in turn recruit phosphatidylinositol (PI) 3 kinase (PI3K). Conversion of PI 4,5-biphosphate (PIP_2) to PI 3,4,5 triphosphate (PIP_3) leads to activation of phosphatidylinositol dependent kinase (PDK) 1. One branch of PDK1 action leads to Protein Kinase B (PKB)/Akt activation which results in the regulation of different aspects of metabolism; i.e. increased glycogen synthesis, gene regulation, phosphorylation of the Akt substrate (AS) 160 and possibly fatty acid metabolism. AS160 phosphorylation leads to Glucose Transporter (GLUT) 4 translocation and glucose uptake.

Other downstream targets of the PI3K-PDK1 signalling cascade are members of the atypical Protein Kinase C (PKC) family [25, 26]. Activation of PKC ζ may also be required for glucose uptake [27] via Glucose-Transporter (GLUT) 4 translocation, which is the predominant glucose transporter isoform expressed in skeletal muscle [28, 29]. A final signalling protein in the regulation of glucose uptake might be Akt-Substrate of a size of 160 kD (AS160), which has been implicated in the control of the translocation and/or internalization of GLUT4 vesicles to the plasma membrane [30, 31]. PKB also signals to downstream targets regulating glycogen-synthesis [32], protein expression [33, 34] and lipid synthesis [35].

Mitogenic control

IRS phosphorylation leads to gene-expression changes via Mitogen Activated Protein Kinases (MAPK). The mitogenic control due to insulin is controlled by activation of MAPK family members Extracellular signal Regulated Kinases (ERK) 1 and 2, c-Jun N-terminal Kinase (JNK) and in part the p38 MAPK. Cytokines also activate p38 and JNK in response to cellular-stress [36]. One target of p38 MAPK is the transcription factor Myogenic Enhancer Factor (MEF) 2C [37, 38](Figure 6).

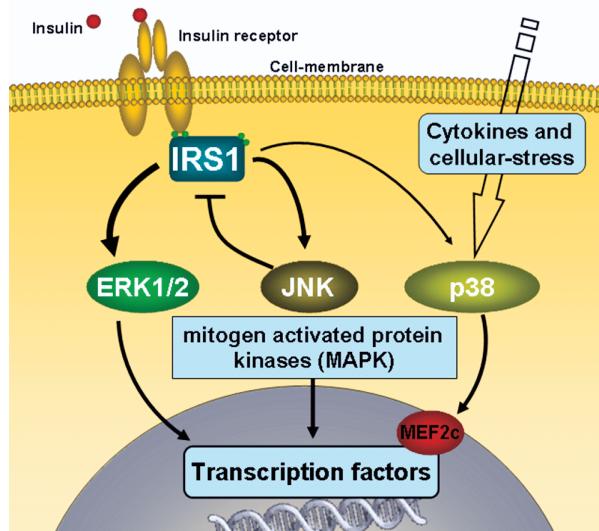


Figure 6: Insulin signalling to mitogenic end points. Activation of insulin receptor substrate (IRS) 1 and Shc leads to activation of the mitogen activated protein (MAP) kinases: Extracellular signal-Regulated Kinases (ERK)1/2, and to a lesser extent, c-jun N-terminal Kinase (JNK) and p38 MAPK. MAP kinases are also activated by cellular stress. MAPK activation leads to regulation of gene expression, in skeletal muscle partly via the Myogenic Enhancer Factor (MEF) 2C.

While there is evidence for a role for the different MAPK isoforms in insulin regulation of gene transcription, a role in glucose uptake is unresolved. ERK1/2 MAPK is not required for the acute effect of insulin to promote glucose uptake, since pharmacological inhibition of ERK1/2 does not reduce glucose transport in skeletal

muscle [39]. Conversely, activation of JNK interferes with insulin action via serine phosphorylation of IRS1 and subsequently prevents full tyrosine phosphorylation of IRS1 by the insulin receptor [40]. The role of p38 MAPK in the regulation of glucose uptake is unclear. However, recent evidence has challenged earlier claims of a role for p38 MAPK in the regulation of glucose uptake [41].

Insulin signalling defects and insulin-resistance

IRS1 phosphorylation on several serine residues suppresses insulin signalling [17, 19]. This attenuation of insulin action via desensitisation of IRS1 also occurs under normal physiological conditions. However, aberrant serine phosphorylation of IRS-1 has been noted in T2DM patients [42] and this seems to be concomitant with decreased tyrosine phosphorylation of IRS-1 [17, 19].

The increased serine phosphorylation of IRS1 leads to reduced insulin receptor and IRS1 protein interaction. This could be a key mechanism explaining the reduced PI3K activity in skeletal muscle from people with T2DM [43, 44] and obese insulin resistant individuals [45]. These impairments are also correlated with decreased GLUT4 translocation, and reduced appearance of GLUT4 at the plasma membrane, and impaired glucose transport. In contrast, protein content of insulin receptor and IRS-1 is unaltered in skeletal muscle from people with T2DM [18, 43, 46]. Furthermore, IRS-1 polymorphisms [47, 48], impaired PKB phosphorylation [49], impaired IRS-2 associated PI3K activation, reduced activation of atypical [50], novel and conventional PKC isoforms [51, 52] and defects in GLUT4 trafficking [53] have been demonstrated in skeletal muscle or isolated skeletal muscle cells from T2DM patients. However, neither alterations in GLUT4 expression [54] nor GLUT4 polymorphisms [55] appear to be of primary importance in T2DM.

Defects in the mitogenic side of the insulin network, (i.e. the MAPK signalling cascades) have been observed in T2DM. In skeletal muscle, insulin action on p38 MAPK and ERK1/2 is unaltered in T2DM patients [44], while JNK phosphorylation/expression is abnormally elevated in various tissues [56]. Increased JNK may lead to increased serine-phosphorylation of IRS-1 [40]. Genetically engineered knockout mice against the *JNK*-gene are protected from obesity-induced insulin resistance [57].

The large number of currently identified signalling defects may partly be due to the complex interactions within the pathway. This further complicates the attempt to dissect primary defects from secondary defects arising following the resultant hyperglycaemia. However, it appears reasonable to assume that not all T2DM patients suffer from identical defects. Instead different combinations of defects may activate a variety of different mechanisms, and lead to T2DM. Interestingly, defects in insulin action are maintained in isolated skeletal muscle cells from subjects with T2DM. Thus, primary human muscle cell cultures provide a useful tool for studies of signalling defects in T2DM.

LIPID-METABOLISM AND INSULIN-RESISTANCE

Excessive fat-depots and increases in circulating free-fatty acids appear to contribute to insulin resistance in skeletal muscle [58-60]. Free-fatty acids mediated activation of JNK has been noted, and this may lead to diminished insulin action [61].

Abdominal visceral fat appears to be more dangerous than lower-body adiposity [62]. The reason for this could be a larger contribution of the more readily available abdominal fat to the circulating amount of free-fatty acids [58, 59, 63]. Hence, the regulation of lipid metabolism is an important aspect to consider when attempting tackling mechanisms causing insulin resistance (**Figure 3**). A class of transcription factors that are of interest in this regard are the PPARs. PPARs are major regulators of various aspects of lipid metabolism, with PPAR δ the most predominant isoform expressed in skeletal muscle. Thus, the role of PPAR δ in skeletal muscle metabolism is a central theme in this thesis.

Regulation of lipid metabolism

Adipose tissue stores fatty acids in the form of triacylglycerols, which are the main storage form of energy in the body, accounting for 15% and 25% of the body mass in healthy men and women, respectively [64]. The elevation in fatty acids arises from either dietary lipid intake or from the lipogenic process, i.e. de novo synthesis of lipids from glucose in the liver and adipose tissue. While adipose tissue plays a major role in lipid metabolism in terms of storage, skeletal muscle is the largest consumer of lipids. This is especially true under physically demanding conditions such as prolonged medium intensity exercise (50-75% VO₂ max), or fasting, when skeletal muscle utilises approximately 50% of energy from fatty acid oxidation [65, 66]. About half of these fatty acids are derived from the circulation and the remainder portion from intra-muscular lipid stores [67, 68].

Interactions between glucose- and lipid-metabolism

Randle and colleagues postulated in 1963 a potential interaction between lipid- and glucose-oxidation [69]. They argued that increased lipid availability could interfere with skeletal muscle glucose metabolism and contribute to insulin resistance, for example in obesity and T2DM. Several studies support the concept that elevation of free-fatty acids produces an impairment of insulin-stimulated glucose metabolism [58, 59, 70].

Metabolic inflexibility in insulin resistance is another concept, explaining interactions of lipid- and glucose-metabolism. In the fasting condition, skeletal muscle predominantly utilises lipid-oxidation for energy production [71]. Upon insulin stimulation in the fed condition, skeletal muscle from healthy people rapidly switches to increased uptake, oxidation and storage of glucose and, at the same time, lipid oxidation is suppressed [72]. In contrast, low glucose levels lead to glucagon release which results in increased lipolysis in adipocytes and subsequent fatty acids release and oxidation. Concomitantly glucagon triggers release of glucose from the liver, as described before (**Figure 1**). Obese individuals and individuals with T2DM manifest higher lipid oxidation during insulin-stimulated conditions as compared to control subjects [73], despite lower rates of lipid oxidation during fasting conditions. This suggests that a key feature in insulin resistance is an impaired ability of skeletal muscle to switch between fuels. Hence regulators of fatty acid metabolism are of interest for the treatment of T2DM. Again, an interesting family of such molecular regulators are the PPARs.

NUCLEAR RECEPTORS

Peroxisome proliferator-activated receptor (PPAR)

PPARs are nuclear receptors and transcription factors, which play central roles in substrate utilisation and have therefore become interesting as pharmacological targets for the treatment of metabolic disease [74, 75].

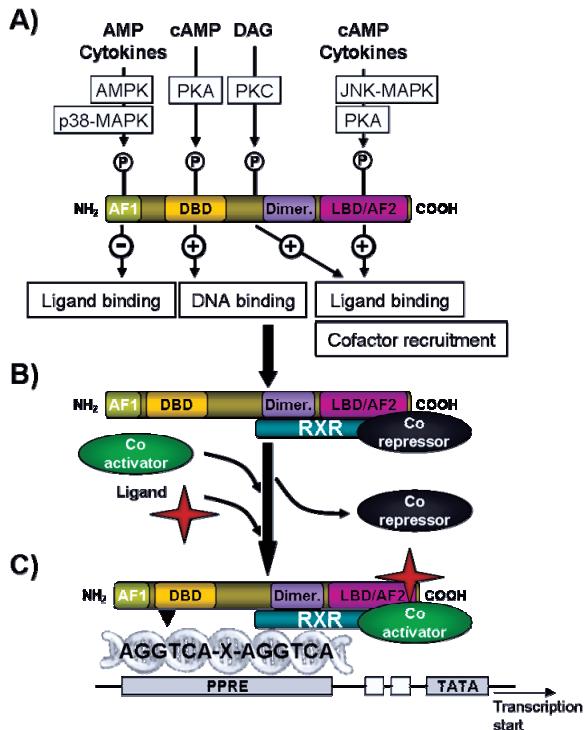


Figure 7: PPAR structure and function. (A) The DNA binding domain (DBD) and the ligand binding domain (LBD) are the most highly conserved regions across receptor isoforms. The LBD is located in the C-terminal half of the receptor. Part of the LBD is the ligand-dependent activation function (AF)-2. This region is involved in the generation of the receptors' co-activator binding pocket. A ligand-independent activation function, AF-1, is found in close proximity to the N terminus (NH_2) of the receptor. PPAR activity and properties can also be regulated by phosphorylation (P) of different parts of the receptor. (B) Upon ligand binding, the co-repressor is released and a co-activator recruited instead. The dimerisation domain (dimer.) assists in the binding of retinoid X receptor (RXR). (C) The DBD recognises a specific promoter region called PPRE. The basal transcriptional machinery, including the RNA Pol II initiation complex, is recruited to the accessible TATA-box promoter region and transcription is initiated.

PPARs form heterodimers with Retinoid X Receptors (RXR) and bind to consensus DNA sites. PPAR-RXR heterodimers are capable of recruiting co-repressors when no agonist is available and silence transcription by active repression [76-78]. Ligand binding induces a conformational change in PPAR-RXR complexes, which releases the co-repressor and instead recruits co-activators such as PPAR γ co-activator (PGC)-1 α [79] (Figure 7). The resultant complex binds to a DNA consensus sequences

(PPRE), after release of Histone H1 from the DNA, and initiates transcription resulting in enhanced gene expression. PPARs regulate lipid homeostasis via transcriptional regulation of genes involved in lipid metabolism, storage, and transport. Another role of PPARs appears to be in inflammation, where they have been shown to be involved in mechanisms that release anti-inflammatory factors or repress inflammatory response [80, 81].

There are three described PPAR isoforms that are expressed in a tissue-specific manner to different degrees. All three isoforms are expressed in skeletal muscle, with PPAR α and PPAR δ being the most abundant and PPAR γ appearing to play a secondary role [75, 82]. The different PPAR isoforms bind to lower-affinity ligands and are activated by various dietary lipids, including saturated and unsaturated fatty acids and an array of their metabolites. PPARs are therefore considered to be nutritional lipid sensors and control lipid homeostasis in a target-tissue specific manner via their transcriptional activity [75]. PPARs can also be controlled via phosphorylation, which alters the ability to bind substrates or influence the DNA-binding capacity [83] (Figure 7). Some activators of PPARs (thiazolidinediones, fibrates, statins) are used to treat lipid disorders by increasing high-density lipoproteins (HDL), lowering triglyceride levels, and enhancing insulin sensitivity [84].

PPAR α

PPAR α was the first PPAR isoform to be cloned as a novel murine receptor. PPAR α is activated by amphiphatic acids such as fibrates that are able to induce a strong hepatic peroxisome proliferation [85]. Fibrates are exclusive activators of PPAR α and do not activate the other PPAR-isoforms. Clofibrate, fenofibrate, and bezafibrate and their derivatives have been widely used to characterise PPAR α functions [85, 86]. PPAR α is highly expressed in hepatocytes, cardiomyocytes, the kidney cortex, and skeletal muscle (i.e. tissues with a high capacity for fatty acid oxidation). PPAR α binds unsaturated fatty acids with the highest affinity of the three isoforms [79]. Saturated fatty acids have lower affinity for PPAR α . PPAR α activation favours fatty acid oxidation, mainly in the liver and heart, and to a lesser extent in skeletal muscle, thus reducing adiposity and redistributing adipose depots [79] (Figure 8). The increased fatty acid oxidation is one of the mechanisms for the lipid-lowering effect of fibrates and explains why PPAR α ligands can, in some situations, improve insulin sensitivity by reducing lipid accumulation in tissues [85, 86]. In skeletal muscle, starvation and diabetes, which are concomitant with high fatty acid availability, could lead to PPAR α activation and the re-direction of glucose carbons from oxidation to lactate synthesis, thus sparing glucose carbons for hepatic glucose production [74]. In mice with a targeted deletion of the PPAR α gene, mitochondrial beta-oxidation is impaired [87] and expression of many genes involved in lipid metabolism are altered [88]. However, in human and rodent muscle, PPAR δ can compensate for the impairments due to a lack of PPAR α expression [89, 90].

PPAR β/δ

Originally PPAR δ was denominated PPAR β in rodents, while the corresponding isoform in humans was called PPAR δ [89-91]. However, it was later realised that homology of the isoform in rodents and human was large and both names are used

interchangeably for the human isoform nowadays. Despite being the predominant skeletal muscle isoform, the exact role and regulation of PPAR δ is incompletely understood [89-91]. Research has mainly been concentrated on PPAR α and PPAR γ , partly because specific chemical activators of PPAR δ are only recently available [92] (**Figure 8**). The reason for this is that PPAR δ has a much larger binding-pocket than the other isoforms and can therefore be activated by a larger number of agonists [93]. Recently, a specific PPAR δ activator (GW501516) was reported to attenuate plasma glucose and insulin levels when administered to genetically obese *ob/ob* mice and appears to increase insulin sensitivity [94]. The same agonist has been reported to substantially increase HDL-cholesterol levels and reduce triglyceride levels in obese Rhesus monkeys [95].

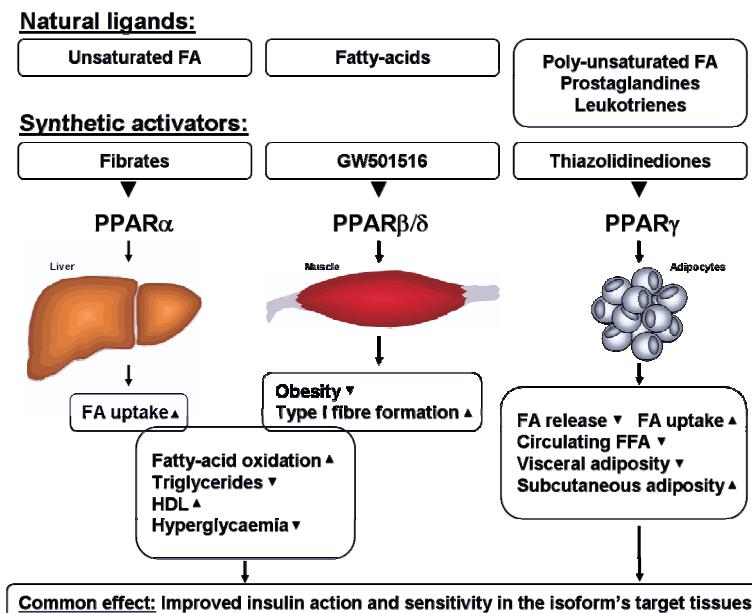


Figure 8: Ligands, synthetic activators and predominant target tissues of PPARs. PPAR α and PPAR δ share similar effects in their respective target tissues. A common property of all isoforms is their insulin sensitising action.

Recent data suggests that PPAR δ serves as a general regulator of fat oxidation and activation of PPAR δ protects from the development of obesity [96]. PPAR δ (*NR1C2*) knockout mice are metabolically less active and glucose-intolerant, whereas receptor activation in *db/db* mice improves insulin sensitivity [97]. Studies performed in humans imply a role for PPAR δ in cholesterol metabolism [98]. PPAR δ has also been shown to play a role in muscle fibre-type determination. Transgenic mice expressing an activated form of PPAR δ have an increased proportion of Type I oxidative fibres [99].

PPAR γ

Natural ligands for PPAR γ are poly-unsaturated fatty acids and prostaglandins and leukotrienes which are synthesized from poly-unsaturated fatty acids and have important roles as mediators and regulators of inflammation [100]. Ectopic expression of PPAR γ induces the differentiation of fibroblastic cells into adipocytes [101]. *In vivo* in rodents, specific activation of PPAR γ induces the differentiation of pre-adipocytes into small adipocytes within a few days [102]. Activation of PPAR γ also induces the differentiation of cultured human pre-adipocytes into mature adipocytes [103]. In addition to effects on pre-adipocyte differentiation that augment adipocyte number, activation of PPAR γ stimulates the storage of fatty acids in mature adipocytes [104].

PPAR γ knock-out mice are insulin resistant [105], but intriguing results have been observed in heterozygous PPAR γ (+/-) mice. When these mice are fed a high-fat diet, they are less insulin resistant and have smaller adipocytes than wild-type mice, and present lower plasma fatty acids and increased levels of the adipokines leptin and adiponectin [106]. Mutations in PPAR γ , resulting in a functionally dominant negative form of the protein, have been associated with severe insulin resistance in a limited number of patients [107]. This fits with the general consensus that PPAR γ is important for maintaining normal insulin sensitivity. A common polymorphism in the *PPARG* gene, Pro¹²Ala, with prevalence of 13% in Caucasians, has been identified. This polymorphism is associated with a decreased transcriptional activity in *in vitro* experiments, and is associated with improved insulin sensitivity [108].

Activators of PPAR γ , such as the Thiazolidinediones (TZDs) are used in the treatment of T2DM. TZDs act by enhancing the sensitivity of tissues to insulin, especially in adipose tissue but may also play a role in skeletal muscle (**Figure 8**). However, despite the predominant role of PPAR γ in adipose tissue, it also plays a role in skeletal muscle. Overexpression of PPAR γ in C2C12 mouse muscle cell lines, in combination with TZD treatment, was suggested to increase insulin sensitivity [109]. Similarly, TZD treatment of L6 rat skeletal muscle cell line leads to the restoration of signal transduction via the AMP-Activated Protein Kinase (AMPK) pathway by restoring AMPK α 2 activity [110]. However, whether the actions of TZDs on AMPK are entirely through the PPAR γ isoform remains doubtful.

Other investigators have reported that treating muscle cells derived from mice with TZDs leads to increases in the AMP/ATP ratio and a concomitant increase in AMPK activity [111]. Similar results were obtained in isolated *extensor digitorum longus* (EDL) mouse skeletal muscle [112]. Others have shown that 15 minutes of stimulation with TZDs in L6 cells, leads to phosphorylation of AMPK, which increases glucose uptake [113]. The short incubation time required for the effects of TZDs on AMPK phosphorylation suggests that changes in gene expression are not required and that it is transmitted via a PPAR γ independent mechanism [113]. A suggested explanation could be that the compound has an effect on mitochondrial uncoupling.

Exercise-mediated regulation of PPARs

PPAR γ expression in skeletal muscle is relatively low [114], however, it is uncertain whether PPAR γ is regulated in response to exercise. In *vastus lateralis* skeletal muscle from healthy young men, PPAR γ mRNA expression was increased 2.7 fold 3 hours following cycling exercise [115]. In rodent skeletal muscle, PPAR γ

mRNA expression was upregulated after 16 weeks of treadmill exercise training [116]. In contrast, other studies have failed to provide evidence for any effect of exercise on mRNA levels of PPAR γ in either rodents or humans [117-119]. Despite this, the PPAR γ_2 Pro¹²Ala variant in humans has been associated with an improved response to exercise. Carriers of the PPAR γ_2 Pro¹²Ala polymorphism have significantly better exercise-mediated improvements in fasting glucose than in a control group, suggesting that PPAR γ plays a role in the exercise response [120]. Differences in mRNA profiles in skeletal muscle have been mapped between groups of subjects who show marked difference in the improvement in glucose tolerance in response to the same amount of 20 week exercise training, demonstrating the existence of “exercise resistance” [121]. Future studies may need to determine activation of PPAR γ ’s transcriptional activity, in addition to changes in mRNA expression.

PPAR α and PPAR δ mRNA is increased in skeletal muscle following an acute three hours exercise bout [122]. Endurance training has also been reported to increase skeletal muscle PPAR α mRNA expression [117, 123]. However, nutritional status at the time of exercise seems to play a role in the post-exercise regulation of PPAR α and δ expression [124]. Fasting dramatically increases PPAR δ expression in skeletal muscle in mice [125]. Given the additional complexity of hormonal and nutrient regulation of these targets, dissecting exercise from nutritional effects on PPAR expression may be a challenge.

Furthermore, single nucleotide polymorphisms (SNPs) of the PPAR δ gene are significantly associated with whole-body insulin sensitivity. Use of positron emission tomography (PET) analysis indicates that PPAR δ SNPs primarily affect insulin sensitivity by modifying glucose uptake in skeletal muscle, but not in adipose tissue [126]. Furthermore, SNPs in the PPAR δ gene modify the conversion from impaired glucose tolerance (IGT) to T2D, particularly in combination with SNPs of PGC-1 α and PPAR γ_2 [127]. Whether the response to exercise is linked to variations within the PPAR δ gene, e.g. as SNPs, or variations in other genes, is currently unknown.

Mitochondrial biogenesis and the PPAR γ Co-activator (PGC) 1

PPARs require co-activators to exhibit actions as transcription factors. The PGC-1 co-factors are important for PPAR δ action in skeletal muscle. PPAR δ and PGC-1 are exercise responsive, and these molecules have been implicated in the regulation of exercise adaptations. Endurance exercise leads to improved oxidative capacity, partly due to an increase in mitochondria density, while strength exercise does not appear to have this effect [128-130]. Furthermore, a reduction in oxidative capacity for fatty acids has been proposed, possibly due to a decrease in mitochondrial density and/or function, as a contributing factor for the onset of T2DM in elderly individuals. Similarly, but much less frequent in occurrence, hereditary mutations that impair mitochondrial function may contribute to the onset of T2DM [131]. The restoration of mitochondrial capacity following endurance exercise has been proposed as a cellular “fix” for diabetes pathology.

Mitochondria encode and express 13 subunits of the respiratory complexes and require a total of approximately 100 proteins to make the mitochondrial machinery function [132]. A key feature of most of the transcription factors involved in mitochondrial biogenesis is binding interaction with the nuclear co-activator PGC-1.

PGC-1 family members do not bind DNA themselves, but interact with DNA-bound transcription factors to regulate gene expression [133]. PGC-1 α was the first of three PGC-1 homologues to be described. PGC-1 β (also called PERC) and PGC-1-related cofactor were initially identified by sequence homology to PGC-1 α , and show a similar tissue distribution as PGC-1 α [134]. Overexpression of PGC-1 β is associated with an increased number of mitochondria, suggesting that PGC-1 β may also play a role in mitochondrial biogenesis [134, 135].

An acute bout of exercise leads to a marked increase in PGC-1 α mRNA immediately following exercise, which then returns to pre-exercise levels within 24 h [136]. Several bouts of exercise training leads to a sustained increase in PGC-1 α [117, 137, 138]. Furthermore, DNA polymorphisms in PGC-1 α have been linked to cardiovascular fitness [139] and the odds of developing T2DM [140]. Whether these polymorphisms are related to the PGC-1 α respond to exercise has yet to be investigated. Interestingly, overexpression of PGC-1 α alone in cultured myoblasts is sufficient to increase in mitochondria biogenesis [141]. In addition PGC-1 is a co-activator of all PPARs, including, PPAR α , and thereby up-regulates genes that are required for the mitochondrial fatty acid oxidation pathway [142]. Overexpression of PGC-1 β in transgenic mice results in increased energy expenditure, and prevention of obesity by increasing fat oxidation [143]. Furthermore, variations in the PGC-1 β gene may contribute to the pathogenesis of obesity in humans [144].

AMP ACTIVATED PROTEIN KINASE (AMPK): A FUEL SENSING MASTER SWITCH

AMPK has been characterised as a fuel-sensor in human skeletal muscle, because it senses changes in the AMP:ATP ratio [145]. AMPK activation reduces energy consuming processes and increases glucose uptake in an effort to restore the cellular energy status [146]. Since AMP levels increase in working skeletal muscle, AMPK is considered an exercise responsive protein. Activation of AMPK has been implicated to partly play a role in insulin independent glucose uptake induced by muscle contraction. However, the function of AMPK reaches beyond the stimulation of glucose uptake through GLUT4, and makes it a master-switch balancing between glucose-and lipid-metabolism, which may ultimately improve insulin sensitivity [147]. AMPK appears to act in concert with PPARs in the regulation of long-term expression-based exercise adaptations.

AMP allosterically activates AMPK, which together with phosphorylation of Thr¹⁷² by an upstream kinase, leads to metabolic and gene regulatory effects [148]. AMPK deactivation of ACC inhibits the conversion of acetyl-CoA into malonyl-CoA [149]. Malonyl-CoA is an inhibitor of Carnitine Palmitoyl Transporter (CPT)-1, a transporter of long-chain fatty acids, into mitochondria for subsequent oxidation. Additionally, pharmacological activation of AMPK with the AMP analogue, AICAraboside (AICAR), leads to an increase of the Fatty Acid Transporters (FAT/CD) and Fatty Acid Binding Protein (FABP) in cardiac myocytes from rodents [150]. AICAR acutely stimulates glucose transport [151, 152], lipid oxidation and induction of GLUT4 mRNA expression [153, 154]. Chronic treatment with AICAR leads to increased GLUT4 expression [155], mitochondrial biogenesis and enhanced lipid metabolism [156-158]. If and where the AMPK- and insulin-signalling pathways converge is unknown; however the AS160 offers a possible point of convergence [159,

160]. Exercise-activated AMPK clearly does not activate the insulin receptor, IRS-1, PI3K or PKB signalling components of the insulin signalling cascade [161-163]. Thus, AMPK acts directly on lipid-metabolism, glucose-metabolism and gene expression (**Figure 9**).

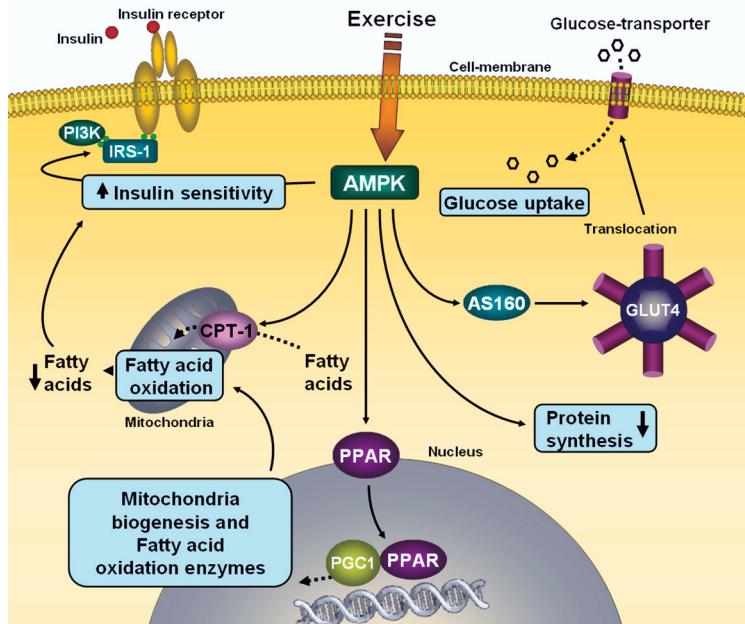


Figure 9: AMPK actions on metabolism and gene expression. Activation of AMPK, e.g. by exercise, increases glucose uptake, presumably via activation of AS160 leading to GLUT4 translocation. Protein synthesis is downregulated, while expression patterns are changed, by alterations of transcription factor activity, like PPARs. This in turn leads to increased mitochondria density and expression changes needed for FA-oxidation. Concomitantly, AMPK inhibits ACC, leading to increased FA uptake into mitochondria. This effect has been proposed to contribute to an increase in insulin sensitivity. Additionally, it has been suggested that AMPK exhibits inhibiting effects on IRS-1 serine phosphorylation, improving insulin sensitivity.

Role of AMPK-isoforms

AMPK is a heterotrimer that consists of the three subunits alpha, beta and gamma [164]. The alpha-subunit is responsible for the catalytic activity of the kinase and contributes to the binding of AMP which activates AMPK [165] (**Figure 10**). The alpha-subunit exists in two isoforms and has a phosphorylation site at Thr¹⁷² on the c-terminus [166]. Activation of the skeletal muscle predominant alpha₂ isoform is three times higher due to AMP activation than alpha₁ [167, 168]. Alpha₁ is also activated by cellular stress, such as hypoxia and high intensity exercise [169, 170]. The alpha-isoforms are activated pharmacologically by AICAR [147].

There are two isoforms of the AMPK beta subunit, but their function within the AMPK heterotrimer is much less defined. The beta-isoform has been suggested to act as a scaffolding protein between the alpha- and gamma-subunit [171, 172].

Additionally, a glycogen-binding domain has been discovered, suggesting a role in glycogen metabolism [173, 174]. There are three gamma-subunit isoforms necessary for the catalytic activity of AMPK. A role of the gamma₃ isoform has been suggested in glycolytic skeletal muscle and a dominant missense mutation at R²²⁵Q, discovered in pigs (R²²⁵Q) leads to enhanced glycogen re-synthesis after exercise and protects against triglyceride accumulation and insulin resistance in skeletal muscle [175-178].

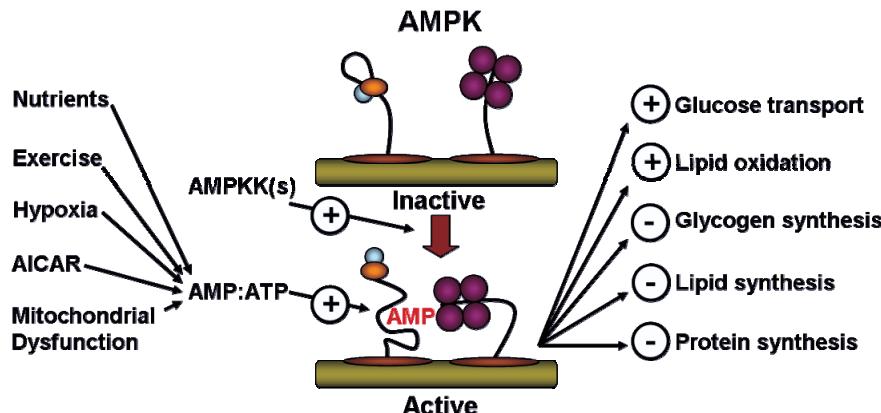


Figure 10: Model of AMPK activation and major metabolic actions. Changes in AMP:ATP ration, caused by the indicated initiators, leads to AMPK activation. Activity of an upstream kinase/ kinases (AMPKK) is required for activation. AMP binding leads to conformational changes of the trimer-protein and regulates a number of metabolic properties.

AMPK in the treatment of insulin-resistance

Due to the protective mechanisms against obesity, and insulin resistance, AMPK has been suggested as a prime target for the treatment of T2DM [179]. Metformin, a drug which has been widely used to treat T2DM is reported to be an AMPK activator [180]. However, AMPK seems to be activated indirectly via changes in AMP:ATP ratio. Metformin improves insulin sensitivity and decreases glucose production by the liver, thus improvement in glucose homeostasis [181]. Two interesting targets of AMPK activation are PPARs [83, 182](Figure 8) and PGC-1 α [183]. Additionally, studies in myotubes have shown that AMPK might have direct effects on IRS serine phosphorylation, thereby improving insulin sensitivity [184].

PHARMACOLOGICAL TREATMENT STRATEGIES FOR T2DM

The pathology of T2DM is complex and various drugs against different physiological targets have been considered. The classic treatment options have been sulfonylureas, a class of substances called secretagogues, which stimulate secretion of insulin from pancreatic beta-cells, Metformin, and insulin [185]. During the last decade, PPAR γ agonists have come into clinical use (Table 1). While TZDs have gained popularity as mono-therapy in the USA, in Europe, use is limited to treatment in combination with other substances, like Metformin and/or sulfonylureas [186, 187].

Metformin has been used in Europe since the 1950s, while it was introduced in the USA much later [188]. In the USA, emphasis on initial therapy has only lately been shifting from secretagogues and alpha-glucosidase inhibitors, to insulin sensitizers, such as Metformin and TZDs [189]. The PPAR δ agonist GW501516 is currently in clinical trials, and expected to become a novel drug-treatment shortly [190].

α-glucosidase inhibitors	Inhibition of intestinal glucose absorption Stimulation of GLP-1 release
Sulfonylureas	Acute stimulation of insulin release
Metformin	Inhibition of hepatic glucose production Increased hepatic insulin sensitivity
	Increased hepatic insulin sensitivity
	Increased muscle insulin sensitivity
Thiazolidinediones (TZDs)	Suppression of non-esterified (free) fatty acids release from adipose tissue
	Fat redistribution (visceral to subcutaneous)
	Modified adipokine release from adipocytes

Table 1: Pharmacological treatment for T2DM. This table summarises drug treatments for T2DM. GLP-1 (glucagon-like peptide), controls blood glucose through regulation of glucose-dependent insulin secretion, inhibition of glucagon secretion and gastric emptying, and reduction of food intake (Reviewed in [191]). Other targets are discussed in the text.

Physical exercise is an efficient alternative and/or supplement to pharmacological treatment. Exercise has been shown to potentially be more efficient than drug treatment in treatment and prevention of T2DM [192].

IMPROVED INSULIN-RESISTANCE DUE TO EXERCISE

Increase physical activity/exercise increases insulin-sensitivity, both following acute and chronic exercise training [193]. The acute effect of exercise on insulin-sensitivity is partly mediated by increased insulin-stimulated IRS-1 associated PI3K activity [194, 195]. In contrast, long-term effects of chronic exercise are possibly mediated by gene-expression changes. Possible mediators of these changes in gene expression include MAPK and AMPK, with effects on gene-expression through PPARs, especially PPAR δ [182].

Regulation of energy homeostasis during exercise

Glycogen utilisation in skeletal muscle increases during exercise in an intensity-dependent manner and decreases over time during prolonged exercise, whereas plasma glucose uptake and subsequent oxidation increase with both intensity and duration of exercise [196, 197] (Figure 11).

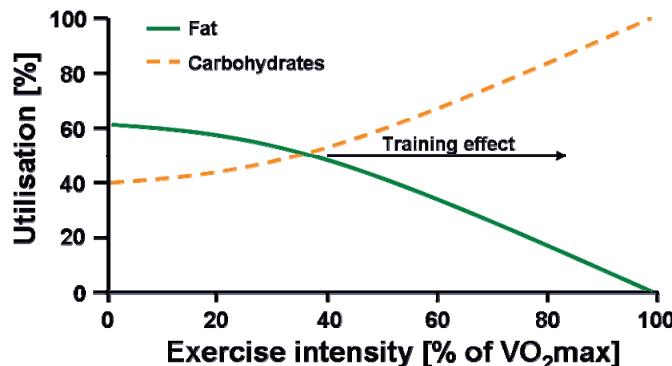


Figure 11: Energy-substrate utilisation during exercise. Fat (lipids) and carbohydrate utilisation is dependent on the intensity of the performed exercise. At lower intensities fat is the predominantly used energy-substrate while at higher intensities a switch to glucose occurs. Exercise adaptation shifts the point of this change to higher exercise intensities, enabling an athlete to utilise less carbohydrates and instead oxidise more fat.

In skeletal muscle, beta-oxidation in mitochondria is increased due to acute exercise. Furthermore, the oxidative capacity of triglycerides is up-regulated [123, 128, 198, 199]. Exercise increases lipolysis of intramuscular lipid stores, thereby increasing free fatty acids available for fatty acid oxidation [123, 200]. AMPK may play a role in the down-regulation of lipid efflux from adipocytes and an increase of intra-muscular lipolysis and beta-oxidation [201, 202]. However, an exclusive dependency on fat oxidation can only supply a metabolic rate of 50–60% of $\text{VO}_{2\text{max}}$ in humans [203]. Intramuscular triacylglycerole (TAG) stores in untrained human muscle constitute about 0.5% of the fibre volume. These stores are doubled with endurance exercise training [204].

In extreme endurance athletes, such as elite cyclists or marathon runners, intracellular lipid stores can make up more than 2% of the fibre volume [205, 206]. Interestingly, intramuscular lipid content is also increased in obesity and is decreased by weight loss; leading to the observation that increase intramyocellular lipid is associated with insulin resistance. However, the relationship between lipid content and insulin resistance appears more complex, since endurance athletes show improved skeletal muscle insulin sensitivity, while obese individuals tend to have decreased insulin sensitivity [62].

Studies in rodents suggest that the increase in lipid oxidation in skeletal muscle can improve insulin sensitivity despite high levels of plasma and intra-muscular fatty acids [97]. Similarly in humans, insulin sensitivity was related to a higher capacity for lipid oxidation [207]. However, the mechanisms behind these effects are poorly

understood, but may involve increased mitochondrial volume, as well as hypertrophy of oxidative muscle fibres and even changes in muscle fibre composition [208].

Muscle fibre composition and muscle phenotype

Mammalian skeletal muscle consists of a variety of different fibre-types, defined by the expression of myosin heavy chain (MyHC) isoforms [209, 210] and physical properties such as speed of contraction which is determined by the mixtures of energy producing mATPase-isoforms the muscle possesses and the muscle-neurological input [211-213]. This diversity of properties has led to a wide range of definitions muscle fibre-type; hence the term “fibre-type” is not being used with coherence. (**Table 2**) Additionally, the nomenclature varies somewhat between species like rodents and human [214].

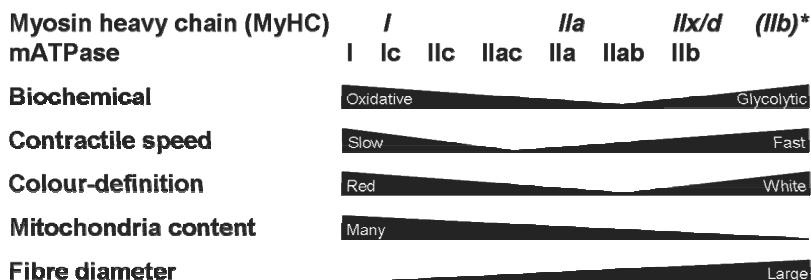


Table 2: Muscle fibre-type classification systems. Skeletal muscle classification is based on myosin heavy chain (MyHC) and Adenosinetriphosphate phosphatase (ATPase) expression, biochemically, by their substrate utilisation capability, by contractile speed, colour, mitochondrial content and the diameter of the individual fibres. Although classification systems are sometimes used interchangeably with each other (e.g. red and white, slow and fast twitch, glycolytic and oxidative), it should be noted that each of these different classification systems are based on different physiological or anatomical properties and are not necessarily analogous. (* MyHC Type IIB exists only in rodents)

Human skeletal muscle is composed of mixture of fast and slow fibre-types, with inter-individual variation in the relative proportion of different fibre-types. Slow-twitch skeletal muscle fibre types are considered to be more oxidative, while fast-twitch skeletal muscles are more glycolytic. Additionally, insulin-stimulated glucose transport is greater in slow-twitch skeletal muscle fibres [215-217], and insulin sensitivity is positively correlated to the proportion of slow twitch oxidative fibres [218].

Fibre type composition is correlated with whole body insulin sensitivity. Insulin resistant T2DM patients and morbidly obese insulin resistant subjects have a lower percentage of type I and elevated type II fibres, particularly type IIB-fibers compared to insulin sensitive subjects [219]. Similar increases in the proportion of type IIB fibres have also been reported in insulin-resistant first-degree relatives of patients with T2DM [220]. These correlative studies offer further support for the hypothesis that whole body insulin sensitivity is partly governed by the skeletal muscle fibre type composition, or more precisely, the oxidative capacity of the individual muscle fibres.

At least some of the effects of fibre type composition of skeletal muscle on insulin sensitivity are attributed to the density of mitochondria in different fibre types.

Reduced mitochondria density has been correlated with T2DM in skeletal muscle of offspring of T2DM [221].

Exercise changes muscle phenotype

Chronic physical activity or chronic inactivity leads to multiple changes in the skeletal muscle phenotype including gene-expression, mitochondrial density and contractile properties [222, 223]. The molecular changes that occur in response to exercise or inactivity ultimately lead to an alteration of the metabolic profile of the skeletal muscle [224-226]. Exercise training promotes a muscle fibre-type transformation from a glycolytic to a more oxidative phenotype. The expression and activity of the Ca^{2+} sensitive enzyme calcineurin plays an important role in muscle phenotype type transformation [227].

Transcription factors of the MEF2 family serve as endpoints for a signalling pathways, whereby calcineurin controls muscle hypertrophy and fibre-type [228]. Additionally, signalling initiated by the PPAR transcription factors also controls the skeletal muscle phenotype [99, 125]. Transgenic expression of activated PPAR δ increases the proportion of type I fibres in mice, thereby setting the contractile and metabolic properties of skeletal muscle to an slow-twitch oxidative phenotype from embryonic development on [99, 125].

Similar results have been obtained in mice with transgenic overexpression of the transcriptional PGC-1 α , whereby type IIA and type I skeletal muscle fibres are predominant, compared to wild-type mice. Thus, in rodents, calcineurin, MEF2, PGC-1 α and PPAR δ form the basis of a signalling network controlling skeletal muscle fibre phenotype and metabolism.

Exercise in the treatment of insulin-resistance

The overall changes in society, generating a more sedentary lifestyle with increased prevalence of obesity, are thought to underlie much of the current increase in T2DM. Thus life-style modification is an attractive non-pharmacological treatment of T2DM. Physical exercise has been shown to have beneficial effects on the prevention and management of T2DM.

Large scale investigations, like the Diabetes Prevention Program (DPP) showed that an intensive lifestyle intervention reduces the risk of diabetes 58% [229]. While the DPP also showed that some medications may delay the development of diabetes, an intervention of diet and exercise is even more efficient. Although exercise is theoretically “available” to anyone, there may be obstacles for T2DM patients.

Various complications due to T2DM make it harder to pursue intense exercise regimes. Circulation defects associated with T2DM can lead to tissue necrosis and open-wounds [230]. This condition is not just painful but may, in combination with poor healing, go as far as amputation. Additionally, increased age, a history of sedentary behaviour and cardio-vascular complications can impair patients in their attempts to become physically active.

However, recent studies have provided evidence that most of the beneficial effects of exercise can be achieved already with low impact and low intensity exercise regimes [231].

AIMS

The underlying pathology of skeletal muscle insulin resistance and the importance of exercise in the treatment of T2DM are incompletely resolved. The aim of this thesis is to explore the signalling mechanism controlling skeletal muscle metabolism.

In particular the following questions were posed.

1. Does low-intensity exercise in humans lead to alterations in PPAR δ expression?
2. Does expression of the PPAR isoforms and the co-activator PGC1 α and β correlate with the oxidative capacity in human skeletal muscle?
3. How does direct activation of PPAR δ in human skeletal muscle alter lipid and glucose metabolism, and which intracellular signalling pathways are activated as a result?

EXPERIMENTAL PROCEDURES

STUDIES PERFORMED UTILISING HUMAN MATERIAL

Subjects in Study I. Six men with complete chronic lesion of the cervical spinal cord, nine non-trained, able-bodied male subjects (normally active) and eleven elite athletes (cyclists) participated in this study. The spinal cord injured (SCI) individuals were five male subjects with complete chronic lesion of the cervical spinal cord. All study participants received a thorough clinical examination, including routine blood and urine chemistry analysis and X-rays of the chest, spinal column, and extremities. Clinical and anthropometric characteristics of the study participants are presented in [232]. The study protocol was reviewed and approved by the institution's ethics committee. SCI subjects were treated against muscle spasm with baclofen (20-25 mg x 2-4) and one subject received additional treatment with diazepam (5 mg x 2). For bladder emptying, condom drainage was used in all SCI subjects. The procedures were explained and informed consent was obtained from each subject prior to participation in the study. Samples were taken from normally active subjects and elite cyclists at least 24 hours after the last bout of exercise training, in order to avoid acute effects of the last exercise bout. The study protocols were reviewed and approved by the institutional ethics committee. The study was conducted according to the principles expressed in the Declaration of Helsinki. None of the study participants were tobacco users. None of the able-bodied subjects were taking any medications. Subject characteristics of the spinal cord injured and able-bodied participants are presented in **PAPER I; Table 1**.

Subjects in Study II. Patients with type 2 diabetes were enrolled from two different primary care practices in two suburban communities outside Stockholm, Sweden. Selection and recruitment of the complete population is described in [233]. Exclusion criteria were severe angina pectoris or other severe disability and insulin treatment. Public meetings, advertised in the local press, were arranged in the two communities and attendants were invited to participate. The intervention group and the control group both consisted of 26 patients. We chose to invite the patients to the two groups from different communities in order to diminish the possibility that the control group patients would start exercising as they learned about the activities in the intervention group. This was therefore not a randomized study. A sub-group (n=15) of the study participants agreed to participate in skeletal muscle biopsy experiments. Patients with insulin treatment and symptomatic coronary heart disease were excluded. Subjects were divided into subgroups depending on increment change in physical activity and the resulting clinical improvements, as described in the results section. Participants were treated with the following drugs and medication and pharmacological treatment regimes were unaltered throughout the study: Anti-diabetic therapy; Metformin (n=5), Sulfonyl urea (Glibenklamid, Glipizid, Glimepirid n=7); Antihypertensive therapy; Betablockers (Atenolol, Metoprolol) (n=5, respectively), ACE/ AII inhibitors (Enalapril, Cilazapril, Losartan, Valsartan) (n=7), calcium channel blockers (Felodipin, Isradipin) (n=5), Diuretics (Hydrochlorthiazide) (n=4) Lipid lowering therapy: Statins (Simvastatin, Pravastatin, Atorvastatin) (n=5), as well as Acetyl salicylic acid (n=3), Nitroglycerin (Glyceryltrinitrate) (n=1), Levotyroxin (n=1), Proton pump inhibitor (Omeprazol) (n=2), VitaminB12 (Cyanokobalamin) (n=1), Estrogen (Estradiol) (n=1), Inhalable steroids (Budesonide) (n=1). All subjects were given advice regarding the positive effects of physical exercise and instructed to increase their exercise duration in the form of brisk walking, 45 minutes three times

weekly, during four months. Participants were asked to keep an exercise diary throughout the study. At the start and end of the study (four months duration), participants also responded to a questionnaire that focused on daily physical activity. Walking support groups were provided four times per week. No recommendations were given concerning changes of dietary habits. The study protocol was approved by the Local Ethical Committee and conducted according to principles of the Declaration of Helsinki. Subject characteristics are presented in **PAPER II; Table 1 and 2**.

Clinical Parameters in Study II.

Resting systolic (SBP) and diastolic (DBP) blood pressure in the supine position and body mass index (BMI) were assessed. Blood samples, obtained in the fasting state were analyzed for plasma glucose, insulin, glycated hemoglobin (HbA1c), and lipid levels (total cholesterol, HDL and LDL cholesterol and triglycerides).

Insulin Sensitivity. Insulin sensitivity was calculated using the homeostasis model assessment (HOMA) index (fasting serum insulin (μ U/ml) \times fasting plasma glucose (mmol/l) \times (22.5) $^{-1}$). Serum insulin levels were analyzed using the Auto DELFIA method (Perkin Elmer TM).

Aerobic Capacity. Age-adjusted physical fitness was determined by bicycle ergometry (Monark 839E ergometer; MONARK EXERCISE ABTM), during a submaximal exercise test. The patients performed six minutes of cycling at a resistance (50-150 W) that resulted in a steady-state heart rate. Heart rate, sex and age were used in the Åstrand nomogram to calculate the predicted maximum oxygen uptake ($VO_{2\text{max}}$).

Muscle biopsies

Muscle biopsies in Study I. Biopsies in normally active subjects and cyclists were taken in the morning after an overnight fast. Muscle biopsies were obtained at rest from the *vastus lateralis* muscle using the percutaneous needle technique similar to the procedure described for Study II. The muscle tissue samples were frozen in isopentane pre-cooled in liquid nitrogen and stored at -80 °C until analysed. Prior to the study, the procedure was explained in both oral and written forms to all subjects. The Ethics Committee of the Karolinska Institutet approved the study. Muscle biopsy specimens from spinal cord injured individuals were obtained using an open biopsy technique, under local anaesthesia (mepivacaine chloride 5 mg/ml). Muscle biopsies were taken from the *vastus lateralis* portion of the *quadriceps femoris*. Briefly, a 4-cm incision was made 15 cm above the proximal border of patella, and muscle fascia was exposed. A skeletal muscle biopsy (~2 g of muscle) was excised and placed in oxygenated Krebs-Henseleit buffer (KHB) containing 5 mmol/l HEPES and 0.1% BSA (RIA Grade).

Muscle biopsies in Study II. Skeletal muscle biopsies were obtained from the Type 2 diabetic subjects before and after the exercise training program (Exercise-Trained Subjects; n=9, Control Subjects n=6). After local anesthesia (Lidokain hydrochloride 5 mg/ml), an incision (5 mm long/10 mm deep) was made in the skin and skeletal muscle fascia, and a biopsy (20-100 mg) was obtained from the *vastus lateralis* portion of the *quadriceps femoris* using a Bergström needle. Biopsies were immediately frozen and stored in liquid nitrogen until analysis.

Skeletal muscle biopsies in Study III and IV were obtained from healthy individuals who underwent general surgery. None of the subjects had known metabolic disease. The ethical committee at Karolinska Institutet approved protocols.

Muscle fibre type determination

Muscle Fibre Typing. Serial transverse sections (10 µm) were cut with a microtome at -20°C and stained for myofibrillar adenosinetriphosphatase (ATPase) activity. The sections were pre-incubated at different pH values in acid (pH 4.3. or 4.6) or alkaline (pH 10.3) buffers. Muscle fibre types were classified by the mATPase-isoforms I– IIC- IIA– IIB based on the myofibrillar ATPase staining characteristics [234](Table 2). We did not examine MHC characteristics of the muscles examined and therefore report the fibre type IIB whereas the equivalent in the MHC system would be denominated type IIX or IID [235, 236].

NADH dehydrogenase staining

Nicotinamide adenine dinucleotide (NADH) dehydrogenase was assessed as described [237]. Briefly, following procedures were used: muscle sections were rinsed in water, incubated in tetrazolium medium, rinsed in water again, then dehydrated in graded alcohols, briefly treated with acetone at room temperature, and mounted with glycerogel, after clearing in xylol.

Preparing biopsies for protein/ mRNA analysis

Gene Expression in Study II was performed, using portions of skeletal muscle biopsy (20-35 mg). The material was removed from liquid nitrogen and RNA extracted, and cDNA synthesized as described below.

Protein Expression in Study II was performed, using skeletal muscle biopsies (40-50 mg). Muscles were freeze-dried overnight and then dissected under a microscope to remove visible blood, fat and connective tissue. Samples were subsequently homogenised in buffer (50 mM Hepes pH 7.6, 150 mM NaCl, 1% Triton X 100, 1 mM Na₃VO₄, 10 mM NaF, 30 mM Na₄P₂O₇, 10% (v/v) glycerol, 1 mM benzamidine, 1 mM DTT, 10 µg/ml leupeptin, 1 mM PMSF and 1 µM microcystin) and protein concentration was determined as described below.

In *Study III and IV*, cell monolayers were cultured in 100 mm dishes, washed once in ice-cold PBS and harvested directly by scraping into 400 µl ice-cold Buffer A for harvest. Homogenates were rotated for 60 min at 4°C and subjected to centrifugation (20000g for 10 min at 4°C).

Isolation of skeletal muscle satellite cells. Muscle biopsies (*rectus abdominus*, other muscles were from the shoulder joint, hip and knee area; 4-3 g) were collected in cold phosphate-buffered saline (PBS) supplemented with 1% PeSt (100 units/ml penicillin/100 µg/ml streptomycin). Satellite cells were isolated and cultures were established based on protocols for human foetal skeletal muscle [238]. Skeletal muscle biopsies were dissected free from visible connective and fat tissues, minced finely, transferred to a digestion solution (0.015 g Collagenase IV (Sigma), 8% 10x trypsin, 0.015 g BSA, 1% PeSt, in Ham's F-10 medium), and incubated with gentle agitation at 37°C for 15-20 min. Thereafter, undigested tissue was allowed to settle and the supernatant containing liberated cells (Satellite cells) was collected and mixed with 1:1 growth medium (Hams F-10 medium containing 20% FBS, 1% PeSt). The remaining tissue was digested for a further 15-20 min at 37°C with fresh digestion solution. The resultant supernatant was then pooled with the previous cell suspension and centrifuged for 10 min at 350g. The cell pellet was resuspended in 5 ml growth medium. The cell suspension was incubated in a non-coated (bacteriological) petri dish for 1 hour, to selectively promote adherence of non-myogenic cells. The supernatant was then transferred and cells were seeded and grown in 150 cm² Costar culture flasks™.

Medium was changed every 2-3 days. At confluence (>80%) cells were trypsinised and subcultured. This first flask (after the first trypsinization) was designated "passage 0". For experimental assays, myoblasts were allowed to reach >80% confluence before initiation of the differentiation protocol.

MANIPULATION OF CELL CULTURES

Culture conditions

Human Skeletal Muscle Cells (HSMC) Cells were seeded at a density of $2-3 \times 10^4$ cells/cm 2 and grown in HAM-F10 (1000 mg/L glucose) with 20% FBS and 1% Penicillin/Streptomycin (PeSt) in non-coated dishes. In order to differentiate human myoblasts into myotubes, dishes with a cell density of 80-90% were grown in DMEM with 4% FBS for 2 days to induce myotube formation, then grown in DMEM with 2% FBS for 2 days. Before utilisation, the cells were controlled optically for formation of elongated myotubes and serum-starved over-night.

Mouse fibroblast 3T3L1 cell cultures in *Study III* were grown in DMEM (4500 mg/L glucose) with 10% FBS and 1% Penicillin/Streptomycin (PeSt). Cells were cultured until 80-90% confluency for passage. Differentiation was initiated at day 2 after 100% confluency. For initiation of differentiation, 0.25 μ mol/L dexamethasone, 0.5 mM isobutylmethylxanthine and 167 nM insulin were added to DMEM. After 72 hours of cultivation, medium was changed to DMEM containing 167 nM insulin. After 2 more days, medium was switched to the initial culturing medium of DMEM (4500 mg/L glucose) with 10% FBS and 1% PeSt. The cells were used after approximately 12 days after completion of the differentiation protocol, when >90% of the cells expressed the adipocyte phenotype (i.e. filled with fat droplets). Prior to experimentation, the cells were washed and pre-incubated with DMEM containing 5 mM glucose, 25 mM Hepes (pH 7.4), 1% PeSt without FBS.

Mouse C2C12 myoblasts in *Study III* were cultured in DMEM (1000 mg/L glucose) containing 20% FBS. For initiation of differentiation the medium was changed to a 2% FBS content when cells were 100% confluent. Cells continued to grow until day 5-7 for formation of myotubes and then used for experimentation.

Giems/Wright staining

To assess the extent of differentiation, myotubes were fixed in methanol (10 min), 1:10 Giemsa (15 min) and 1:10 Wright (20 min). Cells were washed with double distilled H₂O and mono- or multinucleated cells were observed under phase contrast invert light microscope.

Pharmacological inhibition and stimulation

In *Study III*, cells were pre-incubated with ERK1/2 and p38 MAPK inhibitors PD98059 (50 μ M), or SB203580 (10 μ M). The inhibitors were added to Krebs-buffer with either GW0742 in DMSO or DMSO alone (for baseline measurements) for incubation at 37°C for 15 minutes and insulin added as indicated.

siRNA transfection

Myotubes were transfected using Lipofectamine 2000 (Invitrogen, Sweden). Differentiation media were changed to antibiotic-free growth media on day 2 of myotube differentiation. On day 3, individual siRNAs (1 μ g/ml) were mixed in serum/antibiotic-free DMEM (50 μ l) for 5 min and 1 μ l of the transfection agent,

Lipofectamine 2000 (Invitrogen, Sweden) was mixed and incubated with 49 µl DMEM in a separate tube for 5 min. The two mixtures were combined and mixed gently with agitation at room temperature for 30 min (incubating time >16 h). Myotubes were then washed with PBS and 2 ml of DMEM containing 2% of FBS was added in each well. On day 5, cells were used for experiments. Control cultures were similarly prepared, using random siRNA constructs.

siRNA constructs

siRNA controls (random) and against PPAR isoforms were purchased from Dharmacaon (Perbio Science Belgium) Order numbers were
siCONTROL RISC-free siRNA #1 D-001220-01-20,
siGENOME SMARTpool M-003435-01-0010, Human PPARD, NM_006238,
siGENOME SMARTpool M-003434-00-0010, Human PPARA, NM_005036.
siRNA for AMPK $\alpha 1$ and $\alpha 2$ were from Ambion (Austin, Tx) and were designed, sequences were:

AMPK $\alpha 1$

Primer sequences (sense): AGU GAA GGU UGG CAA ACA Utt
Primer sequences (anti-sense): AUG UUU GCC AAC CUU CAC Att

AMPK $\alpha 2$

Primer sequences (sense): UAU GAU GUC AGA UGG UGA Att Utt
Primer sequences (anti-sense): UUC ACC AUC UGA CAU CAU Att

METABOLIC READ-OUTS IN CELL-CULTURES

Glucose uptake

HSMC and C2C12 muscle cells. Six-well cultures from day 5 days post-differentiation, were pre-incubated in serum-free DMEM for >18 h. Cells were then incubated in serum and glucose free DMEM without or with insulin (120 nM) for 40 min and without or with GW501516 for 16-18 h at 37°C with 5% CO₂, followed by addition of 5 mM [³H]2-deoxyglucose (0.33 mCi per well), for 10 min. After incubation with [³H]2-deoxyglucose, medium was rapidly aspirated and cells washed three times with ice-cold PBS and lysed in 1 ml 0.5 M NaOH of which 0.5 ml was transferred to scintillation liquid. Radioactivity was determined by liquid scintillation counting (1214 Rackbeta; Wallac, Turku, Finland). The remaining suspension was used for protein concentration determination using a commercial kit. To determine non-specific uptake, parallel incubations were performed in the presence of 50 mM cytochalasin B, and results were subtracted from the respective incubations in the absence of cytochalasin B. Cytochalasin B exposure reduced glucose uptake by approximately 60%. Each experiment was carried out on triplicate wells.

Glucose uptake in 3T3-L1 adipocytes. For measurements of glucose transport rates in 3T3-L1 adipocytes, cells were grown in serum-free DMEM for 16 h and then incubated in the absence or presence of insulin or GW501516 for 6 h at 37°C. Transport was started by adding 50 µmol/l [³H]2-deoxy-D-glucose (NEN, Boston, MA) and 1 µCi in 1 ml of Krebs-Ringer phosphate buffer (pH 7.4) for 5 min at 37°C and stopped by placing the cells on ice and rapidly washing them three times with ice-cold buffer. Cells were lysed in 1 ml of lysis buffer containing 0.1% Triton X-100 for 45 min. Aliquots of the cell lysates were used for liquid scintillation counting and determination of protein

content (as described below), respectively. Non-specific transport was assayed in the presence of 10 $\mu\text{mol/l}$ cytochalasin B.

Glucose incorporation into glycogen in HSMC

Myotubes (5 days post-differentiation) were grown in six-well plates, serum starved for 16–18 h, stimulated without or with 120 nM insulin for 30 min, and/or GW501516, for 16–18 h min at 37°C and incubated with 5 mM glucose DMEM, supplemented with D-[U- ^{14}C] glucose (1 mCi/ml; final specific activity 0.18 mCi/mmol) for 90 min. Following incubation, monolayers were washed with ice-cold PBS, and lysed in 1 ml 0.03% SDS. 0.85 ml of the suspension was transferred to 10 ml tubes and 100 ml (2 mg/sample) carrier glycogen was added. The remaining cell suspension was used for protein concentration determination. Samples were heated to 95°C for 30 min. Glycogen was precipitated by addition of 95% ethanol and incubated overnight at 4°C with slight agitation. Glycogen pellets were collected by centrifugation for 35 min at 1700 g, washed once with 70% ethanol and resuspended in 200 ml distilled water. Radioactivity was determined by liquid scintillation counting (1214 Rackbeta; Wallac). Each experiment was carried out on triplicate wells.

Intracellular fatty acid accumulation and β -Oxidation in HSMC

Cells were cultured in 25 cm² flasks and differentiated to myotubes. Myotubes were treated for 120 min with 0.4 μCi of [1- ^{14}C] palmitate in 2 ml serum-free DMEM with or without insulin (120 nM) at 37°C, in 5% CO₂-95% O₂. Thereafter, 300 μl of 70% perchloric acid was injected through to the medium. Flasks were laid down with slight agitation for 1 h at room temperature. The filter was removed to a scintillation tube and 200 μl of ice-cold methanol was added. The trapped $^{14}\text{CO}_2$ in the filter was counted in a liquid scintillation counter. To measure the free fatty-acid uptake after CO₂ trapping, the flasks were washed five times with TBS-Tween (0.02%) and cells were lysed with 2 ml of 0.03% SDS for 2 hr at room temperature with slight agitation. Lysates (400 μl) were transferred to 4 ml scintillation fluid and the amount of [1- ^{14}C] in the lysate was counted in a liquid scintillation counter. Protein content of each sample was determined by the BioRad method. Results reported as cpm mg⁻¹ of protein.

HPLC measurement of ATP, ADP and AMP

Cells were grown to confluence in 6 cm dishes and differentiated before experimentation. After treatment, cells were rapidly washed with PBS (twice) and scraped into 450 μL PBS. After transfer to eppendorf tubes containing 150 μL PCA-EDTA (PCA 10%, EDTA 25 mM) on ice, the lysate was vortexed briefly and kept on ice for 30 min. Following centrifugation, the supernatant was transferred to new eppendorf tubes and neutralised to pH 6.5–7.5 with KOH/MOPS (KOH 2 mol/L, MOPS 0.3 mol/L). Lysates were kept at -20°C until usage for High Pressure Liquid Chromatography (HPLC) measurement. Adenine nucleotides were separated by high-performance liquid chromatography using a Spherisorb column 5 μm ODS (0.46 cm x 18 cm). Elution was done with 25 mM sodium pyrophosphate/pyrophosphoric acid, pH 5.75, with a flow rate of 1 ml min⁻¹. Absorbance was measured at 254 nm.

PROTEIN BASED ASSAYS

Protein concentration measurements

Protein concentrations for western blot application was determined in the supernatant of the lysates (cell or muscle biopsies) using the Bradford method (Bio-Rad, Richmond, CA), as described by the manufacturer.

Immunoblotting techniques

For analysis of protein expression, in *Study II* an aliquot of muscle lysate of 40 µg protein, in *Study III and IV* 20 µg cell-lysate, was mixed with Laemmli buffer containing β-mercaptoethanol. Proteins were separated by SDS-PAGE, transferred to polyvinylidenedifluoride (Immobilon-P, Millipore) membranes and probed with the appropriate primary antibody and secondary horseradish peroxidase-conjugated antibodies. Proteins were visualised by enhanced chemiluminescence and quantified by densitometry. To correct for loading, the lower part of each membrane was blotted for Histone H3 in all studies.

Creatine kinase activity

Creatine kinase activity was determined a Creatine Kinase Kit (47-UV) from Sigma Diagnostics, (St. Louis, MO). Cells were grown on 100 mm petri-dishes until the day of use. Glycyl-glycine buffer (400 µl of 0.5 M, pH 6.8) was added and the cells were solubilised by scraping with rubber policeman. Cell suspensions were transferred to microtubes and sonicated 2 times 20 s at 4°C. Creatine kinase activity was determined after protein measurement by evaluating reaction products at 340 nm UV lamp-spectrophotometer.

PI3-Kinase activity

Muscle cells were grown as described above, treated with insulin and then scraped into ice cold homogenising buffer (50 mM Hepes pH 7.6, 150 mM NaCl, 1% Triton X 100, 1 mM Na₃VO₄, 10 mM NaF, 30 mM Na₄P₂O₇, 10% (v/v) glycerol, 1 mM benzamidine, 1 mM DTT, 10 µg/ml leupeptin, 1 mM PMSF and 1 µM microcystin). An aliquot of the supernatant was immunoprecipitated overnight (4°C) with anti-phosphotyrosine antibody coupled to protein A-sepharose (Sigma, St Louis, MO, USA). The lysates were cleared by centrifugation and incubated with anti-IRS-1 antibody (1 in 100 dilution) and 50 ml of protein A-Sepharose (50 mg/ml pre-equilibrated in lysis buffer A) by tumbling end over end at 4°C for 2 h. The immunoprecipitates were then washed 3 times with homogenising buffer, 2 times in buffer B (500 mM LiCl, 100 mM Tris-HCl, pH 8.0, at 4°C), once in buffer C (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.6, at 4°C), and once in buffer D (20 mM Hepes, 1 mM dithiothreitol, 5 mM MgCl₂, pH 7.6, at 4°C). The beads were then resuspended in 40 ml of buffer E (10 mM b-glycerophosphate, 5 mM Na₄P₂O₇, 30 mM NaCl, 1 mM dithiothreitol, pH 7.2, at 4 °C). 20 ml of phosphatidylinositol/cholate solution (3 mg/ml in 1% (w/v) sodium cholate) was added to each tube, and the reaction was started by the addition of 5 mCi of [γ -³²P]ATP in 40 ml of reaction mix (3 mM Na₂ATP, 7.5 mM MgCl₂) and incubated at 37 °C for 15 min. Reactions were terminated by the addition of 450 ml of CHCl₃:CH₃OH (1:2 v/v). The product was then extracted by the addition of 150 ml of CHCl₃ and 150 ml of 0.1 M HCl and then again by the addition of 300 ml of CHCl₃ and 300 ml of 0.1 M HCl. Extracted lipid was dried down under vacuum before redissolving in 25 ml of CHCl₃, CH₃OH, 0.1 M HCl (200:100:1). Reaction products were separated by thin layer chromatography (run in a pre-equilibrated tank containing methanol:chloroform:ammonia:water, 300:210:45:75).

Reaction products were resolved by thin layer chromatography and quantitated using a PhospholImager (Bio-Rad).

List of antibodies

Diacylglycerol kinase (DGK) δ antibody was from Matthew K. Topham (Huntsman Cancer Institute and Department of Internal Medicine, University of Utah, Salt Lake City, UT).

Commercially available antibodies. Were from the following companies:

Company	Protein
Santa Cruz Biotechnology (Santa. Cruz, CA):	UCP, PPAR δ (- β), MEF2A
Upstate (Lake Placid, NY):	AMPK α 1 and α , ACC, 2, Histone 3 (H3), CAP
New England Biolabs (Beverly, MA):	Phosphospecific PKB (Ser ⁴⁷³) and ERK1/2 MAP Kinase (Thr ²⁰² and Tyr ²⁰⁴), protein p38 MAPK and PKB
Cell Signalling Technology (Beverly, MA):	AMPK pan α -subunit (Thr ¹⁷²), p38 MAPK (Thr ¹⁸⁰ and Tyr ¹⁸²), MEF2C
Transduction Laboratories (Lexington, KY):	pan ERK1/2
Biogenesis (Poole, UK):	GLUT1
Genzyme (Cambridge, MA):	GLUT4
Invitrogen (Carlsbad, CA):	Expression respiratory-chain complex I (NADH-ubiquinol oxidoreductase (CI) and complex IV (cytochrome C oxidase (COX) I)
Chemicon (Temecula, CA):	PGC-1 α
Bio-Rad Laboratories (Richmond, CA):	Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G secondary antibodies

Table 3: Source of antibodies

mRNA-EXPRESSION ASSAYS

mRNA extraction and cDNA synthesis

Cells were cultured in 100-mm dishes as described. Cultures were washed three times with RNase-free PBS, and harvested directly for RNA extraction (RNAeasy mini kit; Qiagen, Crawley, UK). All RNA was DNase treated before reverse transcription (RQ1 RNase-free DNase; Promega, Southampton, UK).

Quantitative Polymerase Chain Reaction (PCR)

Multi Fluidic Card® (MFC) gene expression assay. In Study II, gene expression analysis was performed utilising a Taq-Man based Multi Fluidic Card® (MFC) gene expression assay (Applied Biosystems, Foster City, CA). The MFC was specifically designed to assess the expression of 24 genes (5 endogenous controls and 19 target genes), where all genes were analysed in duplicate. Primer and probe sets from Applied Biosystems were lyophilised in the MFC well. Expression of peroxisome proliferative activated receptor (PPAR) δ was determined by real time Taqman PCR.

Quantitative Real-time PCR was used in Study I, III and IV was used for quantification of specific mRNA expression (ABI-PRISMA 7000 Sequence Detector, Perkin-Elmer Applied Biosystems, Foster City, CA). Data was collected and analysed by ABI Prism 7000 SDS Software version 1.1. All reactions were performed in 96-well MicroAmp Optical plates, with a sample volume of 25 μ l. Amplification mixes (25 μ l) contained the diluted cDNA sample, 2x TaqMan Universal PCR Mastermix, forward and reverse primers, and probe for the target gene. Thermal cycling conditions included

2 min at 50°C and 10 minutes at 95°C before the onset of the PCR cycles, which consisted of 40 cycles at 95°C for 15 seconds and 65°C for 1 min. Samples were analysed simultaneously in one assay (i.e. one 96-well plate per gene of interest). The efficiencies of primers and probes for the target genes and for GAPDH were within a similar range allowing for use of the comparative CT method, whereby the relative quantities of different mRNA transcripts were calculated after normalisation of the data against endogenous control.

List of primers and probes

Oligonucleotide primers and TaqMan probes (FAM-MGB) used in *Study II* and *IV* were purchased as Assays-on-demand® from Applied Biosystems (Assay IDs):

Protein name	Order No.	Protein name	Order No.
human calcineurin A α	Hs00174223_m1	human calcineurin β	Hs00236113_m1
PPAR α	Hs00231882_m1	PPAR δ	Hs00602622_m1
PGC-1 β	Hs00370186_m1	Cytochrome c, somatic	Hs01588973_m1
PDK4	Hs00176875_m1	FABP3	Hs00997360_m1
CPT1B (muscle)	Hs00993896_g1	NRF1	Hs00602161_m1
DGK δ	Hs00177552_m1	CONTROL RISC-free	D-001220-01-20
β -actin VIC-MGB	4326315E	18s rRNA VIC-MGB	4310893E
GAPDH VIC-MGB	4326317E		

Table 4: Primers and probes order numbers

Name: PGC-1α (Study I) Forward: 5'CCAAACCAACAACATTATCTCTTCC Reverse: 5'CACACTAAGGTGCGTCAATAGTC Probe: AGTCACCAAATGACCCAAGGGTTCC FAM-TAMRA	Name: HumPGC1 (Study III) Forward: 5'AGAGACAAATGCACCTCCAAAAA Reverse: 5'AAAGTGTGTTGGCTTGCTTAAGT Probe: 5'AAGTCCCACACACAGTCGCAAGTCACAA FAM
Name: HumGLUT1 (Study III) Forward: 5'CCTGTGGGAGCCTGCAAA Reverse: 5'TCTATACACAAACAGGGCAGGAGTCT Probe: 5'CACTGCTAACAGAAC FAM	Name: HumGLUT4 (Study III) Forward: 5'GCTACCTCTACATCATCCAGAACATCTC Reverse: 5'CCAGAAACATCGGCCCA Probe: 5'CTGCCAGAAAGAGTCTGAAGCGCCT FAM
Name: HumPPARA (Study III) Forward: 5'GGGACAAGGCTCAGGCTAT, Reverse: 5'AAGCCCTTGCGACGCTTCA Probe: 5'ATTACGGAGTCCACGCGT FAM	Name: HumPPARD (Study III) Forward: 5'CACACGGCGCCCTTG Reverse: 5'CCTTCTCTGCC TGCCACAA Probe: 5'ATCCACGACATCGAGAC FAM
Name: HumPPARG (Study III and Study IV) Forward: 5'TGGAGATAAACGCTCTGGATTCAC, Reverse: 5'CCGGAAGAACCCCTTGCAT Probe: 5'ATGGAGTTCATGCTTG FAM	Name: HumSREBP1c, Hum SREBP1a (Study III) Forward (1a): 5'CCATGGATTGCACTTCGAA Forward (1a): 5'TGCTGACCGACATCGAAAGAC Reverse: 5'CCAGCATAGGGGGTCAA Probe: 5'TATCAACAAACCAAGACAGTGACTTCCCTGGC FAM

Table 5: Designed primers and probes sequences

Selection of endogenous controls

Expression of ribosomal 18s, β -actin, and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA was determined in order to select an endogenous (“housekeeping”) reference gene to correct for potential variation in cDNA loading and quantity (*Study I*). Expression of GAPDH was unchanged with training status and was therefore selected as an endogenous control in Study I and II. 18s rRNA was selected as endogenous control for Study III and β -actin in Study IV. In Study II five endogenous controls were investigated and GAPDH used for normalisation of expression values (**Figure 12**).

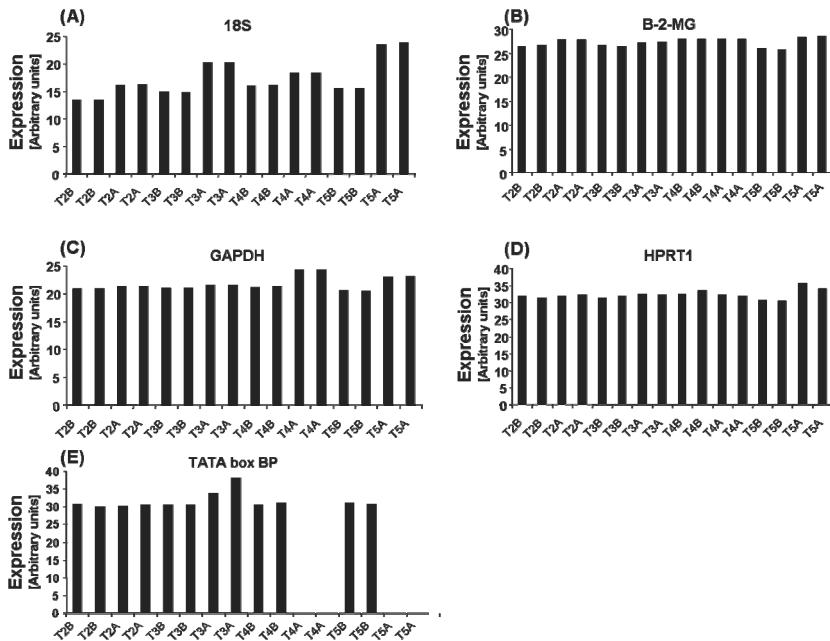


Figure 12: mRNA expression of “housekeeping”genes. mRNA expression of (A) ribosomal 18s, (B) beta-2-microglobulin (beta-2-MG), (C) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (D) hypoxanthine guanine phosphoribosyltransferase (HPRT)-1, (E) TATA-box binding protein was analysed using quantitative real-time PCR. Expression variations of the individuals’ samples (B) before and (A) after the study and inter-individual variations were the basis for selecting the most suitable housekeeper gene. Selected for final standardisation calculations was GAPDH (figures show a representative subset of subjects).

ANIMAL PROCEDURES

Animal care

Male Wistar rats (110 – 120 g) were purchased from B & K Universal (Sollentuna, Sweden) and housed at the animal facility at the Karolinska Institute. Rats were maintained on a 12 h light-dark cycle and given free access to standard rodent chow and water. Rats were studied after a 4-5 hour fast. The regional animal ethical committee approved all experimental procedures.

Muscle preparations

The animals were anesthetized with sodium pentobarbital (5 mg/100 g body weight) injected intraperitoneally. The skin of the front leg was removed and the intact *epitrochlearis* muscle was dissected out and placed in 2 ml of Ice-cold Krebs-Henseleit bicarbonate buffer (KHB) for 5 min. During the dissection procedure, which took about 1 min, ice-cold KHB was applied repeatedly to moisten the muscle. After a 5 min recovery in ice-cold KHB, muscles were trimmed while moistened with cold KHB and then incubated as described below.

Glucose transport

All incubation medias were prepared from a pre-gassed (95% O₂/ 5% CO₂) stock of Krebs-Henseleit bicarbonate buffer (KHB), supplemented with 5 mM HEPES and

0.1% RIA grade bovine serum albumin (fraction V, RIA grade, Sigma). Pre-incubation media contained 5 mM glucose and 15 mM mannitol. Rat *epitrochlearis* muscles were pre-incubated in the presence or absence of insulin (0.6 nM or 12 nM) for 40 min and GW501516 (10 nM) for 6 h, then rinsed in media containing 20 mM mannitol for 10 min followed by transfer to hot media containing 8 mM 3-O-methylglucose with 438 μ Ci/mmol [3 H]-3- O-methylglucose for 12 min. Muscles were incubated in 2 ml of media in a shaking water bath at 30°C. The gas phase in the incubation vial was maintained at 95% O₂ and 5% CO₂.

Muscle extraction procedure

After incubation, muscles were rapidly blotted on filter paper, freeze-clamped with aluminium tongs, cooled to the temperature of liquid nitrogen and then stored at -80°C. Before freeze-drying the muscles were inspected, remaining pieces of tendon were cut off, and they were weighed (all at -20°C). After the muscle had been dissected free from connective tissue, it was extracted. Approximately 2 mg of dry tissue was extracted in 250 ml of ice-cold 300 mM perchloric acid with 1 mM EDTA for 20-30 minutes, while occasionally poking with a small glass rod to ensure that the extraction medium penetrated the muscle tissue. After centrifugation (10 min at 5000 g; 0°C) the major portion of the supernatant was pipetted off and neutralized with a solution containing KOH, imidazole base and KCl, with such proportions (2:l:l) that 80% of the perchloric acid was neutralized by KOH and the rest by imidazole, leaving excess imidazole to buffer the solution at pH 7. The KCl was added in order to favour precipitation of the perchlorate. After centrifugation at 0°C the supernatant was transferred to glass tubes and stored at -80°C until analysis. Radioactivity of aliquots of the samples was measured using a beta-counter (1214 Rackbeta; Wallac) and glucose transport rates calculated.

CHEMICALS

Dulbeccos minimum essential medium (DMEM), Ham's F-10 medium, foetal bovine serum (FBS), penicillin, streptomycin, and Fungizone were obtained from GibcoBRL (Life-Technologies, Stockholm, Sweden). Radiochemical, 2-[G- 3 H]deoxy-D-glucose (6.0 Ci/mmol/l), D-[U- 14 C]glucose (310 mCi/mmol/l), were from Amersham (Life Science, Sweden). All other chemicals were analytical grade and from Sigma-Aldrich Sweden AB (Stockholm, Sweden). Reagents for enhanced chemiluminescence (ECL) were from Amersham (Arlington Heights, IL). For *Study I*, GW501516 was synthesized by Synthelec AB, Lund, Sweden and selectivity of this compound has been described (Oliver WR, Jr., Shenk JL, 2001). GW501516 in *Study IV* was a commercially available product, purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). GW0742 was a gift from Glaxo Smith Kline (Sznaidman ML, Haffner CD, 2003). All GW-compound materials were dissolved in DMSO at 1 μ M concentration. Aliquots were kept for up to 6 month at about -80°C.

STATISTICS

In all studies, data is presented as mean \pm SEM. Statistical differences were determined by ANOVA multiple comparison using Fisher's LSD test or Student's t-test (paired and unpaired), as appropriate. In *Study I*, correlations were calculated by simple linear regression. Significant differences were accepted at p<0.05.

RESULTS AND DISCUSSION

A wide range of animal models have been studied to develop to understand mechanisms accounting for Type 2 Diabetes Mellitus (T2DM). Despite the success of animal models, a thorough understanding of the human condition is not always achieved solely using animal models [239, 240]. Rodents show molecular differences in the insulin signalling pathway as compared to humans, e.g. rodents express an IRS3 isoform that humans lack [241]. Furthermore, rodents have a muscle fibre composition that does not parallel that of humans.

Thus direct investigations in healthy and diabetic human subjects are valuable, while at the same time experimentally challenging, due to ethical and practical issues. Direct investigations in human subjects are also limited by the range of investigations possible and tend to be correlative in nature. For the study of skeletal muscle, cell lines have been used as a complement.

There are several available skeletal muscle cell lines. Although a skeletal muscle cell line derived from humans is unavailable. Furthermore, cell lines are genetically homogeneous, because they are usually derived from one animal, and may accumulate unwanted mutations over the large number of passages [242]. Cell lines also possess specific properties that make them immortal, and often cancer-like, which may interfere with normal biochemical function [243, 244]. Therefore primary human skeletal muscle cell (HSMC) cultures have proved to be a useful and valuable model to investigate human insulin action. [24, 245].

PRIMARY HUMAN SKELETAL MUSCLE CELL CULTURE

Satellite cells: from muscle biopsies to myotubes

HSMC cultures are established utilising the muscle's own "stem cells" (i.e. the so called satellite cells). Satellite cells are thought to play a role in muscle regeneration and are in a quiescent and non-proliferative state until they become activated, by for example myotrauma [246]. When activated, they are released from indentations between the sarcolemma and the basal lamina [247]. Satellite cells proliferate and fuse with either existing cells or together, in order to repair muscle damage [248-252].

For culturing purposes, satellite cells are obtained by partly digesting the connective tissue of a muscle biopsy, which releases satellite cells from the sarcolemma (see "Experimental Procedures"). HSMC cultures can then be established and the satellite cells (also called myoblasts) differentiated into skeletal muscle-like cells. The differentiation process that leads from single-nucleated myoblast to fused multinucleated and elongated myotubes coincides with typical changes in the biochemical, genetic and molecular properties of the cell [253].

Recent research has provided evidence that the fibre type of the mature adult skeletal muscle determines the molecular properties of the satellite cells, at least under certain culture conditions [254]. Hence the final HSMC culture carries an imprint of the original muscle fibres. Therefore, defining the molecular properties of the HSMC system is important.

In order to validate the HSMC model used in this thesis, several investigations to characterise the molecular and signalling properties, as well as the metabolic status of the cultured cells were performed.

Characterisation of the HSMC culture model

Skeletal muscle differentiation follows a highly regulated programme of gene expression [255, 256]. Several proteins have been identified as essential for differentiation, including the transcription factor myocyte enhancer factor (MEF) 2. Transcription factors act at multiple points in the myogenic lineage to establish myoblast identity and to control terminal differentiation [257-260]. We investigated the molecular changes that occur with differentiation of myoblasts into myotubes [253]. Our aim was to determine if the HSMC cultures acquired skeletal muscle-like properties during the differentiation process utilised. Cultures were grown to >80% confluence and then induced to differentiate as described in “Methods”. Fused multinucleated cells were apparent by day 3 post-differentiation. By day 5 post-differentiation, cultures were >90% differentiated (Figure 13A). Concomitantly, as a biochemical marker of differentiation, activity of the muscle-specific creatine kinase was assessed in cultures following addition of differentiation media. Creatine kinase activity increased 6.3 ± 2.3 -fold ($p<0.05$) by day 3 post-differentiation and remained elevated at all further time points tested (Figure 13B). Peak activity was noted at day 5 (94 ± 31 U g⁻¹ protein at day 0 vs. 607 ± 200 U g⁻¹ protein at day 5; $p<0.05$), although there were no significant differences between activity levels observed from days 3 to 14.

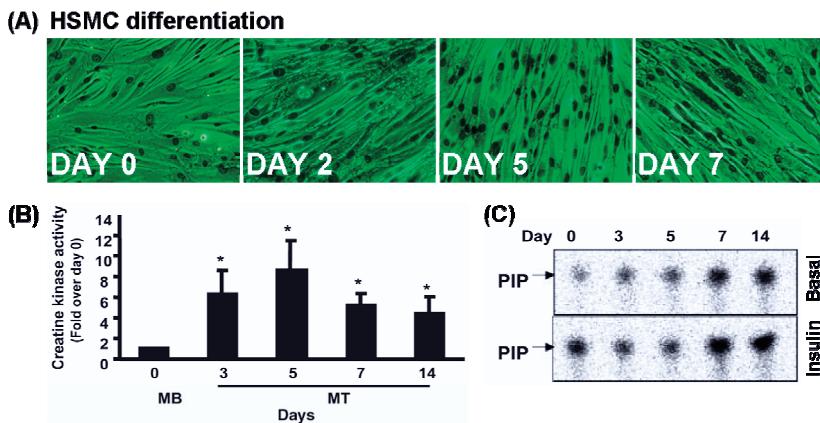


Figure 13: Changes in HSMC during differentiation. (A) Morphological changes during differentiation from mono-nucleated myoblasts to multinucleated myotubes. (B) Creatine kinase activity during differentiation process (* $p<0.05$ vs. day 0). (C) PI3-kinase activity during the differentiation process; visualisation of a representative activity assay showing incorporation of 32 P to generate phosphatidyl inositol phosphate. Top panel shows basal and bottom panel shows insulin-stimulated PI3-kinase activity. Figures B and C reproduced with permission.

Muscle-cell differentiation correlates with increased insulin sensitivity

When studying insulin signalling, the sensitivity of the cell model to an insulin stimulus is important. We therefore investigated whether changes in insulin signalling occurred in HSMC. Insulin significantly increased PKB phosphorylation (data not shown). More importantly, PI3-Kinase activity increased with differentiation, and

peaked at day seven of differentiation (**Figure 13C**). Thus, three separate parameters, morphology, creatine kinase- and PI3-Kinase-activity, indicate that cell differentiation occurs, and that differentiation is completed between day five and seven.

Protein expression of the Insulin Receptor (IR), Hexokinase (HK) II, Insulin Receptor Substrate (IRS)-1 and IRS-2, did not change during differentiation to myotubes (data not shown). In contrast, protein expression of the skeletal muscle marker desmin, Extracellular signal-Regulated Kinase 1 and 2 (ERK1/2 MAP kinase), Insulin Responsive Amino Peptidase (IRAP) and Glycogen Synthase (GS) increased during differentiation to myotubes ($p < 0.05$ vs. expression at day 0; data not shown).

The differentiation into multinucleated myotubes is associated with changes in creatine kinase activity, expression of various signalling and transcriptionally active proteins. Taken together with metabolic markers, this indicates that the satellite cells differentiate to form more insulin sensitive skeletal muscle-like myotubes.

Optimising culture conditions for HSMC

Like all cultured cells, HSMC require specific nutritional conditions. These include a supply of nutrients, salts and hormones, provided by pre-formulated media in combination with Foetal Bovine Serum (FBS). FBS is rich in hormones, including amongst others, thyroid hormone (T3) and insulin which promote cell growth (**Table 5**). Reduction of the FBS content reduces growth promotion and allows differentiation.

Biochemical Component	Units	Fetal Bovine Serum		Horse Serum		Calf Serum		Newborn Calf Serum	
		mean	(range)	mean	(range)	mean	(range)	mean	(range)
Total Protein	g/dl	4.2	4.0 - 4.3	7.0	6.3 - 8.2	6.9	6.1 - 7.5	6.4	6.0 - 7.0
pH	units	7.3	6.7 - 7.3	7.8	7.4 - 8.1	7.6	7.3 - 8.1	7.5	7.3 - 7.7
Osmolality	mosm/kg	314	290 - 335	284	279 - 291	292	273 - 310	292	290 - 310
Glucose	mg/dl	130	107 - 144	75	56 - 86	105	90 - 118	69	59 - 79
Creatinine	mg/dl	3.1	2.8 - 3.3	1.1	0.8 - 1.5	1.1	0.7 - 1.6	0.9	0.6 - 1.1
Cholesterol	mg/dl	34.2	19 - 54	83.6	77 - 91	78.3	95 - 121	70.0	66 - 75
Low Density Lipoprotein	mg/dl	2.8	0 - 7.0	22.6	10 - 35	56.7	51 - 72	18.0	12 - 23
High Density Lipoprotein	mg/dl	6.5	5.0 - 9.0	55.0	48 - 62	36.7	41 - 63	45.0	37 - 52
Triglycerides	mg/dl	219.5	73 - 1720	18.6	11 - 28	16.3	15 - 17	36.7	26 - 53
Growth Hormone	ng/ml	131	126 - 138	not tested		26	14 - 35	36	23 - 37
Insulin	uU/ml	4.3	2.9 - 5.5	11.8	5.8 - 18.5	5.4	4.2 - 6.8	3.9	3.4 - 4.5
Estradiol	pg/ml	13.8	11.2 - 17.5	32.5	12.9 - 90.8	32.9	22.2 - 39.2	36.9	20.3 - 46.1
Progesterone	ng/ml	0.03	0.01 - 0.06	6.8	1.12 - 4.56	1.65	.88 - 2.20	0.02	0.1 - .03
Testosterone	ng/ml	0.40	0.38 - 0.45	0.35	0.28 - 0.46	1.05	0.79 - 1.19	0.49	0.19 - 0.90
T4 (Thyroxine)	ug/dl	14.8	13.9 - 15.8	1.9	1.7 - 2.5	9.2	7.8 - 10.0	9.6	7.0 - 12.2
T3	ng/ml	1.2	0.9 - 1.4	0.6	0.3 - 0.8	1.9	1.5 - 2.5	2.2	1.7 - 2.7

Table 6: Contents of animal sera used for cell-propagation. A selection of contents of some of the most frequently applied animal sera in cell culturing. Highlighted are insulin and T3 concentrations. Source: Personal communication from Invitrogen Corporation.

In response to insulin, HSMC show only modest increases in glucose uptake, ranging from 30-90% compared to basal [245]. This is in contrast to intact skeletal muscle from animals or humans studied *ex vivo*, where insulin stimulation of glucose uptake is 3-5 fold basal [261, 262]. For some study purposes, the increase in glucose

uptake in cultured skeletal muscle gives a small fold insulin response, making identification of reduced uptake a challenge.

We aimed to improve the insulin-mediated glucose uptake noted in HSMC. One hypothesis was that the low expression of GLUT4 was a key issue, and better optimisation of culture conditions could perhaps improve GLUT4 expression level.

Culturing conditions and impact on morphology, gene expression and insulin sensitivity

Treatment with T3 and insulin alters HSMC phenotype

Thyroid hormone T3 has been implicated in altering the expression of key enzymes in skeletal muscle and muscle cells. $\text{Na}^+ \text{-K}^+$ -ATPase expression is enhanced following T3 treatment in HSMC [263]. Thyroid hormones were also shown to regulate mitochondrial content and oxidative capacity in rodent models [264]. We speculated that supplementing media with T3 could enhance the differentiation of the myotubes. Additionally we hypothesized, that metabolic read-outs such as glucose transport would be affected.

HSMC were cultured as described in “Experimental Procedures”, with or without addition of the hormones T3 and insulin (20 nM each). In response to 5 days exposure to insulin and T3, there was a noticeable change in morphology, with an increased number of nuclei per cell, as compared to cells cultured under standard conditions (**Figure 14A**).

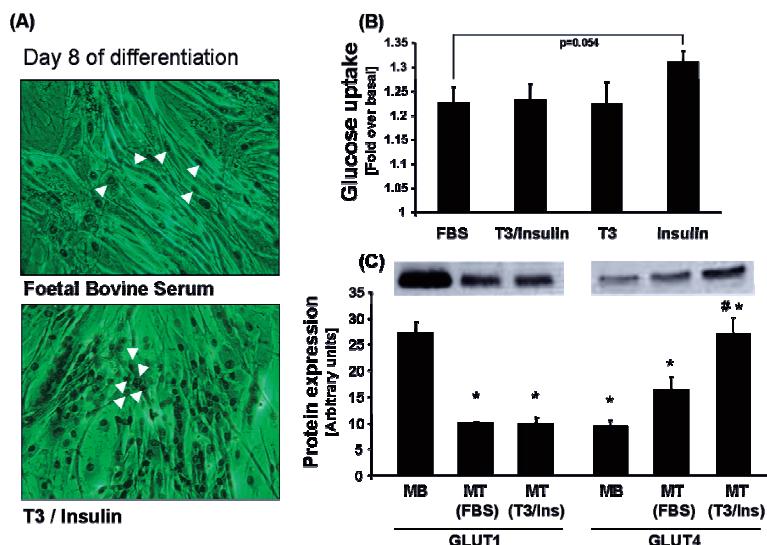


Figure 14: Effects of T3/insulin supplementation on HSMC differentiation. (A) Microscope pictures of HSMC differentiated with T3/insulin or FBS supplemented DMEM at day eight of differentiation. Nuclei were visualised by giemsa-wright staining (Proximity of five selected nuclei is indicated by arrows). (B) Glucose uptake performed after differentiation with either T3/insulin or FBS supplemented DMEM. (C) Protein expression of GLUT1 and GLUT4 of undifferentiated myoblasts (MB) and myotubes (MT) after differentiation with either T3/insulin or FBS supplemented DMEM. Representative western blot images are shown. (*p<0.05 vs. MB; #p<0.05 FBS vs. T3/insulin).

We investigated differentiation related expression of glucose transporters and alterations in insulin stimulated glucose uptake post-treatment. Expression of GLUT1 was decreased 63% ($p<0.05$), while GLUT4 expression was increased 1.7 fold ($p<0.05$) with the standard differentiation protocol, similar to the previously reported 2 fold increase of GLUT4 and a 50% reduction of GLUT1 [245]. With T3/insulin supplement, GLUT1 protein expression was down-regulated 64% ($p<0.05$) and GLUT4 expression increased 2.9 fold ($p<0.01$). Hence, the T3/insulin differentiation protocol increased GLUT4 expression significantly more than the standard protocol ($p<0.05$) (Figure 14C). Despite this, insulin-mediated glucose uptake was not increased significantly when using the T3/insulin protocol, as compared to the standard protocol (Figure 14 B). Thus despite enhanced myotube differentiation, and increased GLUT4 content insulin-stimulated glucose uptake was not increased.

The effects of supplementation of either insulin (20 nM) or T3 (20 nM) added separately on HSMC differentiation was also assessed. Differentiation with insulin supplementation lead to a 31% increase in subsequent insulin-stimulated glucose uptake (120 nM Insulin.), as compared to cells on standard protocols (22% increase $p=0.054$) (Figure 14 B). T3 supplementation did not enhance insulin-stimulated glucose uptake, as compared to standard protocols. Changes in protein expression were not evaluated in cells exposed to either T3 or insulin supplementations (only the combined effect was studied).

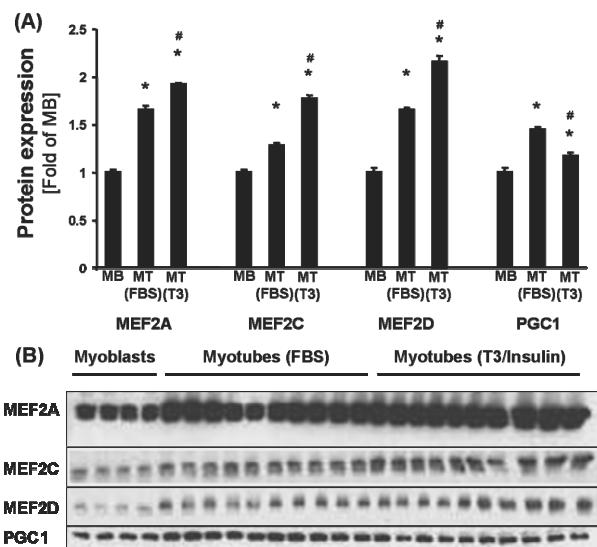


Figure 15: Effects of T3/insulin supplementation on MEF2 and PGC1 expression. Expression of MEF2A, MEF2D, MEF2C and PGC1 following differentiation with either T3/insulin or FBS supplemented DMEM, was determined by Western blotting (* $p<0.01$ vs. MB; # $p<0.01$ FBS vs. T3/insulin). (A) Summary of results for $n=4$ (B) Representative images.

We then investigated the effects on the expression of several differentiation related genes including the MEF2 protein family, PGC-1 and PKB. While MEF2 A/C and D expression was increased with the standard differentiation conditions, a further increase in the expression of these proteins was noted when T3 was added to the insulin treatment. Thus MEF2A expression in cells differentiated under standard protocols was

65% higher than day 0, while T3/insulin differentiated cells showed a 96% increase versus day 0 ($p<0.01$) (Figure 15 A and B). MEF2C expression was increased 28% during differentiation in cells studied using the standard protocol and 77% with T3/insulin supplementation ($p<0.01$), while for MEF2D the values were 65% and 115% for standard and T3/insulin protocol respectively ($p<0.01$). Interestingly, PGC-1 expression increased 44% ($p<0.01$) with the standard differentiation protocol and 17% ($p<0.01$) with T3/insulin supplementation, as compared to basal. Thus PGC1 α expression was significantly lower with T3/insulin than observed with the standard protocol ($p<0.05$).

GLUT4 expression in HSMC following Dexamethasone supplement

In order to increase expression of GLUT4, HSMC were treated with dexamethasone (DEX) since this hormone has previously been shown to increase GLUT4 expression in C2C12 murine skeletal muscle cell lines [265].

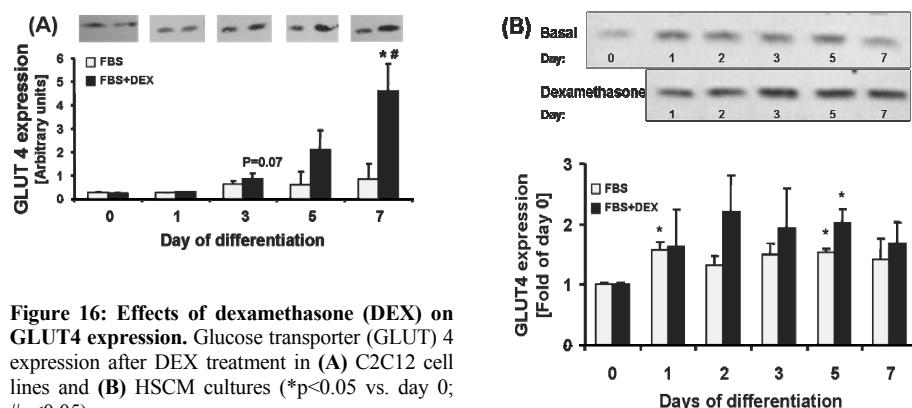


Figure 16: Effects of dexamethasone (DEX) on GLUT4 expression. Glucose transporter (GLUT) 4 expression after DEX treatment in (A) C2C12 cell lines and (B) HSCM cultures (* $p<0.05$ vs. day 0; # $p<0.05$).

HSMC and C2C12 cells were exposed to 1 μ M DEX and protein expression of GLUT4 during differentiation was assessed by Western blot analysis. In C2C12 cells, GLUT4 protein expression increased significantly at day 5 of the differentiation process, as compared to cells studied using standard differentiation media (Figure 16 A). However, even though GLUT4 expression was significantly increased at day 5, when treating HSMC with DEX, a 12% increased was noted ($p<0.05$) (Figure 16B). There was a trend towards increased insulin-stimulated (120 nM) glucose uptake, in cells supplemented with 37% DEX versus 22% in untreated cells ($p=0.13$) (Summarised in Figure 19). Thus, the upregulation of GLUT4 expression by DEX is not sufficient to significantly increase insulin-stimulated glucose uptake. However, it is possible the dexamethasone treatment may directly reduce insulin signalling, as has been reported for glucocorticoids [266].

FBS substitution with platelet lysate (Plysate)

FBS is a necessary supplementation to culture medium, providing essential nutrients and hormones. FBS has ethical and scientific drawbacks [267, 268]. The ethical issues stem from the collection procedure; that is from foetal serum harvested

from unborn, yet living, calves. The scientific challenge lies in its varying (non-defined) composition and quality (**Table 6**). Furthermore it is also an expensive supplement. Naturally, a substitute that addresses the ethical, scientific and economical concerns would be welcome. One possible substitute currently under development is platelet lysate (Plysate), which is harvested from platelets extracted from blood obtained from slaughterhouses [269, 270]. Plysate has been successfully used in various cell lines [271]. We investigated the effects of platelet supplementation on the metabolism, morphology and molecular properties of the HSMC model [272].

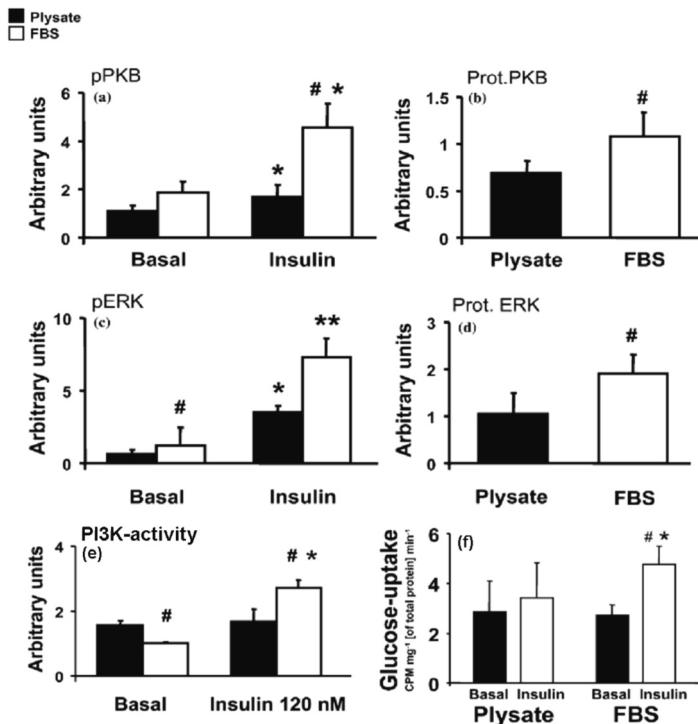


Figure 17: Effects of Plysate on expression and metabolic properties of HSMC. (A) PKB phosphorylation, (B) PKB expression and (C) ERK1/2 phosphorylation (D) ERK1/2expression (E) PI3-kinase activity and (F) Glucose uptake in HSMC propagated in medium supplemented with either Plysate or FBS. Insulin stimulation was 120 nM, 30 min. (**p<0.01 vs. basal; *p<0.05 vs. basal; #p<0.05 Plysate vs. FBS). Figures reproduced with permission [272].

Cells were grown in 20% Plysate-containing growth medium until 90% confluence. Cells were then differentiated as described in "Experimental Procedures", with DMEM containing 2% Plysate instead of FBS. Insulin-induced PI3-Kinase activity was significantly reduced in cells cultured using Plysate, while basal activity was increased (p<0.05)(**Figure 17E**). This was concomitant with a loss of insulin-stimulated glucose uptake (**Figure 17F**). Insulin-stimulated (120 nM) glucose uptake was 1.2 fold and (NS). In FBS treated cells (standard protocol) insulin-stimulated glucose uptake was increased 1.9 fold compared to basal (p<0.05). Furthermore, cells grown with Plysate had reduced expression and insulin-stimulated phosphorylation of

PKB (**Figure 17 A and B**) ($p<0.05$). Similarly ERK1/2 MAPK expression (**Figure 17D**) and phosphorylation (**Figure 17C**) was significantly reduced ($p<0.05$). ERK1/2 MAPK is crucial for myoblast growth [273]. Interestingly, Plysate was associated with an approximately 30% reduction in the proliferation of the HSMC (**Figure 18A**). ERK1/2 has also been described as indispensable for differentiation of myoblasts into myotubes [274]. Since Plysate supplementation also resulted in incomplete differentiation and reduced cell size, as compared to cells grown with FBS supplement (**Figure 18B**), reduced ERK1/2 might be a key explanation for the suboptimal growth noticed in cells grown in Plysate supplemented media.

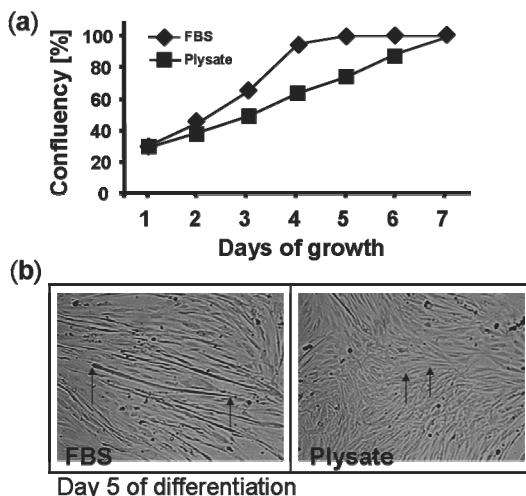


Figure 18: Effects of Plysate on growth and morphology of HSMC. (A) Growth rate of myoblasts in medium supplemented with FBS or Plysate (content 20%). (B) Microscopy capture of HSMC morphology after 5 days of differentiation (2 days at 4% serum or FBS-treated cells are shown on the left and Plysate treated cells on the right. Arrows indicate the ends of one myotube for size comparison. Figures reproduced with permission [272].

We concluded that Plysate was not a suitable substitute for FBS supplementation when culturing HSMC for insulin signalling and metabolic studies. Either a lack of necessary components, or a direct toxic effect of the Plysate, could explain our results. Although satellite cells from muscle of various organisms have been utilised extensively for cell culture, little is known about the actual factors needed for the process of growth and differentiation [275]. Thus, key factors promoting differentiation may be absent from the Plysate-conditioned media. Platelet derived growth factors (PDGF) are the major components of Plysate [271] and this may contribute to the compromised phenotype.

Although the effects of PDGF on cell-proliferation varies depending on species, cell type, method of stimulation, and level of receptor expression (reviewed in [276]), PDGF is known to have differential effects on proliferation in primary human cells in culture, with measurable increases in growth in some, but not all cell-types [277]. PDGF acts on a variety of proteins involved in cell-repair and differentiation (reviewed in [276]). The orchestration of cell growth and differentiation processes initiated by PDGF may be counter-productive for myoblasts differentiation into myotubes.

Interestingly, platelet-derived growth factor receptor (PDGFr) signalling is down-regulated during muscle differentiation [278]. Moreover, growth factor signalling is impaired in differentiated cells, due to the withdrawal of growth factors or due to transcriptional down-regulation of their receptors [278]. Therefore the lack of differentiation in the Plysate-treated cells may occur in response to an abundance of PDGF. These results clearly show that Plysate is not suitable as a FBS substitute in our cell system.

Summary- development of optimal growth conditions for HSMC

The experiments investigating and validating the HSMC system highlighted the importance of growth conditions and medium composition on cell growth and metabolism (**Figure 19**).

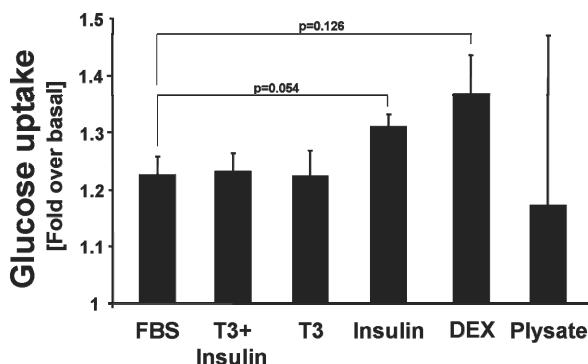


Figure 19: Summary of manipulations of culturing conditions on insulin stimulated glucose uptake. Results are expressed as increased fold over basal. (FBS) Conventional differentiation protocol with FBS. (T3+Insulin) FBS differentiation medium supplemented with thyroid hormone T3 and insulin. (T3) FBS differentiation medium supplemented with thyroid hormone T3. (Insulin) FBS differentiation medium supplemented with insulin. (DEX) Supplement with dexamethasone during differentiation. (Plysate) Instead of FBS the serum-substitute Plysate lysate (Plysate) was applied, in the same concentration and with the same time-course as with the FBS differentiation protocol (20% Plysate for growth until 90% confluence; 2% Plysate for differentiation for 5 days).

We did not see a clear correlation between GLUT4 protein expression and insulin-sensitivity. Previous results have indicated that manipulations leading to increased GLUT4 expression enhance insulin-mediated glucose uptake *in vivo* [279]. In cultured HSMC, increased glucose incorporation into glycogen has been shown to be associated with increased GLUT4 expression [280], suggesting a higher glucose influx. Although DEX-induced expression of GLUT4 resulted in a trend towards improved insulin-mediated glucose uptake, this was not significant. Supplementation with T3/insulin enhanced differentiation, as indicated by morphological analysis and changes in protein expression, including increased GLUT4 expression. However, this did not lead to increased insulin-mediated glucose uptake.

Interestingly, insulin supplementation, in the absence of T3, during differentiation resulted in increased insulin-mediated glucose uptake. Understanding the exact underlying mechanisms of glucose uptake in HSMC requires further investigation of GLUT4 expression, and interaction with other signalling molecules.

NUCLEAR RECEPTOR AND CO-FACTOR EXPRESSION IN SKELETAL MUSCLE

Correlation of gene expression patterns with skeletal muscle phenotype

Human skeletal muscle is composed of mixture of fast and slow fibre-types, with considerable inter-individual variation in the relative proportion of the different fibre types. Metabolic and functional properties (i.e. speed of contraction) vary between fibre types and there are also possible variations within a fibre-type category [281, 282].

Recent evidence from rodent studies has highlighted different molecules which are implicated in the regulation of the skeletal muscle fibre-type and metabolic profile. Various candidate genes have been proposed to play a role in the regulation of oxidative fibre-type expression. Transgenic expression of activated PPAR δ increases the proportion of type I fibres in mice [99, 125].

Skeletal muscle from transgenic mice specifically overexpressing PPAR α exhibit a range of metabolic changes, and PPAR α appears to regulate expression of muscle specific markers including MEF2A [283].

Results obtained in mice with transgenic overexpression of the PGC-1 α reveal increased proportion of type IIA and type I skeletal muscle fibres [225], and PGC-1 α has been proposed as a master regulator of type I muscle fibres. PGC-1 β is a recently cloned homologue of PGC-1 α [284-286], although a clear role for PGC-1 β in fibre-type regulation remains to be established.

The expression and activity of the Ca²⁺ sensitive enzyme calcineurin has been implicated as playing an important role in fibre-type transformation in animals and humans [287]. Given the substantial differences between rodent and human skeletal muscle [288], in particular as regards skeletal muscle adaptation and fibre -type transformation, it is important to evaluate results in human material.

In order to address this issue we have determined the expression of PPAR δ , as well as PPAR α , PGC-1 α , PGC-1 β , calcineurin A α and - β and MEF2 in three separate groups of subjects (**PAPER I**)

Oxidative phenotype correlates with type I muscle fibres

Skeletal muscle biopsies were obtained from normally active subjects, elite athletes (cyclists) and spinal cord injured subjects. These groups had significant differences in their oxidative fibre type composition and oxidative capacity ($\dot{V}O_{2\max}$) (**PAPER I; Table 1**). Cyclists had an increase, and spinal cord injured subjects had a decrease in the proportion of type I fibres and an increase in the proportion of mATPase type IIB fibres, as compared to the normally active subjects and cyclists.

The proportion of type I fibres also correlated with NADH dehydrogenase staining indicating differences in metabolic potential, i.e. oxidative capacity between the three groups (**PAPER I; Fig 1**)

Our hypothesis was that the physiological and pathological variations in skeletal muscle fibre type and metabolism represented by different modes of activity or inactivity would be coupled to mRNA expression of genes implicated in controlling skeletal muscle fibre type transformation and metabolism from rodent studies.

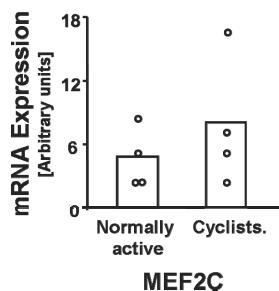
EXPRESSION DIFFERENCES OF KEY-GENES IN OXIDATIVE MUSCLE-FIBRE PHENOTYPE

Expression of MEF2C, Calcineurin, PPAR and PGC-1

Calcineurin A α and calcineurin A β mRNA expression was similar between the cyclists, normally active subjects and spinal cord injured subjects (**PAPER I; Fig 2**). Calcineurin A α expression was slightly increased in spinal cord injured; compared to normally active subjects, (NS). Previous reports in rodents suggest that calcineurin activity, rather than total expression, may drive fibre type transformation [289, 290]. Interestingly exercise may lead to a transient increase in calcineurin A α and β expression in rodent skeletal muscle [291], while in human subjects performing one-legged exercise, a transient increase is noted in the non-exercising leg but not in the exercised leg [136].

Recent evidence suggests, that calcineurin does not mediate exercise-induced changes in skeletal muscle mitochondrial content or GLUT4 in adult differentiated muscle [292]. Thus calcineurin may serve to regulate muscle metabolic profile when activated during development, but may be less important in the adaptation to external stimuli, such as exercise or denervation. Our results suggest calcineurin mRNA expression is unrelated to fibre type composition in humans. In support of this, expression of MEF2C, a downstream target of calcineurin, was similar in normally active subjects and cyclists (**Figure 20**).

Figure 20: MEF2C expression in normally active subjects and elite cyclists. Bars represent the mean of each group with circles showing individual measurements of expression.



Expression of the transcriptional co-activator PGC-1 α was 1.9 fold ($p<0.05$) higher in cyclists, as compared to normally active subjects (**PAPER I; Fig 4**). In spinal cord injured subjects, mRNA expression of PGC-1 α was reduced 74 % ($p<0.01$), as compared to normally active subjects. Expression of the related co-activator PGC-1 β was 4.1 fold ($p<0.05$) higher in cyclists, as compared to normally active subjects (**PAPER I; Fig 4**. In spinal cord injured subjects, mRNA expression of PGC-1 β was reduced 73 % ($p<0.05$), as compared to normally active subjects).

Given the substantial differences between rodent and human skeletal muscle in regard to homogeneity, regulation and degree of skeletal muscle fibre type transformation [288, 293, 294, 295], PGC-1 α may also be important in regulation of human fibre type characteristics. Although overexpression of PGC-1 α in rodents increases type I muscle fibre content, targeted deletion of PGC-1 α does not reduce the proportion of type I muscle. Despite the role of PGC-1 α to induce type I and IIA fibre formation when expressed ectopically, PGC-1 α is not absolutely necessary for type I fibre formation in rodents [296].

PGC-1 β gene expression is decreased in skeletal muscle from patients with type 2 diabetes mellitus [297], and in aged healthy subjects [298]. Whether the observation that PGC-1 β mRNA expression is increased more than four fold in skeletal muscle from elite athletes compared to normally active subjects is a reflection of muscle fibre type composition, or a direct response to the exercise training remains to be determined.

The expression profile of the nuclear receptors PPAR α and PPAR δ was similar to that of PGC-1 α and β (**PAPER I; Fig 3**). Thus mRNA expression of PPAR α ($p<0.05$) and PPAR δ ($p<0.05$) was 2-fold higher in cyclists, as compared to normally active subjects. In spinal cord injured subjects, mRNA expression of PPAR α and PPAR δ was reduced to 15% and 35% ($p<0.01$) of the expression-level of normally active subjects (**PAPER I; Fig 3**).

To date the effect of exercise on expression of PPAR α or PPAR δ in humans is incompletely resolved. An acute three hour exercise bout is associated with an increase in PPAR α and PPAR δ mRNA expression in skeletal muscle [122], whereas endurance training is associated with an isoform-specific increase in PPAR α [117, 299], but not PPAR δ [117]. PPAR α and PPAR δ mRNA expression was increased in cyclists versus normally active subjects providing further evidence that in humans, PPAR α and PPAR δ mRNA are related to muscle activity and/or fibre type specific gene-expression.

Expression of PPAR and PGC-1 correlates with an oxidative muscle phenotype

In order to further test the hypothesis that PPAR α/δ and PGC1 α/β expression is associated with an oxidative slow-twitch muscle fibre phenotype, individual gene expression data was correlated with individual fibre type composition.

Percentage of type I muscle fibres was positively correlated with mRNA expression of PPAR α ($r=0.66$; $p<0.01$), PPAR δ ($r=0.61$; $p<0.01$) and PGC-1 α ($r=0.86$; $p<0.0001$) (**PAPER I; Figure 5 A, B and C**). mATPase Type IIB muscle fibre type content was negatively correlated with mRNA expression of PPAR α ($r=-0.70$; $p<0.001$) (**Figure 21A**), PPAR δ expression ($r=-0.65$; $p<0.01$) (**Figure 21B**) and PGC-1 α ($r=-0.80$; $p<0.0001$) **PAPER I; Figure 5D**.

Type IIA muscle fibre type content was negatively correlated with mRNA expression of PGC1 α ($r=-0.57$; $p<0.01$) (**Figure 21C**). Additionally, PGC-1 α mRNA expression in the combined groups of cyclists and normally active subjects was positively correlated with type I fibres ($r=0.54$; $p=0.036$) (**Figure 21D**). Other correlations in the combined group of able-bodied subjects (cyclists and normally active subjects) were not significant. PGC-1 β expression was not correlated with individual fibre type compositions.

Although ATPase staining has been shown to correlate well with oxidative capacity in type I fibres, this is not always the case for type IIA fibres [300]. A subgroup of type IIA fibres can have the same high oxidative capacity as Type I fibres, as determined by staining for Succinate Dehydrogenase (SDH), a marker of oxidative activity and Glycerol-3-Phosphate Dehydrogenase (GPDH), a marker of glycolytic activity. Hence the actual amount of type I fibres does not represent all highly oxidative fibres, since also a subgroup of IIA fibres is highly oxidative.

The NADH staining procedure we performed adequately estimates the oxidative capacity in type I fibres, but may underestimate the amount of other oxidative fibres. In light of these findings, the correlation analysis could have been improved if used additional staining methods were employed to assess oxidative capacity. SDH-staining would possibly have offered a more accurate measurement of oxidative fibres. However, this remains speculative, since the fibre type definition describes a continuum, rather than sharply distinguished groups, which makes fibre type determination a difficult task.

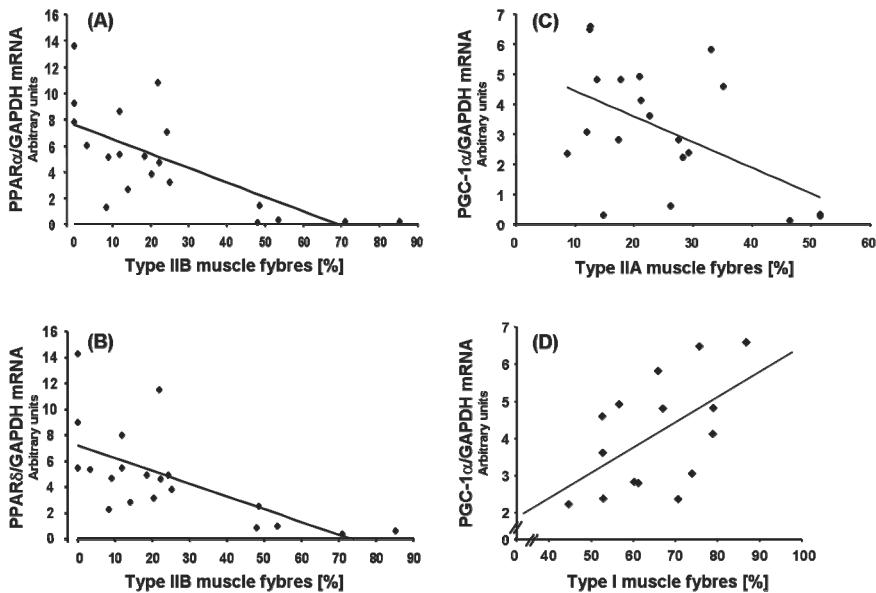


Figure 21: Correlations between expression of PPAR α or δ or PGC-1 α and skeletal muscle fibre type. Individual subject data is represented by diamond-shaped points and mathematical correlation between expression and fibre type for the combined groups is indicated as a trend line. (A) PPAR α expression of normally active, spinal cord injured individuals (SCI) and elite cyclists versus type IIB muscle fibres. (B) PPAR δ expression of normally active, SCI individuals and elite cyclists versus type IIB muscle fibres. (C) PGC-1 α expression normally active, SCI individuals and elite cyclists versus type IIA muscle fibres. (D) PGC-1 α expression of normally active and elite cyclists versus type I muscle fibres. mRNA expression was standardised against GAPDH expression.

The groups studied represent physiological and pathological extremes in fibre-type variation, exercise training, and skeletal muscle oxidative capacity, respectively. The mRNA differences observed between the groups may be related to fibre type composition, degree of skeletal muscle physical activity, or both. PPAR δ expression is increased in response to exercise and changes in metabolism [122, 301]. However, exercise-induced changes in expression are usually evaluated under intense and tightly controlled training conditions (see chapter “Background”). Furthermore, such studies are performed in young healthy volunteers. In the next study we address the question of exercise-mediated regulation of expression changes using a different experimental set-up and asked how a self-supervised low-intensity exercise program in T2DM patients may impact the range of metabolically relevant genes.

EXERCISE EFFECTS IN THE TREATMENT OF T2DM

Exercise versus pharmacological treatment

The Diabetes Prevention Program (DPP) provided clinically-based evidence that intensive lifestyle intervention profoundly reduced the risk of developing T2DM [192]. While anti-diabetic therapy can delay the development of type 2 diabetes, the DPP revealed that conventional therapy with diet and exercise was more efficacious in preventing T2DM progression and maintaining blood glucose control [192]. Beneficial effects of exercise on insulin action may be noted even in elderly subjects, who are generally characterized by glucose intolerance and insulin resistance [302].

The molecular mechanism for the beneficial effects of exercise on glucose homeostasis remains incompletely resolved. Additionally, previous studies performed to evaluate the effects of exercise in T2DM patients required intense life-style changes with stringent control of the exercise program, as well as caloric and fat-intake throughout the study [303, 304]. These conditions are a challenge to create in outpatient clinical practice and often impracticable for most practitioners. A self-supervised, low intensity exercise regime of approximately 150 min walking was therefore developed and applied for patients with T2DM (**PAPER II**).

Low-intensity exercise leads to improvements of clinical parameters

The self-supervised low-intensity aerobic exercise in patients with type 2 diabetes improved several clinical parameters including systolic and diastolic blood pressure, total plasma cholesterol and body mass index (BMI), as previously reported [305]. Insights regarding the effects of regular exercise training on gene expression in skeletal muscle from patients with T2DM are lacking. Our aim was therefore to determine how changes in skeletal muscle gene expression correlate with clinically measurable parameters (**Figure 22**).

Muscle biopsy material was obtained before and after training from a sub-group of the participants described by Fritz et al [233]. Age and BMI were similar between the subjects in this sub-group (**PAPER II; Table 1**). Physical fitness, as assessed by $\text{VO}_{2\text{max}}$ was similar between the groups and was unchanged during the study. This level of physical activity would not be expected to lead to a measurable change in $\text{VO}_{2\text{max}}$. Metabolic control, as assessed by HbA1c was good in both groups. Fasting blood glucose was similar between the groups and unchanged during the study. Medication was unchanged during the study.

The physically active group was further divided into “exercise responders” and “non-responders” based on changes in insulin sensitivity, including decreased plasma insulin levels and HOMA levels, and blood pressure (**PAPER II; Table 1 and 2**). Blood pressure has been described as a reliable indicator of response to low-intensity exercise [306, 307].

There were no differences in clinical parameters at the outset of the study between these groups, except that the non-responders were significantly younger. Interestingly, the non-responders showed a tendency for a greater increase in amount of physical activity during the study period, than reported by the responders (**PAPER II; Table 1**).

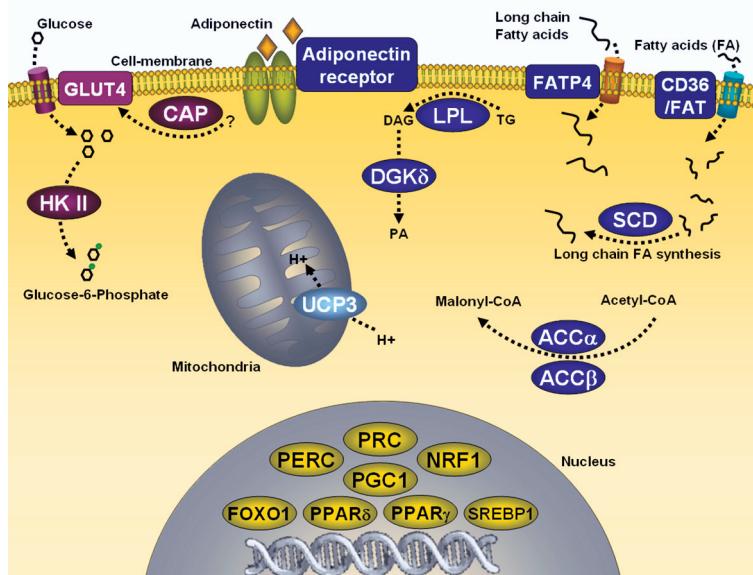


Figure 22: Genes analysed in skeletal muscle before and after 4 month exercise training. Genes investigated were the Glucose transporter protein GLUT4; CAP, a protein thought to be involved in GLUT4 translocation in adipocytes and Hexokinase (HK) II, the rate-limiting protein for glucose uptake. Lipid metabolism genes: Adiponectin receptor; Fatty acid transporter proteins FATP4 and CD36/FAT; Lipoprotein Lipase (LPL), converting triglycerides to diacylglycerol; Diacylglycerol Kinase (DGK) δ , a membrane protein converting diacylglycerol to phosphatidic acid; Stearoyl-CoA delta-9-Desaturase (SCD) is involved in long-chain fatty acid synthesis and Acetyl-CoA Carboxylase (ACC) α and β are proteins controlling fatty acid transport into mitochondria. Uncoupling Protein (UCP) 3 is a marker of mitochondrial activity. Proteins regulating transcription: Forkhead box O1A (FOXO), Nuclear Respiratory Factor (NRF)-1, Peroxisome Proliferator-Activated Receptor (PPAR) γ , PPAR δ , PPAR γ coactivator (PGC)-1 α , PGC-1-related estrogen receptor alpha coactivator or PGC1 β (PERC), PPAR- γ coactivator 1 related protein (KIAA0595 protein) (PRC) and Sterol Regulatory Element Binding Protein (SREBP) 1. Colour code: Genes involved in glucose metabolism are in purple, genes involved in lipid metabolism are blue, and transcription factors are yellow.

Low-intensity exercise changes gene-expression

mRNA expression

mRNA expression of adiponectin receptor, Cbl-Associate Protein (CAP), Fatty Acid Transporter (FAT) 4, Forkhead box (FOX) O1A, Hexokinase (HK) 2, Lipoprotein Lipase (LPL), PPAR γ , PGC-1 α and - β isoforms, PGC-1 related co-activator (PRC), Stearoyl-CoA Desaturase (SCD) and Sterol Regulatory Element Binding Protein (or transcription factor) (SREBP/SREBF) 1, were unaltered between exercise responders and non-responders. In the exercise responders, increased physical activity was associated with a tendency for increased mRNA expression of Diacyl Glycerol Kinase (DGK) δ ($p=0.16$), Uncoupling Protein (UCP)3 ($p = 0.16$), PPAR δ ($p=0.13$), Nuclear Respiration Factor (NRF)-1 ($p=0.14$) (**PAPER II; Figure 1A**). However, these findings were not significant and we investigated changes on the protein level as well, focusing on the genes that showed a trend for an increase after exercise. mRNA

expression of the investigated targets was unaltered in exercise non-responders. However, mRNA expression of Acetyl-CoA Carboxylase (ACC)- α ($p=0.08$) and - β ($p=0.12$), Collagen type I receptor/ Fatty Acid Translocase (CD36/FAT) ($p=0.02$) and Glucose Transporter (GLUT) 4 ($p<0.01$) tended to decrease in exercise non-responders, but remained unchanged in control and exercise responders (Figure 23).

GLUT4 and the CD36/FAT, control glucose and fatty acid transport across the cell membrane, respectively. Alterations in the expression and function of GLUT4 and CD36/FAT can contribute to impaired glucose homeostasis in T2DM. ACC is important for regulating the concentration of malonyl-CoA, thereby controlling Carnitine Palmitoyl Transferase (CPT)-1 and the transfer of long-chain fatty acyl CoA into the mitochondria [308]. This signature may provide a molecular mechanism for the failure of these subjects to respond to exercise training. The reduction in CD36/FAT may hinder uptake and oxidation of lipids by mitochondria [309, 310], thereby preventing positive metabolic adaptations to exercise training in the non-responders. Likewise, the reduction in GLUT4 mRNA expression in the non-responders may could prevent improvements in glucose uptake, an otherwise characteristic exercise response [311-313].

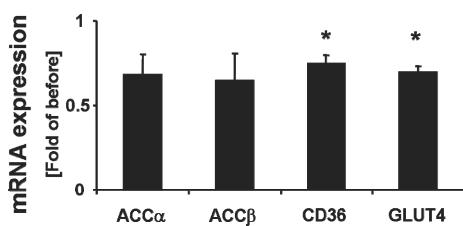


Figure 23: mRNA expression in exercise non-responders. mRNA expression was determined using quantitative real-time PCR. Four selected genes: Acetyl-CoA Carboxylase (ACC) α and β , Fatty Acid Transporter (CD36/FAT) and Glucose Transporter (GLUT) 4
* $p<0.05$.

Protein expression

On the basis of the changes in mRNA, protein expression of DGK δ UCP3, PPAR δ and ACC was assessed. Protein expression of these targets paralleled the mRNA results for the exercise responders (PAPER II; Figure 1B). Protein expression of DGK δ tended to increase, and UCP3 and PPAR δ were significantly increased ($p<0.05$) in the exercise responders (PAPER II; Figure 1 B and C). Consistent with the mRNA analysis, ACC expression was unchanged in the exercise responders.

The improved insulin sensitivity in the subjects responding to increased physical activity, as evidenced by decreased plasma insulin levels and HOMA index, was coincident with increased skeletal muscle protein expression of PPAR δ and UCP3. Furthermore mRNA expression of DGK δ , UCP3, NRF-1 and PPAR δ , genes implicated glucose and lipid metabolism and mitochondrial function, tended to increase.

Recent clinical studies provide evidence that mitochondrial dysfunction plays a role in the development of insulin resistance in the elderly [314] and in insulin-resistant offspring of patients with T2DM [315]. Thus, several genes involved in mitochondrial function and biogenesis have been implicated in regulation of insulin sensitivity.

UCP3, NRF-1 and PPAR δ are markers and initiators of mitochondrial biogenesis, which could also enhance the lipid oxidation capacity in skeletal muscle. UCP3 has been suggested to play an important role in lipid metabolism [316] and is a marker of mitochondria [317]. Importantly, skeletal muscle UCP3 expression is down-regulated

in T2DM [318, 319] and up-regulated after exercise training [317]. NRF-1 expression has previously been shown to increase with aerobic exercise [320]. Moreover, gene expression profiling of skeletal muscle from Mexican American reveals T2DM is associated with reduced expression of multiple NRF-1-dependent genes encoding key enzymes in oxidative metabolism and mitochondrial function [321].

DGK δ is a catalyst for the conversion of diacylglycerol (DAG) into phosphatidic acid (PA) (Reviewed in [322] and changes in expression of this gene are of relevance for insulin action because accumulation of DAG has been associated with reduced insulin sensitivity and impaired glucose-uptake in skeletal muscle [323]. Thus, increased DGK δ expression and protein content after exercise training may possibly reduce DAG content, thereby improving insulin sensitivity in skeletal muscle.

Interestingly, a recent microarray analysis of gene expression profiles performed in *vastus lateralis* muscle biopsies obtained before and after 20 weeks of exercise from individuals participating in the HERITAGE Family Study provides evidence for increased expression of DGK δ in subjects who had enhanced insulin sensitivity after exercise training, i.e. an exercise-responsive group [324]. Collectively, these studies provide evidence to suggest increased skeletal muscle DGK δ expression may be a marker of improved muscle insulin action.

The increased expression of PPAR δ following exercise suggests PPAR δ is a key-player in the mediation of the observed improvements in insulin sensitivity. Previous studies suggested that increased PPAR δ activity improves insulin sensitivity by increasing skeletal muscle oxidation [97]. We hypothesized that PPAR δ may also exhibit direct effects on skeletal muscle glucose metabolism. Our next aim was therefore to investigate PPAR δ effects on glucose metabolism, insulin sensitivity, lipid-metabolism and associated gene expression. These effects were studied in HSMC using a pharmacological activator of PPAR δ ; GW501516.

ACTIVATION OF PPAR-DELTA WITH SPECIFIC AGONIST GW501516

Effect of PPAR δ activation on lipid-metabolism

GW501516 stimulated fatty acid uptake in HSMC requires PPAR δ

To study the effects of PPAR δ activation in human muscle, differentiated HSCM cells were exposed to 100 nM GW501516 for 60 min or over night (16-18 h). Palmitate uptake, as assessed by intracellular accumulation of ^{14}C -labelled palmitate, was significantly increased 37% in response to 18 h treatment with GW501516 (**PAPER IV; Fig 1A**). In contrast, GW501516 did not increase palmitate uptake after 60 min, suggesting that transcriptional effects are required. This data demonstrates that activation of PPAR δ using a synthetic activator increases accumulation of intracellular lipids in HSMC.

Effects on GW501516-mediated fatty acid uptake following siRNA -mediated reduction of AMPK, PPAR α and PPAR δ

In order to determine if PPAR δ is required for GW501516 effects on fatty acid uptake, the expression PPAR δ was specifically reduced using siRNA technology. As an additional control, siRNA against PPAR α was also employed. mRNA expression was

reduced 71% ($p<0.001$) and 78% ($p<0.001$) for PPAR α and PPAR δ , respectively as compared to random siRNA control constructs (**PAPER IV**; **Fig 2**). Interestingly, the siRNA-mediated reduction of PPAR δ abolished palmitate uptake in response to GW501516. The reduction of PPAR α expression did not affect GW501516-mediated palmitate uptake. Furthermore, the siRNA-mediated reduction of AMPK $\alpha 2$ (65%, $p<0.01$; **Fig 2**) was without effect on the GW501516-mediated palmitate uptake (**PAPER IV**; **Fig 1B**). Hence AMPK or PPAR α are not required for the mediation of the GW501516 effect on lipid uptake.

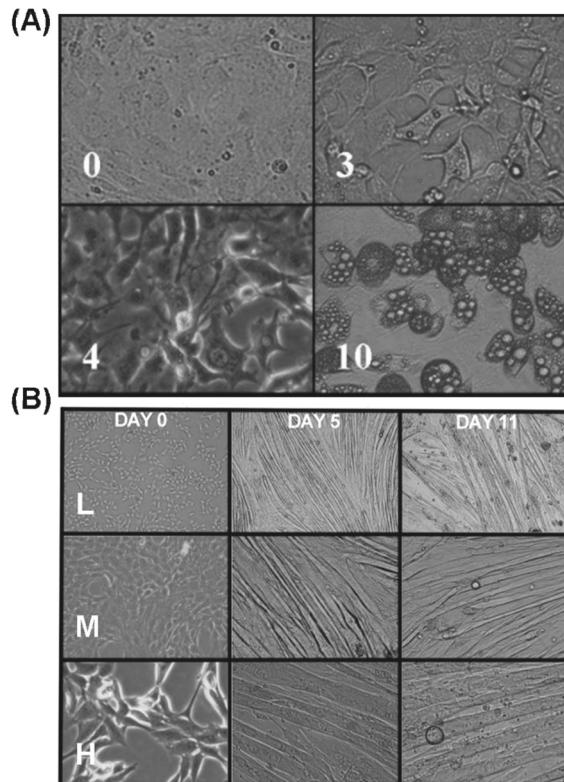


Figure 24: Differentiation of 3T3-L1 fibroblasts and C2C12 mouse myoblasts. (A) 3T3-L1 were differentiated into adipocyte-like cells with fat droplets for 10 days, following the protocol described in Materials and Methods. Microscope captures taken at day 0, 3, 4 and 10 are shown (B) C2C12 were differentiated as described. Microscope images taken at day 0, 5 and 11 are shown for low (L), medium (M) and high (H) resolution settings.

GW501516-induced increase in palmitate oxidation requires PPAR δ and AMPK

In line with the results on palmitate uptake, incubation of differentiated human myotubes with GW501516 over-night resulted in a 50% increase in palmitate oxidation, while no effect was observed after 60 min GW501516 incubation (**PAPER IV**; **Fig 1C**). siRNA mediated reduction of PPAR δ prevented the GW501516-induced stimulation of palmitate oxidation (**PAPER IV**; **Fig. 1D**).

Similar to results for lipid uptake, the siRNA-mediated reduction of PPAR α did not blunt this effect, suggesting that GW501516 is a specific activator of PPAR δ . In contrast to the results for lipid uptake, a reduction in AMPK $\alpha 1/\alpha 2$ expression reduced fatty acid oxidation (**PAPER IV; Fig. 1D**). This response is likely to reflect the key role of ACC in regulating fatty acid oxidation, since ACC phosphorylation was reduced (**PAPER IV; Fig. 4B**) as a result of siRNA-mediated reduction in AMPK and AMPK phosphorylation (**PAPER IV; Fig. 4A**).

Our results suggest that some aspects of PPAR δ -regulated lipid metabolism require functional AMPK and/or ACC signalling.

Effects of GW501516 on glucose metabolism

Insulin sensitivity increases following PPAR δ activation

To assess whether PPAR δ agonists alter insulin-stimulated glucose uptake in cultured human skeletal muscle cells, cultures were incubated in the absence or presence of 10 nM GW501516 for 6 h, followed by addition of [3 H] 2-deoxyglucose for a further 10 min. Insulin exposure (1 and 120 nM for 1 h) led to a modest 1.3- and 1.5-fold increase in glucose transport ($p<0.01$) and a combined exposure of myotubes to GW501516 and insulin resulted in a partial additive effect on glucose uptake (**PAPER III; Figure 2B**). Interestingly, myotubes treated with 10 nM GW501516 alone for 6 h, over-night or 4 days increased [3 H] 2-deoxyglucose uptake ($p<0.01$) (**PAPER III; Figure 2A**).

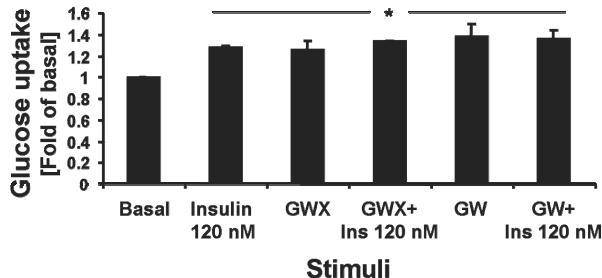


Figure 25: Effect of GW501516 or GW0742 on glucose uptake in mouse C2C12 myotubes. Glucose uptake was measured in differentiated C2C12, following insulin (120 nM), GW501516 (100 nM) or GW0742 (GWX; 100 nM) stimulation or a combination of the GW/GWX stimulus with insulin (* $p<0.05$ vs. basal).

The limited magnitude of glucose uptake HSMC cells makes it difficult to determine if the increased glucose uptake in response to insulin and GW501516 is an additive, or an insulin sensitizing effect. We therefore performed similar experiments in a more insulin sensitive cell system, the murine fibroblast cell-line 3T3-L1, differentiated into adipocyte like cells (**Figure 24A**).

3T3-L1 adipocyte cultures were incubated in the absence or presence of 10 nM GW501516 for 6 h, followed by addition of [3 H] 2-deoxyglucose for a further 10 min. Incubation of 3T3-L1 adipocytes with GW501516 did not alter basal glucose uptake (**PAPER III; Figure 1**). Submaximal (1 nM) and maximal (120 nM) insulin-stimulated glucose uptake was enhanced ($p<0.01$).

This result indicates that the effect of GW501516 on PPAR δ results in enhanced insulin sensitivity. Furthermore, this effect is not restricted to skeletal muscle, but also evident in adipocyte cell lines. Insulin-sensitising effects of PPAR δ activation have also been noted in both primate [95] and rodent [96].

In contrast to HSMC, in 3T3L1 adipocytes, GW501516 did not alter basal glucose uptake. To test if this effect was restricted to cells of human origin, murine C2C12 muscle cells were incubated with GW501516. We confirmed our results using a different PPAR δ activator, GW0742. Differentiated C2C12 myotubes (**Figure 24B**) were exposed to either 10 nM GW501516 or 10 nM GW0742 (GWX) over night hours. Exposure of cells to either agonist resulted in a modest but significant increase in glucose uptake (1.4 fold p<0.05 and 1.3 fold p<0.05 over basal, respectively **PAPER III; Figure 2C**). Thus, PPAR δ agonists enhance glucose uptake in cultured primary human skeletal muscle, as well as mouse skeletal muscle cell lines.

Unexpectedly, neither GW501516 nor GW0742 (GWX) exhibited any insulin sensitising effect in C2C12 myotubes. Insulin stimulation of glucose uptake after 6 h pre-incubation with GW501516 or GW0742 (GWX) lead to a significant ~30% increase of glucose uptake in all conditions (n=3) (**Figure 25**).

GW501516 has no effect on glucose incorporation into glycogen

In order to investigate whether glucose uptake is accompanied by increased glycogen synthesis, we determined if glucose incorporation into glycogen was increased. Glucose incorporation into glycogen was measured by determination of ^{14}C glycogen content in differentiated human myotubes incubated in the presence or absence of insulin (1 nM or 120 nM) and with or without 100 nM GW501516. 120nM insulin increases glycogen synthesis (75%; p<0.01) as compared to control, with a trend noted at 1 nM insulin (**Figure 26A**).

GW501516 treatment over-night did not alter basal glycogen synthesis, and tended to reduce insulin-stimulated glycogen synthesis (12%; p=0.055). Similarly, in C2C12 mouse myotubes, GW501516 did not increase basal glucose incorporation into glycogen, and a similar trend for increased insulin-stimulated glycogen synthesis was noted (**Figure 26B**).

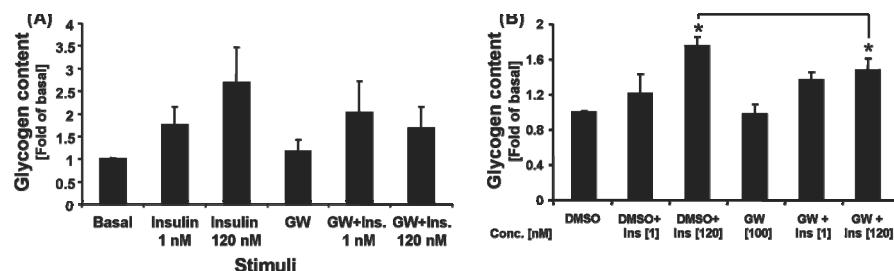


Figure 26: Effect of GW501516 on glucose incorporation into glycogen in C2C12 and HSMC. (A) Glucose incorporation into glycogen in differentiated mouse C2C12 was measured following stimulation with either insulin (1 nM/120 nM) or GW501516 (100 nM), or a combination of both stimuli. (B) A similar experiments performed in HSMC (*p<0.05 vs. basal). The vehicle for GW501516 or GW0742 in all experiments was Dimethyl sulfoxide (DMSO). DMSO was added to other conditions (basal, insulin).

Signalling events that lead to increased glucose uptake following GW501516 exposure

GW501516 effect on glucose uptake does not recruit the insulin signalling network

In an effort to resolve the signalling mechanism by which PPAR δ agonists increase glucose uptake, we determined phosphorylation of several signalling molecules implicated in the regulation of glucose metabolism. Insulin increased PKB phosphorylation 4.2 fold ($p<0.05$) (**PAPER III; Figure 4**). In contrast, GW501516 was without effect on either basal or insulin-stimulated PKB phosphorylation (**PAPER III; Figure 5**). These results exclude a role for PKB signalling in glucose transport in response to PPAR δ activation.

GW501516 leads to AMPK and MAP kinase phosphorylation

Treatment of differentiated human myotubes with GW501516 resulted in a 1.8 fold increase in expression of AMPK $\alpha 2$ (similar results were seen for AMPK $\alpha 1$, data not shown) and a 1.9 fold increased phosphorylation of AMPK ($p<0.05$). In contrast, insulin was without effect on either AMPK expression or phosphorylation (**PAPER III; Figure 4B and PAPER IV figure 4**). Insulin and cellular stress activate MAPK signalling (25, 26). Thus, we determined whether the PPAR δ agonist increased either ERK1/2 (**PAPER III; Figure 4C**) or p38 MAPK (**PAPER III; Figure 4A**) expression and phosphorylation. Similar to results for AMPK, exposure of myotubes to the PPAR δ agonist increased ERK1/2 expression (1.7 fold $p<0.05$) and phosphorylation (2.2 fold, $p<0.05$).

Expression of p38 MAPK increased 1.4 fold ($p<0.05$) and phosphorylation increased 1.2 fold, ($p<0.05$). However, ERK1/2 is unlikely to be involved in glucose uptake [325], as this signalling pathway has been implicated in gene regulatory responses in cultured myotubes [326]. p38 MAPK has been proposed to be a downstream target of AMPK and a required component for AICAR-mediated AMPK signalling to glucose uptake in Clone 9 cells [327]. We therefore used the MAPK inhibitor ERK1/2 and p38 MAPK inhibitors (50 μ M PD98059 or 10 μ M SB203580, respectively) in combination GW501516 in order to investigate the role of these MAP kinases.

Role of MAPK on PPAR δ -mediated glucose uptake in HSMC

The efficiency of the inhibitory effects of the MAPK inhibitors PD98059 and SB203580 was tested using insulin stimulation for ERK1/2 and mannitol for p38 MAPK (**Figure 27**).

Incubation of cells with the MEK inhibitor PD98059 was without effect on either insulin or GW501516 mediated glucose uptake (**PAPER III; Figure 6**). In contrast, pre-exposure of cells to the p38 MAP kinase inhibitor, SB203580 reduced insulin-stimulated glucose-uptake 67% ($p<0.05$), and similarly blunted (65% reduction) the GW501516 stimulated glucose uptake ($p<0.05$).

The additive effect of GW501516 and insulin (120 nM) on glucose uptake was also reduced after exposure to the p38 MAP kinase inhibitor ($p<0.05$). Thus, the p38 MAP kinase inhibitor attenuates insulin and PPAR δ agonist mediated glucose uptake. However, recent data using siRNA mediated reduction of p38 MAPK suggests that p38 MAPK is not involved in glucose uptake and that the inhibition of glucose uptake noted

with SB203580 is due to previously unrecognised inhibition of other kinases [41]. However, the target inhibited by SB203580 that is responsible for glucose uptake inhibition is unknown.

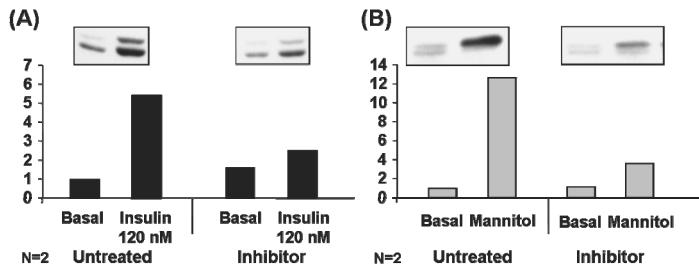


Figure 27: Effect of MAPK inhibitors PD98059 and SB203580. (A) HSMC were pre-incubated with PD98059 or vehicle and the effect of insulin stimulation on ERK1/2 phosphorylation assessed. (B) HSMC were pre-incubated with SB203580 or vehicle and effect of mannitol-induced osmotic stress on p38 MAPK phosphorylation assessed.

GW501516 mediated glucose uptake requires AMPK $\alpha 1$ and - $\alpha 2$ isoform expression

To address whether activation of AMPK is required for the GW501516 effect on glucose uptake we utilised siRNA-mediated downregulation of AMPK $\alpha 1$ and - $\alpha 2$. To demonstrate the specificity for the different PPARs we also applied siRNA directed against PPAR α and PPAR δ . Additionally, we investigated if acute stimulation with GW501516 resulted in increased phosphorylation of AMPK. Exposure of myotubes to 100 nM GW501516 for either 60 min or over-night resulted in a significant 2 fold and 2.3 fold in AMPK phosphorylation, respectively (**PAPER IV; Fig 4A**).

Furthermore, siRNA-mediated down regulation of AMPK $\alpha 1$ and - $\alpha 2$ blocked the GW501516 mediated increase in glucose uptake. siRNA against PPAR δ did not affect the GW501516-induced AMPK phosphorylation, or the glucose uptake, demonstrating that these effects are not mediated by PPAR δ . Similar results were obtained using siRNA directed against PPAR α . Thus we conclude that stimulation of glucose transport by GW501516 is a compound specific effect, mediated via AMPK, and not mediated via interaction with PPAR δ . Attention has recently been drawn to the ability of different synthetic PPAR compounds to induce mitochondrial dysfunction [328]. Thus direct, “non-receptor” effects have been described for TZD-activators of PPAR γ [113].

Mitochondrial uncoupling, resulting in increased AMP:ATP concentrations in the cell leads to activation of AMPK [113, 329]. GW501516, like other chemical compounds including TZDs [112, 330] or metformin [331], could exert some direct or indirect effects on mitochondrial machinery. Changes in the ATP:AMP ratio could activate AMPK and lead to acute mitochondrial changes. We therefore investigated if GW501516 treatment alters the ATP:AMP and ATP:ADP ratios.

ATP:AMP ratio changes account for AMPK activation

In order to further investigate the underlying mechanism of the PPAR δ -independent effect of GW501516 on AMPK phosphorylation, we measured adenine nucleotides concentrations by high pressure liquid chromatography (HPLC) in human skeletal myotubes to determine the cellular ATP:ADP and AMP:ATP ratio.

Following short-term exposure (30 min) to GW501516, ATP levels were significantly reduced (**Figure 28**) and ADP levels were increased in a dose-dependent manner compared to control cells exposed to the vehicle (data not shown). This results in a decrease in ATP:ADP ratio at all concentrations of PPAR δ agonist used (76% at 100 nM, 79% at 1 μ M and 73% at 10 μ M; $p<0.05$, **PAPER IV**; **Fig. 5A**).

In addition, while intracellular AMP concentrations were low and close to the detection threshold, a trend towards an increase in AMP levels and a decrease of ATP levels in presence of GW501516 was noted (**Figure 28**), leading to a concomitant increase in AMP:ATP ratio (**PAPER IV**; **Fig. 5B**).

Taken together, our results suggest that the increase in AMPK phosphorylation observed after GW501516 treatment is due to a decrease in cellular energy status of cultured human myotubes. The GW501516-induced decrease in ATP levels could be due to a specific inhibition of one or more complexes of the respiratory-chain, and/or to an effect on the ATP synthase system (complex V itself, adenine nucleotide translocator and/or inorganic phosphate transporter). Furthermore, an uncoupling effect of GW501516 on the mitochondrial oxidative phosphorylation is also possible, thereby altering the yield of ATP synthesis and leading to AMPK activation [332].

A mitochondrial short-term effect is supported by the fact that a strong AMPK phosphorylation was evidenced together with an ATP drop even following an acute incubation (60 min) with GW501516. Thus, the glucose uptake increase following GW501516 treatment appears compound-specific and PPAR δ independent, and is mediated via AMPK activation.

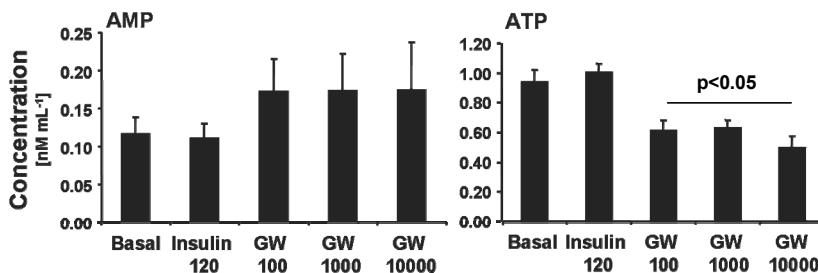


Figure 28: Effect of GW501516 incubation on AMP and ATP concentrations. HSMC were incubated with 100 nM, 1 μ M and 10 μ M GW501516 for 1 h and AMP and ATP contents determined using HPLC. As a negative control cells were incubated with 120 nM insulin.

However, AMPK may affect the ligand binding of PPARs via phosphorylation. Hence, the PPAR δ independent activation of AMPK might ultimately still impact on PPAR δ function, complicating the dissection of direct PPAR δ effects from AMPK induced indirect effects on PPARs.

The PPAR δ independent nature of glucose uptake following GW501516 is supported by the observation that 60 min of incubation with GW501516 had no effect on fatty acid metabolism. This further suggests that the GW501516 effect on glucose uptake is a mitochondrial effect. Interestingly, both PPAR α and PPAR γ agonists have

been shown to exhibit similar effects on mitochondria [333]. These compounds appear to interfere with complex I in the respiratory electron transport chain.

Supporting a role for PPAR δ in skeletal muscle glucose metabolism, there is evidence that *in vivo* glucose utilisation is increased in rodent models following GW501516 treatment [97]. Furthermore, human genetic analysis of PPAR δ SNPs highlights a role in the regulation of insulin sensitivity [127]. Furthermore, we observed that GW501516 increases insulin sensitivity in HSMC and 3T3-L1 adipocyte like cells. We attempted to investigate the extent of the role played by PPAR δ in mediating increased insulin sensitivity, using siRNA. However, the limited range of glucose uptake in HSMC makes it difficult to dissect to which extent this is due to direct effects of GW501516 on AMPK or PPAR δ .

Nutritional status affects GW501516 mediated glucose uptake

Previous reports using GW501516 in isolated rodent muscle indicated that the presence of fatty acids in the incubation media affected the GW501516 effects on glucose metabolism [330]. To investigate whether nutritional status of muscle cells influenced glucose uptake in response to GW501516, serum was removed from differentiated muscle myotubes for different time periods. Cells which were serum-starved over night showed an 18% ($p=0.03$) increase in GW501516-mediated glucose uptake while a 24 h starvation period resulted in a 33% ($p<0.01$) increase (Figure 29A).

In contrast, muscle cells which were not serum starved or starved for 8 h showed no increase in glucose uptake following treatment with 100 nM GW501516. Insulin stimulation (120 nM, 30 min) resulted in a 43% increase in glucose uptake as compared to baseline, with no effect of the length of serum withdrawal noted on the insulin mediated glucose uptake. This finding suggests that fatty acids present in the serum may influence the action of GW501516 on glucose uptake in our human skeletal muscle cell model. The effects of fatty acids on the GW501516 mediated mitochondrial uncoupling and/or AMPK phosphorylation remains to be determined.

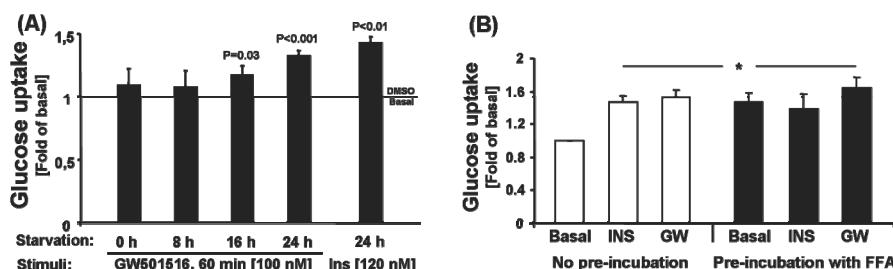


Figure 29: Effect of FBS withdrawal and fatty acid supplementation on GW501516 mediated glucose uptake. (A) Glucose uptake was measured in differentiated HSMC following different time intervals of FBS withdrawal (starvation) with GW501516 or insulin stimulation. Vehicle (DMSO) concentration was equalised for all conditions. (B) Glucose uptake in differentiated HSMC was measured following insulin (120 nM) or GW501516 (100 nM) stimulation. One set of cells were pre-incubated 2 h with oleate (0.5 mM) and then exposed to the same stimuli (*= $p<0.05$).

Fatty acids increase basal glucose uptake in HSMC

In order to test the effects fatty acids on the cellular response to GW501516, myotubes were pre-incubated with oleate (0.5 mM) for 2 hours, followed by 10 nM GW501516 for 1 hour. Rather unexpectedly, oleate alone increased glucose uptake 1.5 fold, similar to the effect of GW501516 (**Figure 29B**). A combination of GW501516 and oleate resulted in a 1.6 fold increase glucose uptake.

Stimulation with insulin (120 nM) for 30 minutes and oleat, increased glucose uptake 1.4 fold, while insulin alone increased glucose uptake 1.5 fold. The effect of oleate on human muscle glucose uptake requires further investigation. However, a similar result has been observed in HSMC exposed to palmitate. Supplementing media with palmitate (0.6 mM) for 24 hours increased glucose uptake to a similar extent as insulin [334]. One possible mechanism explaining these effects would be via a fatty acid-mediated uncoupling of mitochondria [335], leading to increased AMP and activation of AMPK [329].

GW501516 effects on glucose transport ex vivo

The effects of GW501516 on glucose uptake and AMPK was also analysed in intact rat skeletal muscle preparations incubated *ex vivo*. Increased insulin mediated glucose uptake in response to GW501516 has been reported in intact *soleus* muscle incubated *ex vivo* [330]. Rat *epitrochlearis* muscle strips were incubated with or without 10 nM GW501516 for 6 hours. The insulin concentration in the media was 6 nM during the last 30 minutes. As expected, insulin increased glucose uptake ~4.5 fold. In contrast, glucose uptake was unaffected by GW501516 (**Figure 30C**), either alone, or in combination with insulin. Despite this, AMPK phosphorylation was significantly increased (30%) in skeletal muscle in response to GW501516 treatment (**Figure 30A**).

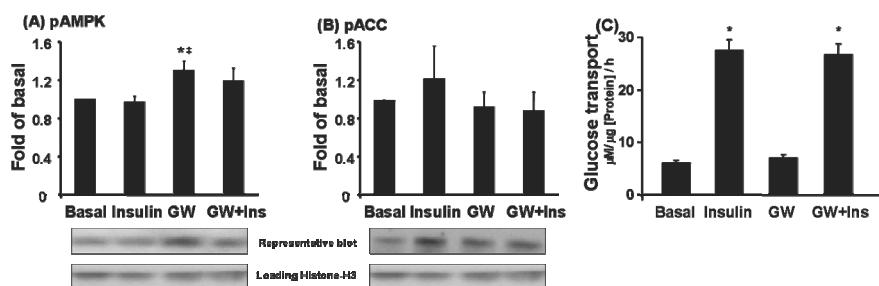


Figure 30: GW501516 effect on ex vivo epitrochlearis muscle from rat. (A) Phospho-AMPK and **(B)** phospho-ACC was measured following insulin, GW501516 (100 nM, 6 h) or a combined insulin/GW501516 stimulus (*p<0.05 vs. basal; **p<0.05 vs. insulin). Loading was controlled for probing for H3 histone expression. **(C)** Glucose transport was measured using the same stimuli (*p<0.05).

Phosphorylation of ACC was unaltered under any of the conditions studied (**Figure 30B**). Thus GW501516 does not appear to have a direct effect on glucose uptake in rat *epitrochlearis* muscles. This is in contrast to reports from other laboratories [330]. However, we used significantly lower concentration of GW501516 (10 nM vs. 1 μM), a different muscle preparation (*epitrochlearis* vs. *soleus*) and a shorter incubation time (6 hours vs. 24 hours).

Although a small increase in AMPK phosphorylation was noted, this was significantly reduced compared to results in HSMC (30% vs. 234%). Thus the compound-specific effects on changes in cellular energy status proposed may require a higher dose and/or longer exposure in order to result in measurable glucose uptake effects.

Non-specific effects have been noted in skeletal muscle exposed to TZDs [333]. In contrast to TZDs, GW501516, is a more potent specific activator of PPAR δ than TZDs are for PPAR γ [336]. Hence the concentrations required for clinical activation of PPAR δ may be below the threshold required to trigger mitochondrial uncoupling.

In line with this, PPAR δ knock-out mice placed on a high-fat diet fail to correct metabolic abnormalities in response to GW501516, as compared to wild type mice, suggesting that PPAR δ -specific effects are required for metabolic improvements [96]. The GW501516 effects on glucose uptake in cultured cells may reflect a faster accumulation of compound in mitochondria in a cell monolayer, as compared to tissue. The PPAR δ independent effects of GW501516 on AMPK will require further evaluation.

Gene- and protein expression following PPAR δ activation or silencing

mRNA expression of genes involved in lipid metabolism

Incubation of differentiated human myotubes with GW501516 over-night resulted in a significant increase in CPT1 (5.5 fold), PDK4 (4 fold) and FABP3 (1.7 fold) expression, compared to vehicle treated cells (**PAPER IV; Fig 3A**). In contrast, expression of a number of other genes, including DGK δ , GAPDH, NRF1 and Cytochrome C were unaltered following 18 hours GW501516 treatment (**Figure 31**).

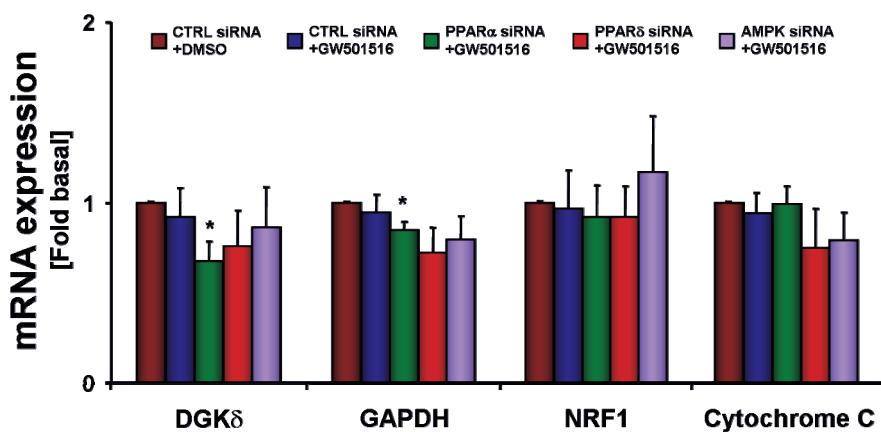


Figure 31: GW501516-induced changes in mRNA expression in HSMC. Differentiated HSMC were transfected with siRNA against PPAR α , PPAR δ , AMPK or a random control (CTRL) sequence siRNA. Transfected cells were cultured in the presence of GW501516 (100 nM, 16 h) and mRNA expression of Diacylglycerol Kinase (DGK) δ , Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH), Nuclear Respiratory Factor (NRF)-1 and mitochondrial Cytochrome C, determined using quantitative real-time PCR (*p<0.05 vs. Untreated CTRL (containing the vehicle DMSO)).

The GW501516-mediated transcriptional effects on CPT1, PDK4, and FABP3 were abolished when PPAR δ expression was reduced by siRNA. siRNA-mediated reduction of PPAR α or AMPK $\alpha 1/\alpha 2$ expression did not alter the GW501516-mediated induction of these genes. Taken together, our results indicate that activation of PPAR δ in cultured primary human skeletal muscle increases lipid metabolism and increases mRNA content of key regulators of fatty acid transport (FABP3) and oxidation (CPT1 and PDK4). Interestingly, inhibition of PPAR α gene expression resulted in a significant reduction of DGK δ and GAPDH expression. This reduction was apparent in PPAR α -reduced cultures after over-night GW501516 treatment. Although there have been no previous reports linking PPAR α and DGK δ expression, the PPAR γ agonist TZD has been reported to increase DGK expression [337]. This induction was completely blocked by a dominant-negative mutant of PPAR γ , indicating a PPAR γ dependent action [337]. The fact that PPAR α inhibition down-regulates GAPDH is interesting in relation to GAPDHs role in glycolysis. These results require further investigation since the effect of reducing PPAR α expression in the absence of GW501516 treatment is unknown.

A six hour exposure to GW501516 reduced mRNA expression of PPAR δ , SREBP-1a and SREBP-1c in skeletal muscle (**PAPER III; Figure 3B and 3C, p<0.05**). This GW501516-mediated reduction in SREBP expression is in contrast to a previous report in rodent adipose tissue, where activation of PPAR δ was without effect on SREBP expression [94]. However, in HEK293 cell lines, SREBP-1c expression was repressed [338]. Whether this discrepancy is due to species- or tissue-specific differences, or reflects differences in the duration of PPAR δ activation is unclear. However, the reduction in PPAR δ expression in myotubes after GW501516 exposure is comparable with the reported TZD-mediated down-regulation of PPAR γ expression in 3T3-L1 rat-adipocytes [339, 340]. Ligand-mediated down-regulation of receptor expression is well-documented for numerous receptor subtypes, including the nuclear receptors [341-344]. In contrast to results for SREBP-1 and PPAR δ , exposure to GW501516 did not alter mRNA expression of PPAR γ , PGC1, GLUT1 and GLUT4 (**PAPER III; Figure 3A to 3C**) or protein (data not shown), similar to previous reports in C2C12 [345]. Since we note increased glucose uptake following 30 minutes of GW501516 exposure, this suggests that the GW501516-mediated increase in glucose uptake is independent of changes in glucose transporter expression.

GW501516 stimulation does not alter expression of mitochondrial marker proteins

Expression of activated PPAR δ in mouse skeletal muscle has been reported to increase expression of several mitochondrial markers and to induce a shift in skeletal muscle fibre type towards type I muscle fibres [99]. In primary cultured human skeletal muscle, protein expression of PGC-1, which is involved in mitochondrial biogenesis [346] and formation of slow-twitch muscle fibre phenotypes [225], was unaffected as a result of over-night stimulation with GW501516 (**PAPER IV; Fig. 3B**).

Similarly, protein expression of CI and COX I, two subunits of the main mitochondrial respiratory-chain complexes, was also not altered by the PPAR δ agonist after either one hour or over-night treatment. These results indicate that the observed changes in FA metabolism are independent of changes in the mitochondrial density and/or efficiency. We have not investigated longer exposures, and it remains to be determined if a long term adaptation to continuous PPAR δ activation would result in mitochondrial biogenesis.

Summary of results from GW501516 effects in cultured human muscles

Specific activation of PPAR δ in cultured human muscle cells provides evidence that PPAR has a crucial role in regulation of lipid-metabolism and gene expression. This regulation is dependent on PPAR δ , since siRNA-mediated down-regulation of PPAR δ blocks these effects (Figure 32). Some PPAR δ -dependent effects on lipid metabolism also require the presence of AMPK. We have also shown that exposure of cells to GW501516 increased glucose uptake and improves insulin sensitivity in HSMC and 3T3-L1 adipocytes cells. The role of PPAR δ in the mediation of glucose uptake in skeletal muscle cells appears more complex. Although there is genetic evidence implicating variations in the *PPARD* gene in regulating human muscle glucose metabolism, *in vitro* experiments suggest that a major effect on glucose uptake is a compound-specific effect on AMPK. The PPAR δ agonist GW501516 results in changes in ATP:AMP ratio and subsequently activates AMPK. Thus GW501516 has similar non-specific effects on mitochondrial function, as have been reported for other PPAR agonists for the α and γ isoforms.

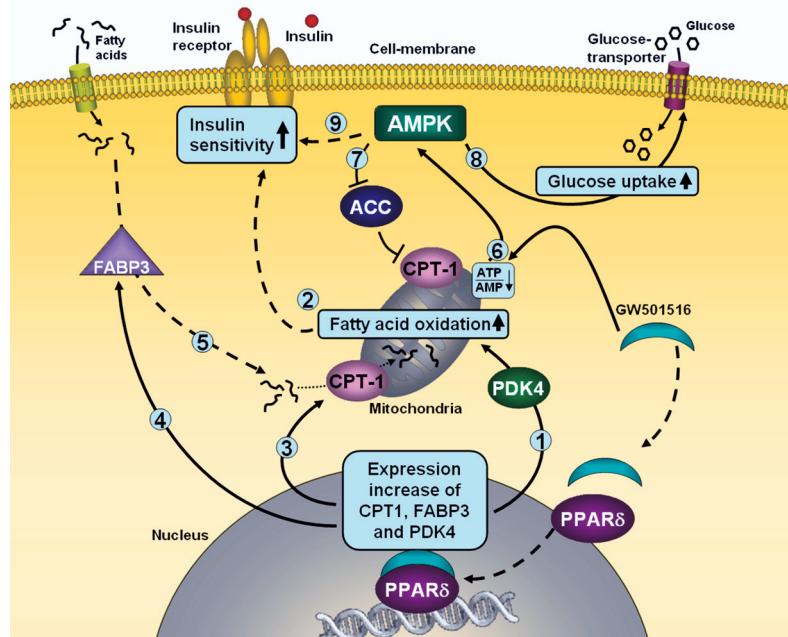


Figure 32: Proposed mechanism of GW501516 action in HSMC. GW501516 binds specifically to PPAR δ leading to changes in mRNA expression. These changes involve (1) PDK4, which, as a switch between glycolytic and oxidative metabolism presumably contributes to the observed (2) increase in mitochondrial oxidation of fatty acids (FA). Increased expression of CPT-1 (3) could contribute to an increase in FA oxidation. Increased expression of the intracellular FA-transporter FABP3 (4) is expected to lead to increased FA transport (5) into the mitochondria, further contributing to improved oxidative capacity. Another observed action of GW501516 is an acute and rapid drop in the ATP:AMP ratio (6), possibly mediated via interaction with mitochondria. This in turn activates AMPK, leading to phosphorylation and inhibition of ACC (7), thus releasing the ACC controlled inhibition on CPT-1, and allowing for more FA-influx into mitochondria. (8) AMPK furthermore leads to an insulin independent increase in glucose transport and has been linked to improved insulin sensitivity (9). (Dashed lines represent literature supported hypotheses; solid lines indicate actions where experimental evidence is provided in this thesis)

SUMMARY

The aim of this thesis has been to determine the role of the nuclear hormone receptor PPAR δ in regulation of human skeletal muscle metabolism. The results presented in this thesis may be summarised as follows:

- Low intensity exercise leads to increased PPAR δ expression in skeletal muscle of Type 2 diabetic patients. This increase is coupled to an improvement of insulin sensitivity and reduced blood-pressure as response to exercise.
- In Type 2 diabetic patients, where exercise fails to enhance the clinical phenotype, skeletal muscle PPAR δ expression is unaltered.
- Expression of PPAR δ is positively correlated with the proportion of oxidative type I skeletal muscle fibres in human skeletal muscle. Expression of PPAR α and PGC-1 α are similarly correlated. Hence, skeletal muscle mRNA expression of these genes is coupled to the oxidative capacity of skeletal muscle in humans.
- Pharmacological activation of PPAR δ in cultured human skeletal muscle leads to an increase in glucose and lipid uptake and enhanced lipid oxidation, as well as changes in expression of key genes regulating lipid metabolism.
- The PPAR δ agonist GW501516 increases the AMP/ATP ratio in cells independent of effects on PPAR δ . This challenges the direct role of PPAR δ on glucose uptake and raises the possibility that changes in cellular energy status mediate glucose uptake.

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis is directed towards understanding the role of PPAR δ in human skeletal muscle.

The result presented in the thesis highlight the role of PPAR δ as a key regulator of skeletal muscle metabolism.

Evidence from genetically manipulated mouse models has implicated a role for PPAR δ in the regulation of skeletal muscle fibre-type and oxidative capacity. Our results, demonstrate that the oxidative phenotype of human skeletal muscle correlates with PPAR δ expression. This observation further underscores a role for PPAR δ as a key-regulator of the oxidative capacity in human skeletal muscle. However, the relative roles of PPAR δ , PPAR α and PGC1 α and PGC1 β need to be explored. Whether PPAR δ plays a direct role in the formation of Type 1 fibres during development, or whether PPAR δ is able to regulate muscle metabolism in mature skeletal muscle requires further evaluation. Thus, the increased PPAR δ expression in skeletal muscle from elite cyclists could be a reflection of a genetically inherited predisposition towards high PPAR δ expression, resulting in an increased proportion of type I fibres, and hence an aptitude and talent for endurance sports such as cycling.

Evidence to support that expression of PPAR δ may be changed following an altered physiological stimulus, is the observation that mild exercise training increased skeletal muscle PPAR δ expression in subjects with Type 2 diabetes. Particularly interesting was the observation that the increased PPAR δ expression was correlated with an improved clinical profile of the patient. The same subjects also increased expression of UCP3, which together with the increase in PPAR δ suggests that improved mitochondrial function/content could be a central factor. Future studies will be directed towards a better understanding of the subjects who did not respond to exercise and who did not increase skeletal muscle PPAR δ expression. The dose of exercise performed in the study reported in this thesis may have been close to the minimum threshold for exercise-derived improvements, and a doubling of the exercise dose may lead to improvements in a greater proportion of subjects. Another explanation would be that genetic differences, at the *PPARD* locus or elsewhere, could determine an individual's ability to respond to exercise. To address this, an analysis of a given exercise response in relation to genotype could be performed.

The PPAR δ agonist GW501516 is currently under evaluation as a possible future treatment for Type 2 diabetes and metabolic disease. The results presented in this thesis show that direct activation of PPAR δ changes gene expression and metabolism in human skeletal muscle. Furthermore, we have shown that this compound alters the energy state of the cell. This property is similar to that of PPAR α agonists (fibrates) and PPAR γ activators (thiazolidine diones). Whether this mechanism is a possible secondary and beneficial effect *in vivo*, as proposed for thiazolidinediones, remains to be determined.

POPULAR SCIENTIFIC SUMMARY

Physical exercise is beneficial for patients with Type 2 Diabetes, mostly due to the fact that exercise leads to increased insulin sensitivity in skeletal muscle. Exactly how exercise induces these changes in skeletal muscle is not known, however, one important component may be a protein called PPAR δ . PPAR δ (Peroxisome Proliferator-Activated Receptor delta) is a so-called transcription factor, that is, a specialised protein which regulates the activity of genes in cells. Transcription factors thus control whether a gene is turned on or off in a particular tissue. Mice where a constitutively activated form of PPAR δ has been artificially introduced have an increased amount of type 1 muscles. Type 1 muscle fibres have an increased endurance, and the PPAR δ mice are able to run twice the distance of their normal brothers. Furthermore, these mice are protected from the metabolic consequences of a high fat diet. They do not become obese, and also do not develop Type 2 Diabetes [99]. The American scientists who described this suggested that PPAR δ increased fat-burning capacity.

In order to investigate if the amount of PPAR δ is coupled to increased endurance performance also in human skeletal muscle, we analysed muscles from elite cyclists, normally active individuals and spinal cord injured subjects [347]. Skeletal muscle obtained from the cyclists had the greatest proportion of the high endurance/type 1 muscle fibres, the normally active an intermediate content, and in spinal cord injured individuals, there were almost no type 1 fibres. When the skeletal muscle content of PPAR δ was determined, this too was highly expressed in elite cyclists. Normally active people had about half the amount of PPAR δ , and this was further reduced in spinal cord injured individuals. Hence, in human muscle PPAR δ content correlated with amount of type 1 muscle, similar to results obtained in mice.

In order to investigate if physical exercise could increase the amount of PPAR δ in skeletal muscle, we analysed muscle tissue obtained from people with Type 2 diabetics, before and after participation in a 4 month exercise programme. During this time, participants carried out approximately two and a half hours per week walking exercise. After four months, half of the participants had clear improvements in their diabetes (lipid profile, cholesterol and insulin sensitivity)[348]. In contrast, the other half of the group did not show these improvements; despite the fact that they had carried out the same amount of exercise, thus this group was “exercise resistant”. Analysis of the muscle tissue revealed that the group that improved their insulin sensitivity also increased the amount of muscle PPAR δ . The exercise resistant group had unchanged muscle PPAR δ content. Thus it appears that PPAR δ is an important component in the adaptive response of humans to exercise and a potential regulator of skeletal muscle insulin responsiveness.

In order to further investigate the impact of PPAR δ in human skeletal muscle, we utilised a pharmacological compound to specifically activate PPAR δ , and added this to human skeletal muscle cells growing in culture. We could directly demonstrate that activation of PPAR δ in human skeletal muscle cells enhances uptake and burning of fat (D. K. Krämer et al., submitted 2006), and increased the response of these cells to insulin [349]. We could also show that PPAR δ changes the expression of several genes important for fat-burning in the cell.

Thus the work in this thesis highlights the transcription factor PPAR δ as playing a key role in regulating skeletal muscle response to exercise and capacity for burning fat. We also link activation of PPAR δ to improved insulin sensitivity. Hence, PPAR δ is an attractive target for treatment and prevention of Type 2 diabetes.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

Sport ist für Patienten mit Typ-2-Diabetes nützlich, weil es den Metabolismus ankurbelt und die Insulinsensitivität in der Skelettmuskulatur erhöht. Eine wichtige Komponente dabei ist möglicherweise das von uns erforschte Eiweißmolekül PPAR-delta.

Das Protein PPAR-delta (Peroxisome Proliferator-Activated Receptor delta) ist ein Steuerelement der Genaktivität in Zellen. Mäuse mit künstlich aktivierten PPAR-delta-Rezeptoren, haben eine ungewöhnlich hohe Menge an sogenannter Typ 1 Muskulatur. Dieser Muskulaturtyp ist besonders ausdauern, weshalb die Mäuse deutlich länger laufen als normale Mäuse. Außerdem waren die Mäuse vor den negativen Auswirkungen einer fettrichen Ernährung geschützt. Sie legten weder stark an Gewicht zu, noch entwickelten sie Typ-2-Diabetes [99]. Die US-Forscher die diese Mäuse untersuchten, glauben, dass dieser Umstand einer gesteigerten Fettverbrennung zu verdanken ist.

Um zu überprüfen ob auch beim Menschen die Typ 1 Muskulatur an Ausdauer gekoppelt ist, untersuchten wir die Menge an PPAR-delta bei Radprofis und Querschnittsgelähmten sowie Gesunden mit normaler körperlicher Aktivität. Radprofis hatten die grösste, normal Aktive mittlere und Querschnittsgelähmte die geringste Menge an Typ 1 Muskulatur. Die Menge des PPAR-delta-Rezeptors im Skelettmuskel war in der Gruppe der intensiv trainierten Radprofis am höchsten, bei Untrainierten geringer und am geringsten bei Querschnittsgelähmten [347]. Die Menge an PPAR-delta korrelierte also auch beim Menschen mit der Menge an Typ-1-Muskelfasern.

Um zu untersuchen, ob Sport eine Auswirkung auf die Menge an PPAR-delta in der Muskulatur von Diabetikern hat, baten wir eine Gruppe von Studienteilnehmer mit Typ-2-Diabetes, insgesamt 2,5 Stunden pro Woche spazieren zu gehen. Nach Ende der Studie hatte die eine Hälfte der Teilnehmer eine deutlich verbesserte Insulinsensitivität, einen niedrigeren Blutdruck und verbesserte Blutfettwerte als zu Beginn der Studie [348]. Die übrigen Teilnehmer hatten dagegen keine verbesserten Werte, obwohl sie pro Woche körperlich genauso viel leisteten wie die erste Gruppe. Die Analyse der Muskelproben zeigte, daß bei den Teilnehmern mit besserer Insulinsensitivität die PPAR-delta Menge zugenommen hatte. Hingegen blieb die Menge an PPAR-delta unverändert bei den Patienten, die keine Verbesserung durch Sport gezeigt hatten. Die Aktivierung von PPAR-delta scheint somit eine bedeutende Komponente bei der Erhöhung der Insulinsensitivität durch Sport zu sein.

Um den Einfluss von PPAR-delta Aktivität weiter zu erforschen, bedienten wir uns einer Substanz, die zur Aktivierung von PPAR-delta entwickelt wurde. Wir fanden heraus, dass die Fettverbrennung in Muskelzellen zunahm (D.K. Krämer et al, submitted 2006) und daß die Zellen zugleich empfindlicher für Insulin wurden, wenn sie mit dem PPAR-delta Aktivator behandelt wurden [349]. Durch Untersuchungen der Genaktivität fanden wir, daß die Aktivität von Genen gesteigert wurde, die die Fettverbrennung ankurbeln.

Wir zeigten zusammenfassend, daß sowohl pharmakologische Aktivierung von PPAR-delta möglicherweise Typ-2-Diabetes vorbeugen kann, aber auch zur Behandlung von Personen eingesetzt werden kann, die bereits an Diabetes erkrankt sind, da es die Empfindlichkeit für Insulin verbessert. Wir zeigen überdies, daß PPAR-delta sowohl auch auf natürliche Weise, als auch durch Sport, angeregt werden kann.

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APPENDIX: ORIGINAL PAPERS AND MANUSCRIPTS

- I** **David Kitz Krämer**, Maria Ahlsén, Jessica Norrbom, Eva Jansson, Nils Hjeltnes, Thomas Gustafsson and Anna Krook.
mRNA expression of PPAR α , PPAR δ , PGC-1 α and PGC-1 β is altered following pathologically and physiologically induced variations in skeletal muscle fibre type.
ACTA Physiol 2006, 188, 207–216
- II** Tomas Fritz*, **David Kitz. Krämer***, Håkan KR Karlsson*, Dana Galuska, Peter Engfeldt, Juleen R. Zierath and Anna Krook.
Low-intensity exercise increases skeletal muscle expression of PPAR δ and UCP3 in Type 2 diabetic patients.
Diabet Metab Res Reviews 2006; 22: 492–498.
- III** **David Kitz Krämer**, Lubna Al-Khalili, Sebastio Perrini, Josefín Skogsberg, Per Wretenberg, Katja Kannisto, Harriet Wallberg-Henriksson, Ewa Ehrenborg, Juleen R. Zierath and Anna Krook.
Direct activation of glucose transport in primary human myotubes after activation of peroxisome proliferator-activated receptor δ .
Diabetes 2005, 54:1157–1163
- IV** **David Kitz Krämer**, Bruno Guigas, Ying Leng and Anna Krook.
Role of AMP kinase in PPAR δ regulation of lipid and glucose metabolism in skeletal muscle.
Submitted 2006