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# INSULIN SIGNALING AND GLUCOSE TRANSPORT IN INSULIN RESISTANT HUMAN SKELETAL MUSCLE

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# ABSTRACT

Insulin resistance in skeletal muscle is a hallmark feature of Type 2 diabetes mellitus. The overall aim of this thesis was to investigate downstream intermediates in the insulin signaling pathway in an attempt to characterize the molecular mechanism of skeletal muscle insulin resistance in Type 2 diabetes.

Skeletal muscle biopsies were obtained from healthy and Type 2 diabetic subjects before and after an *in vivo* hyperinsulinemic infusion. Insulin infusion increased the phosphorylation of several proteins reacting with a phospho-Akt substrate antibody. We focused on AS160, as this Akt substrate has been linked to glucose transport. A phosphorylated protein of 160 kDa was identified as AS160 using an AS160-specific antibody. Insulin-stimulated AS160 phosphorylation was reduced in Type 2 diabetic patients, whereas AS160 protein expression was similar between Type 2 diabetic and control subjects. Impaired AS160 phosphorylation was related to a reduction in Akt $Thr^{308}$-phosphorylation.

To investigate whether pharmaceutical treatment improves glucose uptake due to enhanced insulin signaling in skeletal muscle, biopsies were obtained during a euglycemic hyperinsulinemic clamp from newly diagnosed Type 2 diabetic subjects before and after 26 weeks of metformin, rosiglitazone or placebo treatment. Insulin-mediated whole body and leg muscle glucose uptake was enhanced 36% and 32%, respectively, after rosiglitazone, but not after metformin or placebo treatment. Insulin-stimulated IRS-1 Tyr-phosphorylation, IRS-1 associated PI 3-kinse activity, Akt $Ser^{473}$-phosphorylation, and AS160 phosphorylation were assessed. Gene expression of several targets involved in lipid and glucose metabolism was also determined. Insulin signaling parameters and gene expression were unaltered after metformin or rosiglitazone treatment, despite the improvement in glucose uptake.

Since an altered metabolic milieu may secondarily cause insulin resistance in Type 2 diabetic subjects, we studied a cohort of healthy glucose tolerant first-degree relatives of Type 2 diabetic patients to determine whether early metabolic and molecular defects contribute to insulin resistance in skeletal muscle. Skeletal muscle from first-degree relatives and control subjects were incubated *in vitro* in the absence or presence of increasing concentrations of insulin. Glucose transport, AS160 phosphorylation, and GLUT4 expression were assessed. Insulin-stimulated glucose transport rate at a maximal insulin concentration tended to be reduced in skeletal muscle from first-degree relatives. Insulin increased AS160 phosphorylation in a dose-dependent manner, with no difference between first-degree relatives and control subjects. A tight correlation was observed between insulin action on AS160 and glucose transport in control subjects, whereas a weak correlation was observed in first-degree relatives.

Tanis, a recently described putative receptor for serum amyloid A, has been implicated in inflammatory responses. We determined mRNA expression of the human homologue of Tanis, SelS/AD-015 in skeletal muscle and adipose tissue biopsies obtained from Type 2 diabetic patients and healthy subjects. Expression of Tanis/SelS mRNA in skeletal muscle and adipose tissue biopsies was similar between Type 2 diabetic and control subjects. Adipose tissue Tanis/SelS mRNA expression was unchanged after insulin infusion in control subjects, whereas Tanis/SelS mRNA increased following insulin stimulation in Type 2 diabetic subjects. Skeletal muscle and adipose tissue Tanis/SelS mRNA expression were positively correlated with plasma serum amyloid A.

In summary, defects in insulin action on AS160 may impair GLUT4 trafficking in Type 2 diabetes. Further, insulin-sensitizing effects of rosiglitazone are independent of enhanced insulin signaling of the IRS-1/PI 3-kinase/Akt/AS160 pathway in Type 2 diabetes, suggesting that improvements might be achieved at distal events at the level of GLUT4 translocation. In addition, healthy first-degree relatives of Type 2 diabetic subjects have a tendency of impaired glucose transport at a super-physiological insulin concentration, despite a normal insulin action on AS160, implicating early insulin signaling defects are likely to be a consequence of an altered metabolic milieu. Moreover, the positive correlation of Tanis/SelS mRNA expression and serum amyloid A suggests a potential interaction between immune system responses and Tanis/SelS expression in muscle and adipose tissue. In conclusion, future strategies designed to treat insulin resistance in human skeletal muscle may target multiple sites along insulin signaling and GLUT4 pathways depending on the progression of the disease.

# LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their roman numbers:

I. **Karlsson H.K.R.**, Zierath J.R., Kane S., Krook A., Lienhard G.E. and Wallberg-Henriksson H. Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of Type 2 diabetic subjects. *Diabetes*, 54(6): 1692-1697, 2005.

II. **Karlsson H.K.R.**, Hällsten K., Björnholm M., Tsuchida H., Chibalin A.V., Virtanen K.A., Heinonen O.J., Lönnqvist F., Nuutila P. and Zierath J.R. Effects of metformin and rosiglitazone treatment on insulin signaling and glucose uptake in newly diagnosed type 2 diabetic patients: A randomized controlled study. *Diabetes*, 54(5): 1459-1467, 2005.

III. **Karlsson H.K.R.**, Ahlsén M., Zierath J.R., Wallberg-Henriksson H. and Koistinen H.A. Insulin signaling to glucose transport in skeletal muscle from first-degree relatives of subjects with type 2 diabetes. Submitted.

IV. **Karlsson H.K.R.**, Tsuchida H., Lake S., Koistinen H.A. and Krook A. Relationship between serum amyloid A level and Tanis/SelS mRNA expression in skeletal muscle and adipose tissue from healthy and Type 2 diabetic subjects. *Diabetes*, 53(6): 1424-1428, 2004.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| AS160 | akt substrate of 160 kDa |
| ATP | adenosine triphosphate |
| BMI | body mass index |
| cDNA | complementary DNA |
| DNA | deoxyribonucleic acid |
| FFA | free fatty acids |
| GAP | GTPase activating protein |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GLUT | glucose transporter |
| GSK3 | glycogen synthase kinase 3 |
| GTP | guanosine triphosphate |
| HEPES | N-[2-Hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid] |
| IR | insulin receptor |
| IRS | insulin receptor substrate |
| KHB | Krebs-Henseleit buffer |
| MFC | Multi Fluidic Card |
| mRNA | messenger RNA |
| mTOR | mammalian target of rapamycin |
| OGTT | oral glucose tolerance test |
| PAS | phospho-Akt substrate |
| PDK | 3´-phosphoinositide-dependent kinase |
| PH | pleckstrin homology |
| PKC | protein kinase C |
| PPAR | peroxisome proliferator-activated receptor |
| PTB | phosphotyrosine binding |
| PtdIns | phosphatidylinositol |
| Rab | Ras homologous from brain |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase-polymerase chain reaction |
| SAA | serum amyloid A |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SelS | selenoprotein S |
| Ser | serine |
| SGLT | sodium dependent glucose transporter |
| SH2 | src homology 2 |
| Thr | threonine |
| TZD | thiazolidinediones |

# 1 INTRODUCTION

Overweight and obesity has increased dramatically worldwide during the last decades. This dramatic increase in adiposity is not restricted to developed countries. The World Health Organization (WHO) and International Diabetes Federation (IDF) reported in a joint press release from November 2004 [http://www.who.int/mediacentre/news/releases/2004/pr81/en/index.html], an estimate that more than 22 million children under five years of age are obese or overweight today, and more than 17 million of them are in developing countries. An explanation for this startling number of obese children is the increased availability and promotion of foods high in fat and sugar and to a large extent by lifestyle changes, where less physical activity is performed due to increased urbanization and changes in transport systems, as well as increased hours spent in front of televisions and computers. The lifestyle and diet habits of people are changing rapidly, by comparing the fact that children today do not eat the way their parents did.

The link between obesity and Type 2 diabetes is well-established. According to a recent estimation, in the year 2000, at least 171 million people worldwide were estimated to suffer from Type 2 diabetes, a figure that is expected to rise to 366 million in year 2030 (Wild *et al*, 2004). In the industrialized western countries, Type 2 diabetes has already taken on epidemic-like proportions, and with the rapid increase now seen worldwide, we are facing an extensive future health problem.

In light of these facts, the pathogenesis and metabolic mechanisms of Type 2 diabetes is utterly important to study in order to investigate possibilities to generate pharmacological interventions for treatment.

## 1.1 TYPE 2 DIABETES

At the whole-body level, glucose is used as a source for energy, to provide fuel for skeletal muscle and other tissues. In the postprandial state after a meal, insulin is secreted from the pancreatic β-cells into the circulation to promote glucose uptake into insulin-sensitive tissues and to decrease the endogenous hepatic glucose production. When glucose levels are low, the pancreatic α-cells releases glucagon into the circulation that promotes glucose production from the liver. This system has to be tightly regulated to achieve glucose homeostasis (Figure 1). Type 2 diabetes is a metabolic disorder where the efficacy of insulin is insufficient to regulate glucose uptake and hepatic glucose production. As a result, glucose levels in the circulation increase. When the glucose level in the circulation is chronically elevated, hyperglycemia develops. Chronic hyperglycemia is associated with many serious pathological complications. Deficiently managed hyperglycemia will potentially cause a variety of macrovascular and microvascular complications, such as atherosclerosis, nephropathy, neuropathy and retinopathy. Thus, it is important to maintain euglycemia in order to prevent these complications, which can ultimately lead to very severe coronary heart disease, kidney failure, foot ulcers and blindness.

### 1.1.1 Diagnostic definition of Type 2 diabetes

The diagnostic definition for Type 2 diabetes is a fasting plasma concentration at or above 7 mmol/L or a plasma concentration at or above 11.1 mmol/L 2 hrs post a 75g oral glucose load, as measured by means of the oral glucose tolerance test (OGTT) (Alberti and Zimmet, 1998; Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2004).

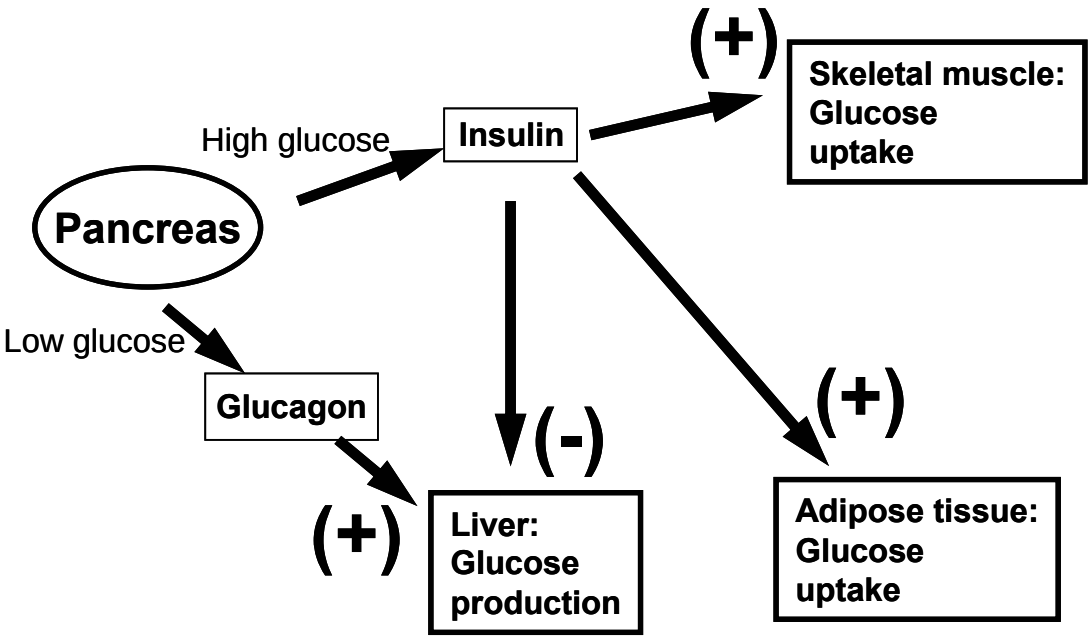


**Figure 1. Maintenance of glucose homeostasis.** High glucose levels in the circulation trigger the pancreas to release insulin. Insulin release leads to an increased (+) glucose uptake in skeletal muscle and adipose tissue, and a decreased (-) glucose production from the liver. Low glucose levels lead to glucagon release to increase (+) glucose production from the liver.

### 1.1.2 Different stages in the development of Type 2 diabetes

In a pre-diabetic state, insulin resistance often exists in the insulin responsive peripheral tissues, namely skeletal muscle and adipocytes. This leads to an impaired glucose tolerance, which means that the insulin secreted from the pancreatic β-cells is insufficient to clear the ingested glucose that is absorbed into the circulation after a meal. In combination with insulin resistance in the liver, which results in an impairment of the ability of insulin to inhibit glucose production, impaired fasting glucose levels arise. Accordingly a plasma glucose level between 6.1 to 6.9 mmol/L is considered impaired fasting glucose (Alberti and Zimmet, 1998). However, the consensus statement for the upper threshold for a normal glucose level is currently under debate, with the upper limit suggested to be lowered to < 5.6 mmol/L (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2004).

In an initial period of pre-diabetes, the pancreatic β-cells have the ability to compensate for peripheral insulin resistance by secreting more insulin, which results in hyperinsulinemia. However, continuous hyperinsulinemia will directly lead to even more severe insulin resistance and this viscous circle will ultimately lead to β-cell failure. In 1981, a mathematical "minimal modeling technique" was proposed which enables a quantitative measurement of the contributions of pancreatic responsiveness and insulin sensitivity to an intravenous glucose tolerance test (Bergman *et al*, 1981). With this minimal model technique the pancreatic responsiveness was shown to have a hyperbolic relationship to insulin sensitivity, such that for any difference in insulin sensitivity, there is a reciprocal and proportionate difference in glucose responsiveness of the pancreas (Kahn *et al*, 1993) (Figure 2).

Thus, several factors contribute to the progression from normal to impaired glucose tolerance and Type 2 diabetes. Diagnosis of Type 2 diabetes is often made at a stage when secondary complications to the metabolic derangements already are manifested. Therefore, it is of great interest to investigate primary causes of the disease,

to be able to gain knowledge about cellular mechanisms underlying insulin resistance and impaired glucose uptake. This could then lead to more accurate clinical investigation of early predictions of high risk individuals, and potentially suitable medication treatment of a more specific nature.

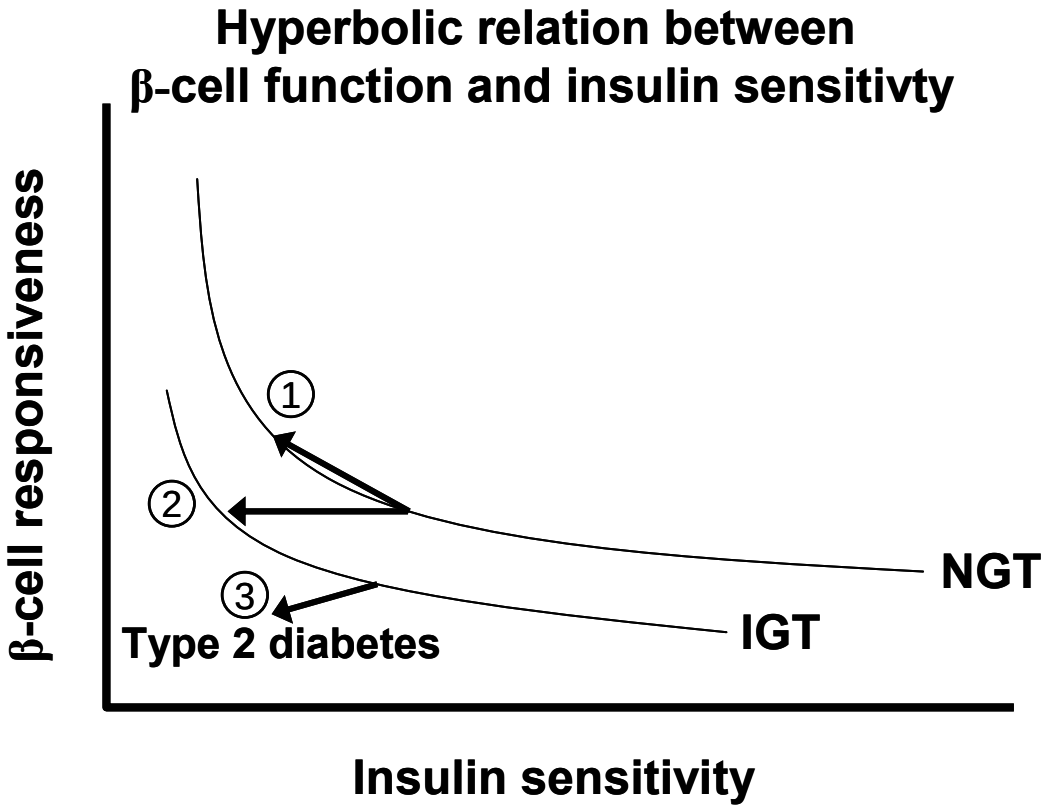


**Figure 2. "Minimal model".** The hyperbolic relationship between pancreatic responsiveness and insulin sensitivity (Modified from (Stumvoll *et al*, 2005)). Examples of stages in the progression from normal glucose tolerance (NGT) or impaired glucose tolerance (IGT) to Type 2 diabetes; 1) insulin resistance with β-cell compensation; 2) insulin resistance without β-cell compensation (deviation from the hyperbola); 3) insulin resistance and impaired β-cell function, leading to Type 2 diabetes.

### 1.1.3 First-degree relatives of patients with Type 2 diabetes

Certain inherited genotypes predispose impairments in glucose metabolism. First-degree relatives of patients with type 2 diabetes, *i. e.* people with one or both parents with Type 2 diabetes, have a higher risk of developing Type 2 diabetes. In one study where first-degree relatives of Type 2 diabetic patients were followed over 25 years, a high proportion of these individuals developed impaired or diabetic glucose tolerance (Nauck *et al*, 2003). Even first-degree relatives with normal responses to oral glucose tolerance tests demonstrate impaired whole-body glucose metabolism, with defects mainly observed in non-oxidative pathways governing glucose metabolism (storage of glucose as glycogen) (Eriksson *et al*, 1989). In Type 2 diabetes, insulin resistance in skeletal muscle largely occurs as a consequence of the altered metabolic milieu caused by hyperglycemia, hyperinsulinemia and dyslipidemia. Therefore, first-degree relatives of Type 2 diabetic patients, which are at a high risk to develop Type 2 diabetes, are an important group to study in order to investigate possible molecular mechanisms that may occur at an early state in the disease. Thus, these subjects offer a unique opportunity to reveal whether genetically inherited defects or environmental factors contribute to the Type 2 diabetic phenotype progression.

### 1.1.4 The metabolic syndrome and Type 2 diabetes

The metabolic syndrome is characterized by insulin resistance, obesity (in particular abdominal adiposity), hypertension and dyslipidemia (reviewed in (Moller and Kaufman, 2005)). The characteristics of dyslipidemia in this context are increased triglyceride concentration and decreased HDL-cholesterol concentration, together with or without elevated LDL-cholesterol. There are several proposals of definitions of the metabolic syndrome; the World Health Organization (WHO) consultation (Alberti and Zimmet, 1998), the National Cholesterol Education Program (NCEP) Expert Panel (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001) and the European Group for the Study of Insulin Resistance (EGIR) (Balkau and Charles, 1999). All of these organizations have come to consensus on the definitions of the essential components of the metabolic syndrome including glucose intolerance, obesity, hypertension and dyslipidemia, but they differ in the details and certain criteria. The definitions and criteria are continuously debated and discussed in publications and at scientific meetings.

The metabolic syndrome encompasses Type 2 diabetes. Given the many metabolic alterations, the pathogenesis of Type 2 diabetes is heterogeneous and multifactorial. In an attempt to determine which of the various components of the metabolic syndrome have the strongest influence in predicting Type 2 diabetes incidence, a large cohort of Pima Indians was studied during a median follow-up of 4.1 years (Hanson *et al*, 2002). In the Pima cohort, hyperinsulinemia and obesity were identified as strong risk factors for the development of overt Type 2 diabetes. Dyslipidemia, *i. e.* the combination of hypertriglyceridemia and low HDL-cholesterol, is a more modest, but still significant risk factor. However, high blood pressure is only weakly, if at all, associated with the incidence of Type 2 diabetes (Hanson *et al*, 2002). Since differences exist in the criteria and definitions of the metabolic syndrome, the outcome from this kind of clinical approach might differ. Prediction of either prevalence or incidence of Type 2 diabetes according to different criteria by WHO or NCEP were compared in a larger cohort of subjects (Laaksonen *et al*, 2002). The result revealed that the criteria by WHO versus that of NCEP, were more sensitive in detecting the prevalence and incidence of Type 2 diabetes, where NCEP had higher specificity than WHO. To date, the molecular mechanisms that precede the conversion from impaired glucose tolerance to overt Type 2 diabetes are unknown.

### 1.1.5 Inflammatory disease and Type 2 diabetes

Recently, great interest has been raised towards the link between inflammation and Type 2 diabetes (Dandona *et al*, 2004). One of the first studies performed in a larger cohort to investigate and identify the connection between inflammatory markers and the development of Type 2 diabetes was part of the larger Atherosclerosis Risk in Communities Study (ARIC) (Schmidt *et al*, 1999). In a continuation study, the investigators could further provide evidence that a low-grade systemic inflammation precedes and predicts the development of Type 2 diabetes in adults (Duncan *et al*, 2003).

Adipose tissue has been identified to function as an endocrine organ releasing a variety of metabolic mediators or adipokines (Kershaw and Flier, 2004). Tumor necrosis factor α (TNF-α) is a cytokine that is released from adipose tissue and is involved in mediating insulin resistance (Hotamisligil *et al*, 1993). Another cytokine that has created recent interest for an involvement in metabolic regulation is interleukin-6 (IL-6). IL-6 is secreted by adipose tissue (Fried *et al*, 1998) and skeletal muscle (Febbraio and Pedersen, 2002) and serum concentrations are elevated in Type 2 diabetes (Pickup *et al*, 1997). Plasma adiponectin concentrations are reduced in

individuals with Type 2 diabetes and levels of adiponectin are closely related to insulin resistance and hyperinsulinemia (Weyer *et al*, 2001). Other adipokines secreted by adipose tissue are leptin and resistin (reviewed in (Kershaw and Flier, 2004)).

Serum amyloid A (SAA) is an acute-phase inflammatory response serum protein that has been proposed to be involved in Type 2 diabetes. Serum concentration of SAA has been demonstrated to be higher in patients with Type 2 diabetes (Kumon *et al*, 1994; Pickup *et al*, 1997; Ebeling *et al*, 1999). Furthermore, elevated levels of SAA in Type 2 diabetes are associated with insulin resistance. For example, troglitazone and rosiglitazone treatment reduces SAA levels in parallel with an increase in insulin sensitivity (Ebeling *et al*, 1999; Dhindsa *et al*, 2005). Tanis, a recently described putative receptor for serum amyloid A, has been implicated to be dysregulated in a rodent model of Type 2 diabetes (Walder *et al*, 2002). However, whether the human homologue of Tanis, SelS/AD-015, is altered in skeletal muscle and adipose tissue from Type 2 diabetic subjects is unknown.

## 1.2 SKELETAL MUSCLE – ROLE IN WHOLE BODY METABOLISM

Glucose can either undergo oxidative or non-oxidative metabolism in skeletal muscle. Glucose is oxidized by glycolytic processes generating pyruvic acid that is subsequently transformed to acetyl-CoA that is further metabolized in the citric acid cycle and that ultimately leads to generation of chemically bound energy in form of ATP. If glucose is not immediately utilized for energy, it is stored as glycogen, *i. e.* non-oxidative metabolism, which can be utilized for energy production later when the muscle cell requires energy.

During a euglycemic hyperinsulinemic clamp, peripheral tissues accounts for approximately 80-90% of glucose disposal, where skeletal muscle tissue is quantitatively the most important tissue (DeFronzo *et al*, 1981; DeFronzo *et al*, 1985). However, in the postprandial period after ingestion of a meal, skeletal muscle accounts for approximately 1/3 of the ingested glucose (Kelley *et al*, 1988; Moore *et al*, 2003). Notably, skeletal muscle is one of the key tissues involved in the maintenance of whole-body glucose homeostasis, and insulin resistance in skeletal muscle is a major contributor to the pathogenesis of Type 2 diabetes.

## 1.3 GLUCOSE TRANSPORT

Glucose is a hydrophilic molecule and thus, cannot diffuse through the lipid bilayer of cell surface membrane. Therefore, glucose entry into the cell needs to be facilitated by membrane transporters. There are mainly two different families of glucose transporters in humans, the sodium dependent glucose transporter family (SGLT) and the facilitative glucose transporters (GLUT) (Joost *et al*, 2002; Scheepers *et al*, 2004). While the sodium dependent glucose co-transporters are mainly involved in glucose absorption in the intestine and the kidneys, GLUT family members are facilitative glucose transporters. At present there are 14 identified genes coding for individual proteins of the GLUT family (GLUT1-14). The GLUT's have different tissue expression and different affinity for glucose (Scheepers *et al*, 2004). In skeletal muscle GLUT4 is the predominantly expressed isoform (Birnbaum, 1989; Fukumoto *et al*, 1989; James *et al*, 1989) and GLUT4 is localized intercellular and is translocated to the surface membrane in response to insulin, exercise or hypoxia (Douen *et al*, 1990; Hirshman *et al*, 1990; Kristiansen *et al*, 1996; Ryder *et al*, 2000).

Impaired insulin-stimulated glucose transport in skeletal muscle is the rate determining step in the reduced glycogen synthesis observed in insulin resistant Type 2 diabetic patients (Cline *et al*, 1999). Total GLUT4 expression is not reduced in skeletal

muscle from Type 2 diabetic patients (Handberg *et al*, 1990; Pedersen *et al*, 1990; Shepherd and Kahn, 1999). Thus, impaired glucose uptake in insulin resistant skeletal muscle cannot be explained by a decrease in production of GLUT4.

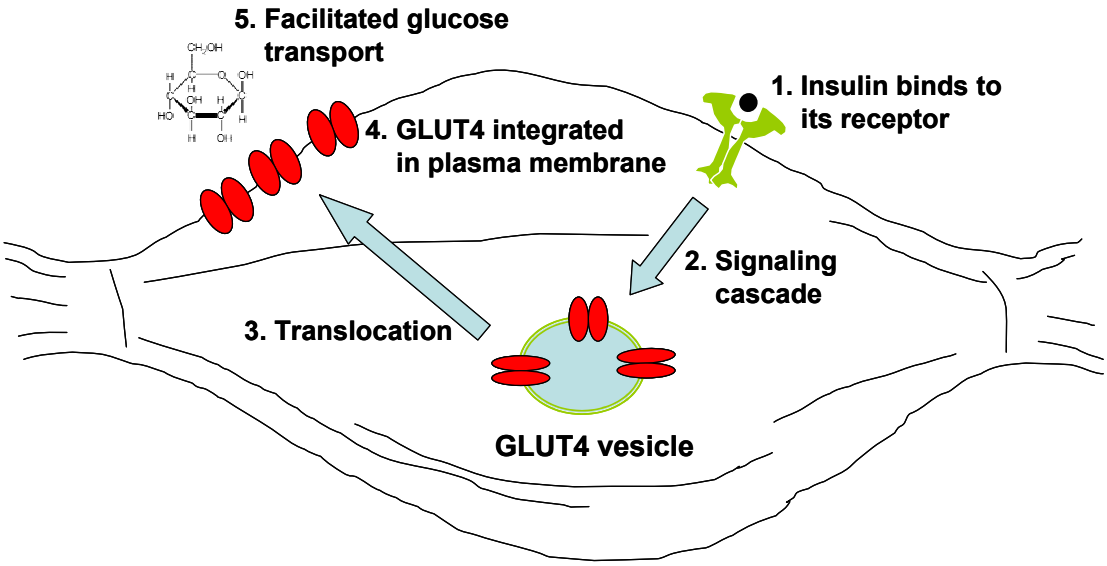


**Figure 3. Principle of insulin-induced glucose uptake in skeletal muscle.** Insulin binds to the insulin receptor (1), thereby initiating a signaling cascade through the insulin signaling pathway (2). The intracellular membrane bound GLUT4-containing vesicles are then translocated to the cell surface membrane (3), where they ultimately integrate GLUT4 in the membrane (4) which then facilitates glucose transport into the muscle cell (5).

### 1.3.1 Cellular events

Early studies in adipocytes revealed that the glucose transport system was translocated from an intracellular compartment to the cell surface membrane in response to insulin (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Later a unique insulin-regulatable glucose transport protein was identified in skeletal muscle and adipose tissue (James *et al*, 1988), which was subsequently cloned and characterized as GLUT4 (Fukumoto *et al*, 1989; James *et al*, 1989). GLUT4 was shown to be the glucose transporter that accounts for the major part of insulin-induced glucose uptake in cultured adipocytes (Holman *et al*, 1990). Using a bis-mannose photolabel technique, a strong correlation between insulin-induced 3-O-metyl-glucose uptake and cell surface expression of GLUT4 was observed (Holman *et al*, 1990). GLUT4 is a membrane protein and resides within intracellular membrane bound vesicles. In response to insulin or exercise these GLUT4-containing vesicles are translocated to fuse with the cell surface membrane to facilitate transport of glucose molecules into the cell (reviewed in (Holman and Sandoval, 2001), Figure 3). Several studies have demonstrated that the translocation and trafficking of GLUT4 containing vesicles in insulin resistant human skeletal muscle is impaired (Garvey *et al*, 1998; Ryder *et al*, 2000). Currently it is unknown whether defects in GLUT4 translocation precede the development of Type 2 diabetes.

## 1.4 INSULIN SIGNALING

Insulin responsive tissues express insulin receptors (IR) at the cell surface plasma membrane. The IR consists of four subunits, two extracellular insulin-binding α-peptides linked with two transmembrane β-peptides. On the β-subunits intracellular

side, there is a tyrosine kinase domain. The β-subunits are activated due to autophosphorylation when insulin binds to the receptor. Phosphorylated tyrosine residues on the activated insulin receptor protein provide docking sites for the binding of several down-stream signaling molecules including Shc, Grb2 and the insulin receptor substrate (IRS) proteins. Insulin transduces signals along metabolic and mitogenic/gene-regulatory pathways.

When the IR is activated, it recruits IRS, which binds to phosphorylated tyrosine residues on the receptor via a phosphotyrosine binding (PTB) domain on IRS. When IRS is bound to IR, the kinase activity of IR can catalyze phosphorylation of tyrosine residues on IRS. IRS's are scaffolding proteins and the two most predominant IRS's involved in metabolic regulation in human skeletal muscle are IRS-1 and IRS-2. These IRS's share a high sequence similarity, but appear to have specific signaling roles. In L6 myotubes, where IRS-1 or IRS-2 protein expression was reduced approximately 75% using small interfering RNA-mediated gene silencing, IRS-1 was demonstrated to be responsible for reduction in glucose uptake and GLUT4 translocation, whereas IRS-2 was without effect on these responses (Huang *et al*, 2005). There is also direct evidence for tissue specific effects of the IRS´s from functional genomics. In transgenic mice models where either a heterozygous null mutation of genes coding IR/IRS-1 or IR/IRS-2 are expressed, the former developed severe insulin resistance in skeletal muscle, while the latter developed severe insulin resistance in liver, with only mild insulin resistance in skeletal muscle (Kido *et al*, 2000). Additional evidence for tissue specific differences in IRS action is provided from studies in which IRS-2 knockout mice develop Type 2 diabetes, partly by becoming insulin resistant, but primarily because of reduced β-cell mass, which therefore prevents an adequate compensation in insulin release (Withers *et al*, 1998).

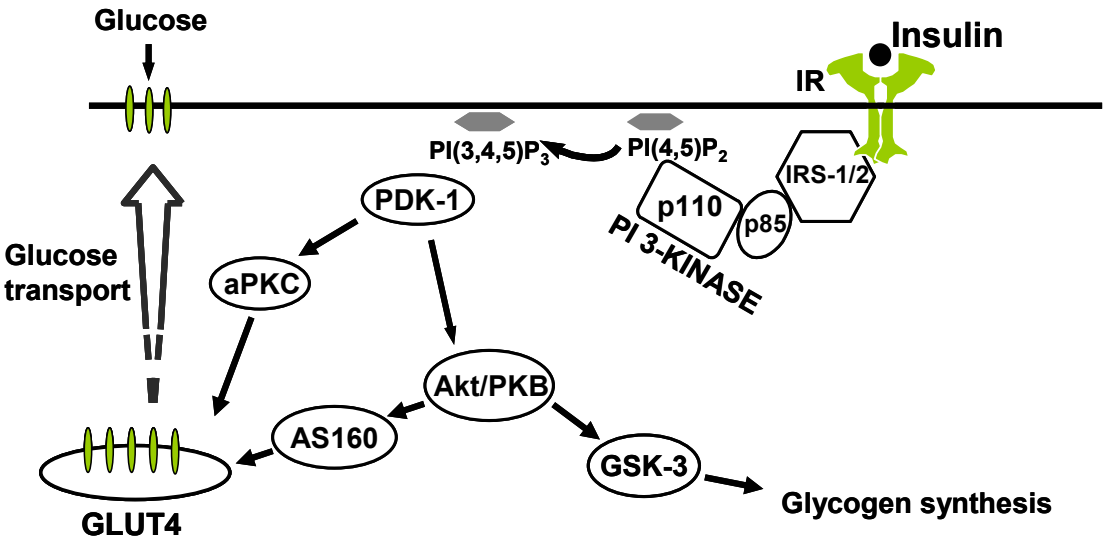


**Figure 4. Insulin signaling along metabolic pathways.**

### 1.4.1 IRS/PI 3-kinase signaling

Tyrosine-phosphorylated IRS-1 mediates the insulin signaling to downstream enzymes by binding to a number of src-homology 2 (SH2) domain-containing signaling proteins. One important signaling intermediate that promotes the insulin signal is the phosphatidylinositol 3 (PI3)-kinase (Cantrell, 2001). PI 3-kinase is composed of one regulatory subunit and one catalytic subunit, which phosphorylates the 3'-OH position of the inositol ring of plasma membrane inositol phospholipids. The substrate products that are generated are phosphatidylinositol-3-monophosphate (PtdIns(3)P), PtdIns-3,4-

bisphosphate (PtdIns(3,4)$P_2$) and PtdIns-3,4,5-triphosphate (PtdIns(3,4,5)$P_3$). These phosphoinositides are specifically recognized by proteins that contain pleckstrin homology (PH) domains which are then redistributed to the plasma membrane.

### 1.4.2 Signaling downstream of PI 3-kinase

The serine/threonine kinase Akt, also known as protein kinase B (PKB), is a central intermediate for many of the insulin and growth factor responses downstream of PI 3-kinase. Akt was identified to be one of the insulin-responsive kinases that phosphorylates glycogen synthase kinase-3 (GSK-3) (Cross *et al*, 1995). Subsequent studies demonstrated that Akt has a role in promoting GLUT4 translocation in adipocytes (Tanti *et al*, 1997) and glucose transport and glycogen synthesis in L6 myotubes (Ueki *et al*, 1998).

Subsequently to insulin stimulation, the generation of PtdIns(3,4,5)$P_3$ by PI 3-kinase is necessary for re-localization of Akt to the cell surface membrane via interaction of its N-terminal PH domain. Akt was characterized to be activated by two phosphorylation steps in response to insulin (Alessi *et al*, 1996). Akt is phosphorylated at $Ser^{473}$ in a hydrophobic motif at the C-terminal tail. The identity of the kinase responsible for the phosphorylation at $Ser^{473}$ has been debated. Recent studies have reported DNA-dependent protein kinase (DNA-PK) (Feng *et al*, 2004) or the mTOR:Rictor:GβL complex (Sarbassov *et al*, 2005) to be responsible for phosphorylation at the $Ser^{473}$ residue. Thus, the true identity of this kinase or complex of protein kinases is unclear (reviewed in (Dong and Liu, 2005)). Akt is then phosphorylated at $Thr^{308}$ in the catalytic domain by 3´-phosphoinositide-dependent kinase 1 (PDK1) to achieve full kinase activity. PDK1 interacts with the second messengers products of PI 3-kinse, PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$, with its PH domain and is believed to localize in the proximity of Akt, where the phosphorylation event occur (Alessi *et al*, 1997; Mora *et al*, 2004).

There are three isoforms of Akt (Akt1, Akt2 and Akt3), which are all expressed in skeletal muscle. Experimental work with knockout mice deficient of either Akt1 or Akt2 demonstrates that there are isoform-specific roles (Cho *et al*, 2001a; Cho *et al*, 2001b). Akt2 deficient mice have an impaired ability of insulin to lower blood glucose because of defects in the action of the hormone on liver and skeletal muscle, thus Akt2 is essential in the maintenance of normal glucose homeostasis (Cho *et al*, 2001a). In contrast, Akt1 is required for normal growth, but dispensable for maintenance of glucose homeostasis in mice (Cho *et al*, 2001b).

Other signaling proteins downstream of PI 3-kinase involved in insulin signaling to glucose transport include the protein kinase C- ζ and λ isoforms (PKC-ζ/λ). PKC-ζ/λ belong to the atypical family of PKC's and are phosphorylated and activated by PDK1 (Chou *et al*, 1998). The involvement of PKC-ζ in insulin signaling to glucose uptake in skeletal muscle was first investigated in L6 myotubes, where immunoprecipitable PKC-ζ activity was inhibited by the PI 3-kinase inhibitor wortmannin (Bandyopadhyay *et al*, 1997). Furthermore, a stable and transient expression of a kinase-inactive PKC-ζ inhibited basal and insulin-stimulated glucose transport in L6 myotubes (Bandyopadhyay *et al*, 1997).

### 1.4.3 The link between insulin signaling and glucose transport

The post-receptor signal transduction events that are initiated when insulin binds to its receptor are mediated primarily by kinase/phosphoryation action and have been rather well established during the recent years. However, there is a high level of complexity within the canonical insulin signaling pathways, implying that signaling networks, rather than signaling pathways, mediate downstream responses. One of the primarily metabolic roles of insulin is to promote translocation of intracellular GLUT4 to the cell

surface membrane to facilitate glucose uptake into the cell. However, the link between the insulin signaling cascade and the translocation of the GLUT4-contaning vesicles to the plasma membrane has been elusive. Different members of the small GTPase protein families have been proposed to be required for membrane trafficking of GLUT4, with members of the Rab family of GTPases under particular investigation (Cormont and Le Marchand-Brustel, 2001).

### 1.4.4 AS160

Recently a novel Akt substrate of 160 kDa (AS160) was identified in 3T3-L1 adipocytes (Kane *et al*, 2002). This protein contains a Rab GTPase activating protein (GAP) domain and is phosphorylated on several serine and threonine residues in response to insulin. In untreated adipocytes AS160 is concentrated in the low density microsomes (LDM) fraction. Stimulation with insulin caused a marked redistribution of AS160 from the LDM compartment to the cytosol. Furthermore, mutational analysis provides evidence that AS160 is required for GLUT4 translocation in adipocytes. Expression of AS160 with two or more of the phosphorylation sites mutated to alanine markedly inhibited insulin-stimulated GLUT4 translocation (Sano *et al*, 2003). This inhibition did not occur when the GAP function was inactivated by a point mutation. These findings strongly indicate that insulin-stimulated phosphorylation of AS160 is required for GLUT4 translocation. The proposed mechanism for this action is that phosphorylation of AS160 in response to insulin leads to either inactivation of the Rab GAP function or redistribution of AS160 from the LDM compartment, which thereby activates the Rab proteins involved in GLUT4 vesicle translocation. Furthermore, insulin stimulation of GLUT4 exocytosis is dependent of AS160 phosphorylation, while the inhibiting effect of insulin on endocytosis is independent of AS160 (Zeigerer *et al*, 2004). The physiological role and regulation of AS160 in human skeletal muscle is unknown.

Several Rab proteins have been suggested to be targets for the GAP activity of AS160 (Miinea *et al*, 2005). When a recombinant GAP domain of AS160 was tested for its activity against several Rab proteins *in vitro,* Rab 2A, 8A, 10 and 14 were identified with the highest GTPase activity (Miinea *et al*, 2005).

### 1.4.5 Insulin signaling defects in insulin resistance

Several studies have provided evidence for defects at different levels in the insulin signaling cascade in human skeletal muscle using *in vivo* and *in vitro* approaches (Table 1; reviewed in (Leng *et al*, 2004)). However, whether insulin signaling defects are a cause or a consequence of skeletal muscle insulin resistance in Type 2 diabetes is unknown. The published results of insulin signaling defects in skeletal muscle are somewhat inconsistent and are mainly focused on assessments of insulin action in people with long-standing Type 2 diabetes. There are obviously many parameters that can influence the outcome from these different studies, the intention here is not to discuss these potential differences in detail, but rather to give examples of reported defects observed in insulin resistant obese and Type 2 diabetic subjects.

Protein expression of IR in skeletal muscle from Type 2 diabetic patients is unaltered (Arner *et al*, 1987; Krook *et al*, 2000). Insulin binding to IR has been reported to be normal (Ciaraldi *et al*, 2002) or impaired (Caro *et al*, 1987; Maegawa *et al*, 1991). Furthermore, tyrosine phosphorylation and/or activity of the IR has been reported to be normal (Caro *et al*, 1987; Klein *et al*, 1995; Krook *et al*, 2000; Meyer *et al*, 2002; Kim *et al*, 2003) or impaired (Arner *et al*, 1987; Maegawa *et al*, 1991; Nolan *et al*, 1994) in skeletal muscle from non-obese and obese Type 2 diabetic subjects, compared with non-diabetic subjects.

In isolated skeletal muscle from non-obese Type 2 diabetic patients and healthy subjects incubated with insulin (0.6-60 nmol/l), tyrosine phosphorylation of IR β-subunit is similar, whereas glucose transport is impaired at all levels of insulin studied (Krook *et al*, 2000). Thus, signaling defects at the level of the insulin receptor are unlikely to fully account for the impairments in glucose transport in skeletal muscle from Type 2 diabetic patients. However, in morbidly obese insulin resistant humans phosphorylation and protein expression of IR is reduced (Goodyear *et al*, 1995). Post-receptor, rather than receptor defects, are likely to account for impairments in skeletal muscle glucose uptake in diabetes.

One of the first post-receptor events in insulin signaling is IRS-1 phosphorylation. Defects in IRS-1 function have been reported in skeletal muscle from Type 2 diabetic patients. *In vivo* and *in vitro* studies reveal that insulin-stimulated tyrosine phosphorylation of IRS-1 is impaired in non-obese and obese Type 2 diabetic patients (Björnholm *et al*, 1997; Cusi *et al*, 2000; Krook *et al*, 2000; Bouzakri *et al*, 2003; Kim *et al*, 2003). This defect is not associated with altered protein expression of IRS-1 (Björnholm *et al*, 1997; Cusi *et al*, 2000; Krook *et al*, 2000; Beeson *et al*, 2003).

A growing body of evidence indicates that increased serine/threonine phosphorylation of IRS-1 can decrease the ability of IRS-1 to be tyrosine-phosphorylated by the insulin receptor. One serine residue located near the PTB domain in IRS-1 is $Ser^{312}$ ($Ser^{307}$ in rat IRS-1). IRS-1 can be phosphorylated by insulin-stimulated or stress-activated kinases, such as JNK on this serine residue (Lee *et al*, 2003), thereby disrupting the interaction between the catalytic domain of the insulin receptor and the PTB domain of IRS-1 (Aguirre *et al*, 2002). IRS-1 $Ser^{636}$ is another residue suggested to be involved in the impaired insulin signaling in Type 2 diabetes. In cultured skeletal muscle cells from Type 2 diabetic patients, increased basal phosphorylation of IRS-1 on $Ser^{636}$ has been observed, providing a putative mechanism for reduced IRS-1 tyrosine phosphorylation (Bouzakri *et al*, 2003).

Impaired insulin-stimulated PI 3-kinase activity in skeletal muscle from Type 2 diabetic subjects has been reported in several studies performed *in vivo* (Björnholm *et al*, 1997; Kim *et al*, 1999; Cusi *et al*, 2000; Beeson *et al*, 2003; Kim *et al*, 2003) and *in vitro* (Krook *et al*, 2000; Tsuchida *et al*, 2002; Bouzakri *et al*, 2003) in skeletal muscle from lean (Krook *et al*, 2000; Tsuchida *et al*, 2002) and obese Type 2 diabetic patients (Kim *et al*, 1999; Cusi *et al*, 2000; Beeson *et al*, 2003; Kim *et al*, 2003).

Little is known about the physiological regulation of PDK-1 in skeletal muscle from Type 2 diabetic patients. In a recent study, insulin action *in vivo* on PDK-1 activity in *vastus lateralis* muscle from lean, obese insulin-resistant and obese Type 2 diabetic subjects has been determined (Kim *et al*, 2003). Insulin increased PDK-1 activity two-fold in skeletal muscle from healthy subjects, with a similar response noted in obese insulin-resistant and Type 2 diabetic subjects. PDK-1 protein expression was similar between lean, obese insulin-resistant and obese Type 2 diabetic subjects. Thus, from this limited available information, insulin action on PDK-1 appears to be normal in insulin resistant skeletal muscle.

Downstream targets of PDK1 include the atypical PKC (aPKC) isoforms ζ and λ and Akt. Protein expression and activity of PKC-ζ/λ has been reported to be impaired in obese Type 2 diabetic patients *in vivo* (Beeson *et al*, 2003; Kim *et al*, 2003). Protein expression and activity of Akt is reported to be normal in obese Type 2 diabetic patients *in vivo* (Kim *et al*, 1999; Beeson *et al*, 2003). In contrast with these *in vivo* experiments, *in vitro* studies indicate that insulin-stimulated Akt activity is reduced in skeletal muscle from Type 2 diabetic subjects with pharmacological levels of insulin (Krook *et al*, 1998). However, Akt activity was normal when skeletal muscle strips were incubated with a lower concentration of insulin (Krook *et al*, 1998). Since three

**Table 1. Regulation of insulin signaling in skeletal muscle from insulin resistant and type 2 diabetic subjects**

| | Insulin receptor | | IRS-1 | | |
|---|---|---|---|---|---|
| | Binding or protein level | Phosphorylation or activity | Protein level | Tyrosine phosphorylation | Serine phosphorylation |
| Non-obese type 2 diabetic | ⇔ (1; 2)<br>⇓ (3) | ⇔ (1; 4)<br>⇓ (2; 3) | ⇔ (1; 5) | ⇓ (1; 5) | |
| Obese type 2 diabetic | ⇔ (2; 6)<br>⇓ (7) | ⇔ (7-9)<br>⇓ (2; 10) | ⇔ (11; 12) | ⇓ (8; 11; 13) | ⇑ (13) |
| Obese | ⇔ (2)<br>⇓ (7; 14) | ⇔ (7-9)<br>⇓ (2; 14) | ⇔ (11) | ⇔ (11) | |

| | PI 3-kinase | | PDK1 | | PKCζ/λ | |
|---|---|---|---|---|---|---|
| | Protein level | Phosphorylation or activity | Protein level | Phosphorylation or activity | Protein level | Phosphorylation or activity |
| Non-obese type 2 diabetic | ⇔ (15) | ⇓ (1; 5; 15) | | | | |
| Obese type 2 diabetic | ⇔ (11; 12; 16) | ⇓ (8; 11-13; 16) | ⇔ (8; 12) | ⇔ (8) | ⇓ (8; 12) | ⇓ (8; 12) |
| Obese | ⇔ (11; 16) | ⇔ (16)<br>⇓ (11) | | | ⇔ (8; 17) | ⇓ (8; 17) |

| | Akt | | GSK-3 | |
|---|---|---|---|---|
| | Protein level | Phosphorylation or activity | Protein level | Phosphorylation or activity |
| Non-obese type 2 diabetic | ⇔ (18) | ⇓ (18) | | |
| Obese type 2 diabetic | ⇔ (12) | ⇔ (12; 16) | ⇑ (19) | ⇔ (19)a<br>⇑ (19)b |
| Obese | | | ⇔ (19) | |

**a**, specific activity of GSK-3α
**b**, total activity of GSK-3

**References for Table 1:**

**1**. (Krook *et al*, 2000); **2**. (Arner *et al*, 1987); **3**. (Maegawa *et al*, 1991); **4**. (Klein *et al*, 1995); **5**. (Björnholm *et al*, 1997); **6**. (Ciaraldi *et al*, 2002); **7**. (Caro *et al*, 1987); **8**. (Kim *et al*, 2003); **9**. (Meyer *et al*, 2002); **10**. (Nolan *et al*, 1994); **11**. (Cusi *et al*, 2000); **12**. (Beeson *et al*, 2003); **13**. (Bouzakri *et al*, 2003); **14**. (Goodyear *et al*, 1995); **15**. (Tsuchida *et al*, 2002); **16**. (Kim *et al*, 1999); **17**. (Vollenweider *et al*, 2002); **18**. (Krook *et al*, 1998); **19**. (Nikoulina *et al*, 2000)

Akt isoforms are expressed in insulin target tissues, a selective isoform specific impairment may be masked by compensation from other isoforms. This has been illustrated in a study where insulin action on Akt isoforms in morbidly obese insulin resistant subjects was assessed (Brozinick *et al*, 2003). In skeletal muscle from lean insulin sensitive subjects, insulin activated all three Akt isoforms, whereas only Akt1 activity was increased in skeletal muscle from obese subjects (Brozinick *et al*, 2003).

## 1.5 ANTI-DIABETIC TREATMENT

At present several therapeutic agents are used in the treatment of Type 2 diabetes and the five main approaches are discussed (listed below):

1. **Insulin.** Insulin treatment is mainly used in a later stage in the progression of Type 2 diabetes, when the pancreatic β-cells can no longer secrete a sufficient amount of insulin to maintain an acceptable glucose concentration in the circulation. Insulin treatment has the adverse effects that it can cause hypoglycemia events and also lead to weight gain.
2. **Sulphonylureas.** Sulphonylureas treatment mainly affects the pancreatic β-cells by acting as an insulin secretagogue, generating an enhanced insulin secretion. Because sulphonylureas increase insulin levels, they also have similar adverse effects as insulin treatment, causing events of hypoglycemia and weight gain.
3. **Metformin.** Metformin belongs to the family of biguanide compounds and has glucose lowering effects. The molecular targets for the effects of metformin are currently unknown, but it mostly affects the liver, where it inhibits endogenous glucose production.
4. **Acarbose.** Treatment with acarbose leads to a reduction of glucose absorption in the gut. Acarbose targets and competitively inhibits α-glucosidase, without directly interfering with glucose uptake (Wehmeier and Piepersberg, 2004). Acarbose is often used in combinational therapy with other drugs and results in reduced post-prandial glucose levels.
5. **Thiazolidinediones.** Rosiglitazone and pioglitazone are the thiazolidinediones currently in clinical use. Troglitazone has been omitted from the market since it showed hepatic toxicity. The molecular target for thiazolidinediones is the peroxisome proliferator-activated receptor γ, where it acts as an agonist. Thiazolidinediones have insulin sensitizing effects on liver, muscle and fat. Paradoxically, treatment with thiazolidinediones often results in increased fat mass, although there is evidence for a redistribution of adipose depots (Kelly *et al*, 1999; Shadid and Jensen, 2003).

Several treatment studies in which large cohorts of patients have been followed during a longer period of time have been performed. One of those is the United Kingdom Prospective Diabetes Study (UKPDS), in which Type 2 diabetic patients were subjected to intensive blood-glucose control with either sulphonylureas or insulin compared to conventional diet treatment and followed during a 10 year period (UK Prospective Diabetes Study Group, 1998). The main outcome of this study was that the group of patients undergoing intensive treatment had a reduced risk for microvascular complications, but treatment was without effect on macrovascular disease. Furthermore, patients in the intensive treatment group had more hypoglycemic episodes and had significant increase in weight gain.

The Diabetes Prevention Program Research Group study is another large scale investigation of non-diabetic subjects with impaired glucose tolerance and thereby a high risk for the development of Type 2 diabetes (Diabetes Prevention Program Research Group, 2002). Subjects were studied during an average follow-up period of 2.8 years. The subjects treated with metformin reduced their cumulative incidence of Type 2 diabetes 31% compared to placebo. Interestingly, in another control group subjected to a life-style intervention program, the cumulative incidence of Type 2 diabetes was reduced 58%, confirming the result from a previous similar life-style intervention study (Tuomilehto *et al*, 2001).

Acarbose treatment also delays the progression of Type 2 diabetes in subjects with impaired glucose tolerance. Results from a large scale study showed that the risk of progression to diabetes over an average follow-up period of 3.3 years was reduced 25% compared to a randomized placebo group (Chiasson *et al*, 2002).

Thiazolidinediones (TZD's) act as agonistic ligands for the transcription factor PPARγ that belongs to the nuclear hormone receptor superfamily. The anti-diabetic insulin-sensitizing effects of TZD's were known before PPARγ was identified as the target of action (Lehmann *et al*, 1995). In a recent report, high-risk Hispanic women with previous gestational diabetes were treated 3 months with troglitazone in a randomized placebo controlled double blind study. Treatment with troglitazone delayed the onset or prevented Type 2 diabetes. The protective effect of troglitazone was associated with preservation of β-cell function that was mediated by a reduced insulin secretory demand, caused by insulin sensitization (Buchanan *et al*, 2002). Troglitazone monotherapy has been reported to decrease fasting and postprandial glucose levels in patients with Type 2 diabetes, primarily by augmenting insulin-mediated glucose disposal after a 6 month treatment period (Maggs *et al*, 1998). Furthermore, pioglitazone monotherapy significantly improves $HbA_{1c}$ and fasting plasma glucose, while producing beneficial effects on serum lipids in patients with Type 2 diabetes (Aronoff *et al*, 2000).

In summary, these treatment studies demonstrate that many of the interventions, especially changes in life-style factors such as diet and exercise, are effective at least in delaying the onset of Type 2 diabetes in high risk individuals with impaired glucose tolerance. Furthermore, treatment interventions of overt Type 2 diabetes can often help maintain glycemic control and delay the progression of pathological complications. However, there are presently no pharmacological interventions identified to cure and reverse the Type 2 diabetic phenotype. Given the overwhelming predicted rise in the incidence of Type 2 diabetes, efforts to identify predictive biomarkers and treatment strategies are desperately needed.

# 2 AIMS

The overall aim of this thesis is to investigate insulin signaling mechanisms leading to impaired glucose uptake and insulin resistance in human skeletal muscle. The following specific aims were addressed:

- To investigate whether insulin action on the Akt substrate, AS160, is impaired in Type 2 diabetic subjects.

- To investigate whether pharmacological treatment improves glucose uptake and enhances insulin signaling in skeletal muscle of Type 2 diabetic subjects.

- To assess whether insulin signaling and glucose uptake in first-degree relatives to subjects with Type 2 diabetes is altered in order to determine whether peripheral defects are a cause or a consequence of insulin resistance.

- To investigate the mRNA expression of SelS/AD-015, a putative inflammatory marker, in skeletal muscle and adipose tissue from Type 2 diabetic subjects.

# 3 EXPERIMENTAL PROCEDURES

## 3.1 SUBJECTS

The subjects in Paper I, III and IV were recruited through local newspapers advertisements. The subjects in paper II were recruited by advertisement and among clients of the occupational health service in Turku, Finland. All healthy and Type 2 diabetic subjects were matched for sex, age and BMI. Except for the anti-diabetic drugs, no other drugs were taken. The subjects were instructed to abstain from any form of strenuous physical activity for a period of 72 h (Paper III and IV), 48 h (Paper I) or 24 h (Paper II) before the experiment. The subjects reported to the laboratory after an overnight fast, and in the case of the Type 2 diabetic patients, before administration of anti-diabetic medication. Subjects received oral and written information regarding the nature of their participation and informed consent was received. All studies were approved by the ethical committees at Karolinska Institutet. The study in Paper II was approved by the local ethical committee at the University of Turku, Finland.

## 3.2 EUGLYCEMIC HYPERINSULINEMIC CLAMP PROCEDURES

The euglycemic hyperinsulinemic clamp technique (DeFronzo *et al*, 1979) was used to determine insulin-stimulated glucose disposal. In brief, a catheter is inserted into the antecubital vein for glucose and insulin infusion and into the brachial artery for blood sampling. After baseline blood samples and biopsy samples are taken, insulin is infused at a set rate to establish hyperinsulinemia. Under these conditions, glucose production from the liver is inhibited. Plasma glucose concentration was kept constant by a variable glucose infusion that was adjusted after plasma glucose measurements every 5 min. Blood samples were obtained frequently for analysis of plasma-free insulin concentration. During the last 60 min a steady state in plasma glucose concentration was achieved. The glucose infusion rate required to maintain euglycemia during the last hour of the clamp was used as a measure of whole-body insulin sensitivity.

In Paper I, a modification of the hyperinsulinemic clamp procedure was used. After a 30 min glucose priming period, a bolus injection of insulin was administered (17.6 nmol $\cdot$ kg$^{-1}$ $\cdot$ min$^{-1}$) for 4 min, and hyperinsulinemia was maintained by means of a continuous insulin infusion (5.5 nmol $\cdot$ kg$^{-1}$ $\cdot$ min$^{-1}$) for 180 min.

In Paper II, the euglycemic hyperinsulinemic clamp consisted of a 160 min intravenous insulin infusion (1 mU$\cdot$kg$^{-1}\cdot$min$^{-1}$) while normoglycemia was maintained by a variable infusion of 20% glucose.

In Paper IV, insulin-mediated glucose utilization was determined using the euglycemic hyperinsulinemic clamp procedure, where insulin was infused at a rate of 40 mU $\cdot$ m$^{-2}$ $\cdot$ min$^{-1}$ for 180 min.

## 3.3 TISSUE BIOPSIES AND PREPARATION

In Paper I, muscle biopsies were removed with a Weil-Blakesly's conchotome (Henriksson, 1979) under local anesthesia (mepivacain chloride 5 mg/ml). A basal biopsy sample was obtained after a 30 min glucose priming period. A second biopsy (insulin-stimulated) was obtained 40 min after the onset of the insulin infusion. Serum insulin levels at the time of the second muscle biopsy were ~600 pM. Each biopsy was obtained from different muscle bundles from the same incision site.

In Paper II, muscle biopsy samples were obtained at each clamp occasion using a Bergström's needle with suction. Skeletal muscle biopsy samples were obtained under local anesthesia (lidocaine hydrochloride 10 mg/ml) before (basal) and 160 minutes after the onset of the insulin infusion in the rested (insulin-stimulated), as well as in the exercised (insulin plus exercise) leg. When two biopsy samples were taken from the same leg, the second sample was taken 3 cm distal from the first incision site.

In Paper III, open muscle biopsies were obtained under local anesthesia (mepivakain chloride 5 mg/ml), as previously described (Zierath, 1995). Briefly, a 4 cm incision was made 15 cm above the proximal border of patella, and muscle fascia was exposed. Thereafter 4-5 muscle fiber bundles (approximately 1 g of muscle) were excised carefully to avoid stretching. Muscle biopsies were placed in oxygenated Krebs-Henseleit buffer (KHB), which contained 5 mM HEPES, 5 mM glucose, 15 mM mannitol and 0.1% bovine serum albumin (BSA).

In Paper IV, muscle biopsies were removed with a Weil-Blakesly's conchotome (Henriksson, 1979) under local anesthesia (mepivacain chloride 5 mg/ml). A biopsy sample was obtained under basal conditions and 180 min after the onset of the insulin infusion. Adipose tissue biopsies were taken by needle aspiration under local anesthesia (mepivacain chloride 5 mg/ml) from subcutaneous abdominal adipose tissue at the level of umbilicus in the basal state and after 180 min of insulin infusion (Koistinen *et al*, 2000).

All muscle biopsy samples were immediately frozen and stored in liquid nitrogen or at -80°C until analysis.

### 3.3.1 Skeletal muscle processing for insulin signaling

Muscle biopsies were freeze-dried overnight and then dissected under a microscope to remove visible blood, fat and connective tissue. Samples were subsequently homogenized in ice cold homogenization buffer containing 20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 % Triton X-100, 10 % (w/v) glycerol, 10 mM NaF, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM Na-pyrophosphate, 0.5 mM Na3VO4, 1 μg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1 μg/ml aprotinin, 1 mM dithiothreitol (DTT), 1 mM benzamidine and 1 μM microcystin. The homogenized samples were then rotated for 30 min at 4°C and thereafter subjected to centrifugation (12 000 g for 15 min at 4°C). Protein concentration was determined in the supernatant using the BCA protein assay kit (Pierce, Rockford, IL, USA).

### 3.3.2 RNA extraction and cDNA synthesis

In Paper II and Paper IV, skeletal muscle and adipose tissue biopsies (Paper IV) were removed from liquid nitrogen and homogenized using a polytron mixer in 1 ml guanidium thiocyantate-phenol solution (Sigma Tri-Reagent, Sigma, St. Louis, MO, USA) and total RNA extracted according to the manufacturer's instructions. Extracted RNA was subjected to DNase I treatment using a DNA-free kit (Ambion, Austin, TX, USA), according to manufacturer's instructions. cDNA was synthesized using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), using random hexamer primers according to the manufacturer's instructions.

## 3.4 ASSAYS AND ANALYSIS

### 3.4.1 *In vitro* skeletal muscle incubations

Skeletal muscle strips (~15-25 mg) were dissected from specimen obtained during the open muscle biopsy surgery. Muscle strips were mounted on plexiglass clamps, and incubated in pre-gassed (95% $O_2$ and 5% $CO_2$) KHB in shaking water bath (35°C). The

gas phase in the vials was maintained during the incubation procedure. After a 30 min incubation in KHB, skeletal muscle strips were incubated for 30 min (35°C) in KHB without (basal) or with increasing concentrations of insulin (0.6 nM, 1.2 nM, and 120 nM). The concentration of insulin was maintained throughout the incubation procedures.

### 3.4.2 Skeletal muscle glucose transport

Skeletal muscle strips were transferred to fresh KHB containing 20 mM mannitol and incubated (35°C) for 10 min. Thereafter, muscles were incubated 20 min in KHB containing 5 mM 3-O-methyl [$^{3}$H]glucose (800 μCi/mmol) and 15 mM [$^{14}$C]mannitol (53 μCi/mmol). Thus, muscle strips were exposed to insulin for a total of 60 min. After the final incubation, the muscle strips were blotted of excess fluid, snap frozen in liquid nitrogen and stored at –80 °C until analysis. Glucose transport was analyzed by the accumulation of intracellular 3-O-methyl [$^{3}$H]glucose, as previously described (Wallberg-Henriksson *et al*, 1987).

### 3.4.3 IRS-1 tyrosine phosphorylation

For IRS-1 tyrosine phosphorylation aliquots of muscle lysates (800 μg protein) were immunoprecipitated with anti-IRS-1 antibody (Martin G Myers, Joslin Diabetes Center, Boston, MA, USA) overnight at 4°C. Thereafter, protein A sepharose beads were added to the lysates and samples were incubated for 2 h at 4°C. The immunoprecipitates were washed three times with homogenization buffer, two times with buffer B (0.1 M Tris (pH 8.0) and 0.5 M LiCl) and one time with buffer C (10 mM Tris (pH 7.6), 0.15 M NaCl and 1 mM EDTA). Pellets were resuspended in Laemmli buffer containing β-mercaptoethanol. Samples were heated at 95°C for 4 min and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and blocked with Tris-buffered saline with 0.02 % Tween (TBST) containing 5% milk. Membranes were incubated with horseradish peroxidase conjugated (HRP) anti-phosphotyrosine antibodies (PY2005, Affiniti, Exeter, UK) overnight at 4°C. Immuno-reactive proteins were visualized by enhanced chemiluminescence (ECL plus; Amersham, Arlington Heights, IL; USA) and quantified using densitometry and Molecular Analyst Software (Bio-Rad, Richmond, CA, USA).

### 3.4.4 PI-3 kinase activity

An aliquot of supernatant (1 mg protein) was immunoprecipitated with anti-IRS-1 antibody and washed, as described above (IRS-1 tyrosine phosphorylation). Immunoprecipitates were then washed one time with buffer D (20 mM HEPES (pH 7.3), 1 mM DTT, 5 mM $MgCl_2$) and thereafter, resuspended in 20 μl of buffer E (20 mM Hepes (pH 7.3), 20 mM β-glycerophosphate (pH 7.2), 5 mM $Na_4P_2O_7$, 30 mM NaCl and 1 mM DTT). The kinase reaction was started adding 30 μl of buffer F (buffer E containing 12.5 μM ATP, 7.5 mM $MgCl_2$, 20 μg phosphatidyl inositol per reaction (Avanti Polar Lipids, Alabaster, AL) and 20 μCi [γ-$^{32}$P]ATP per reaction) and carried out for 15 min at room temperature. The reaction was terminated by addition of 150 μl of 1% perchloric acid. Thereafter, a 2:1 mixture of methanol:chloroform was added followed by two washes with 1% perchloric acid, where the aqueous phase was removed between washes. The reaction product was applied onto a silica gel coated thin layer chromatography aluminum sheet (Silica gel 60; Merck, Darmstadt, Germany) and was separated in a pre-equilibrated tank containing methanol:chloroform:ammonia:water (75:54:20:10) and quantified using a PhosphorImager (Molecular Analyst Software, ver. 1.5; Bio-Rad).

### 3.4.5 Western blot analysis

An aliquot of muscle lysate was mixed with Laemmli buffer containing β-mercaptoethanol. Proteins were separated by SDS-PAGE, transferred to polyvinylidenediflouride or nitrocellulose membranes and blocked in fat-free milk for 2 h in room temperature. Membranes were incubated with respective primary antibody overnight at 4°C. Incubated membranes were washed in TBST and incubated with appropriate secondary HRP-conjugated antibodies (Bio-Rad). Proteins were visualized by enhanced chemiluminescence (ECL or ECL Plus; Amersham) and quantified using densitometry and Molecular Analyst Software (Bio-Rad, Richmond, CA).

### 3.4.6 Real Time PCR

In Paper IV, mRNA expression was determined by means of real time quantitative polymerase chain reaction (PCR) (Heid *et al*, 1996). For Tanis/SelS/AD-015, primers and probe were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA). The human homologue of TANIS nucleotide sequence is SelS/AD-015 with accession no AF157317 (Homo sapiens AD-015), which was obtained from the NCBI database. AD-015 mRNA coding sequence consist of 6 exons and is in total 1209 base pair in length. The probe was designed to overlap the exon 1 / exon 2 boundary, to selectively detect cDNA from coding sequence mRNA and not DNA from potential contamination of genomic sequences.

Probe: 5´- 7AC ACC ACG GTG GGC TCC CTG CT - 3´; 7 = 6-FAM, Reporter: FAM Quencher: TAMRA.

Forward Primer: 5´ - CGA GGG GCT GCG CTT C - 3´.

Reverse Primer: 5´ - GAT GTA CCA GCC ATA GGT GGC - 3´

These primers generate an amplicon of 64 base pairs.

Three µl cDNA (corresponding to 0.15 µg of total RNA) was amplified using 300 nM of both primer and probe in an Applied Biosystems Taqman Universal PCR mastermix (Brachburg, NJ, USA) by real time quantitative PCR, using an ABI PRISM 7900 HT (PE Applied Biosystems). cDNA specificity of each primer pair was verified by RT-PCR using RNA processed by a cDNA protocol with the omission of reverse transcriptase. For normalization of RNA loading, control samples were assessed using β2-microglobulin housekeeping gene. Expression levels were quantified by generating a five-point serial standard curve.

### 3.4.7 Multi Fluidic Card analysis

In Paper II, gene expression analysis was carried out utilizing a Taq-Man based Multi Fluidic Card (MFC) gene expression assay (Applied Biosystems). MFC is a high throughput variant of real-time PCR analysis. The card consists of 8 loading ports connected each by a channel system to 48 wells (384 wells in total). The MFC was specifically designed to assess the expression of 24 genes (5 endogenous controls and 19 target genes) in duplicate. The primer and probe sets from Applied Biosystems were pre-lyophilized in the MFC wells. Briefly, cDNA samples were mixed with Applied Biosystems Taqman Universal PCR mastermix and pipetted into the loading ports of the card. The sample mixes were distributed through the channel system to all 48 individual wells in each system (2 µl reaction volume/well) by centrifugation and thereafter each well was individually sealed to avoid diffusion and cross-contamination. The card is loaded into the 7900 HT real-time PCR for analysis. Determination of

target gene mRNA expression was normalized to mRNA expression of GAPDH as endogenous control.

## 3.5 STATISTICS

Data are presented as means ± SE. Student's paired or unpaired *t*-test or the Wilcoxon's matched-pairs signed-rank test was used to assess differences, as appropriate. Differences within and between groups were determined by ANOVA. Fisher's least significant difference *post hoc* analysis was used to identify significant differences. Pearson's correlation analysis was applied to determine the existence of possible relationships. Differences were considered significant at $P < 0.05$.

# 4 RESULTS AND DISCUSSION

During the last 20 years, the study of the insulin signaling pathway to glucose uptake in skeletal muscle has lead to many breakthroughs, and has provided a better understanding of the mechanisms involved. Improved experimental tools, together with more powerful computational analysis, have contributed to advances within molecular biology and cellular signaling. However, concomitant with identification of novel intermediates within the signaling cascades and discoveries of new protein interactions and signaling feed-back loops, the complexity of intracellular signaling systems has been made apparent. To be able to design pharmaceutical drugs that can eventually correct defects within this signaling system, there are still many questions that remain to be answered. Undoubtedly, treatment efforts for the rapid global increase in obesity, Type 2 diabetes and the metabolic syndrome are one of the major challenges for the medical research field in the nearest future. This thesis work is focused on identifying insulin signaling defects in human skeletal muscle, with a comparative approach where insulin resistant Type 2 diabetic patients or first-degree relatives of Type 2 diabetic patients have been contrasted against well-matched healthy control subjects.

## 4.1 *IN VIVO* INSULIN ACTION ON AS160

There has been great progress in elucidating the insulin signaling pathway leading to GLUT4 translocation and glucose uptake in skeletal muscle cells. In addition, the understanding of mechanisms involved in the trafficking of GLUT4 containing vesicles and their fusion with the cell surface plasma membrane has also been evolving. However, the link between the insulin signaling cascade and the GLUT4 translocation machinery is still elusive.

In 2002, the first report was published whereby a novel Akt substrate was proposed to link the proximal insulin signaling steps to the translocation of GLUT4 vesicles (Kane *et al*, 2002). Akt phosphorylates its substrate proteins at a RXRXXS/T peptide sequence motif. Using antibodies that specifically recognize the phosphorylated Akt substrate epitope, an Akt substrate of 160 kDa, named AS160, was identified in 3T3-L1 adipocytes.

AS160 is phosphorylated on several serine and threonine residues in response to insulin in 3T3-L1 adipocytes. When AS160 is phosphorylated, it redistributes from LDM compartments to the cytosol (Kane *et al*, 2002). When several of the serine and threonine residues, which are phosphorylated in response to insulin, are mutated to alanine, it markedly inhibits GLUT4 translocation in 3T3-L1 adipocytes (Sano *et al*, 2003; Zeigerer *et al*, 2004). These studies reveal that AS160 is required for the translocation of GLUT4 vesicles in adipocytes. Furthermore, mRNA of AS160, also designated as KIAA0603 (TBC1 domain family member 4), is highly expressed in human heart and skeletal muscle tissue compared to other tissues (Matsumoto *et al*, 2004). In addition, AS160 is phosphorylated in response to insulin in a dose-dependent fashion in rat skeletal muscle (Arias *et al*, 2004; Bruss *et al*, 2005). However, there are as yet no reports of insulin action on AS160 in human skeletal muscle. Thus, we investigated whether AS160 is phosphorylated in response to insulin in human skeletal muscle during an *in vivo* insulin infusion. We further hypothesized that defects in insulin action on AS160 are associated with insulin resistance in human skeletal muscle.

Basal and insulin-stimulated skeletal muscle biopsy samples were obtained from Type 2 diabetic patients and well-matched control subjects (Paper I). Immunoblot

analysis with an anti-phospho-Akt substrate (PAS) antibody revealed several insulin-responsive phospho-proteins, where a protein of 160 kDa was the most prominent (Paper I, Figure 1). We confirmed the 160 kDa protein to be AS160 by means of immunoprecipitation experiments using an anti-AS160 antibody (Paper I, Figure 2). Phosphorylation under basal conditions was comparable between Type 2 diabetic patients and control subjects (Paper I, Figure 4B). However, the increment of insulin-induced phosphorylation over basal state was reduced 39% in Type 2 diabetic patients compared to healthy controls (Paper I, Figure 4B). Protein expression of AS160 was similar between Type 2 diabetic patients and healthy control subjects (Paper I, Figure 4A). Therefore, the reduction in AS160 phosphorylation is not caused by reduced protein expression. Our results provide evidence to suggest that impaired insulin-induced phosphorylation of AS160 is likely to play a role for the reduction of GLUT4 translocation and glucose uptake observed in skeletal muscle of Type 2 diabetic patients.

**Insulin action on Akt**

Given that AS160 phosphorylation was impaired in the cohort of Type 2 diabetic patients in the present study, phosphorylation status of the serine/threonine protein kinase Akt was determined. Akt is phosphorylated at residue $Ser^{473}$ and $Thr^{308}$ in response to insulin in order to become fully activated (Alessi *et al*, 1996). Earlier studies have demonstrated early post-receptor insulin signaling defects in human skeletal muscle from Type 2 diabetic patients at the level of tyrosine-phosphorylation of IRS-1 and PI 3-kinase activity (Björnholm *et al*, 1997; Kim *et al*, 1999; Krook *et al*, 2000). Despite the observed early post-receptor signaling defects, the downstream kinase, Akt, has been reported to have normal *in vivo* insulin response in Type 2 diabetic patients (Kim *et al*, 1999; Beeson *et al*, 2003). However, at pharmacological insulin concentrations during *in vitro* incubation of human skeletal muscle, Akt activity is diminished (Krook *et al*, 1998).

We analyzed phosphorylation state of Akt at $Ser^{473}$ and $Thr^{308}$ in the skeletal muscle samples (Paper I). Insulin-induced $Ser^{473}$-phosphorylation was comparable between Type 2 diabetic patients and control subjects (Paper I, Figure 5A). However, the insulin-induced $Thr^{308}$-phosphorylation increment over basal was reduced 51% in Type 2 diabetic patients (Paper I, Figure 5B). The discrepancy between observed unchanged Akt activity (Krook *et al*, 1998; Kim *et al*, 1999), and reduced $Thr^{308}$-phosphorylation in our cohort of subjects is unclear. However, isoform-specific impairments in insulin action of Akt2 and Akt3, but not Akt1, have been observed in skeletal muscle from morbidly obese insulin resistant subjects (Brozinick *et al*, 2003). Thus, Akt isoform-specific defects could potentially account for the differences in insulin action on Akt activity and phosphorylation, as assessed in our moderately obese diabetic cohort. However, an isoform-specific analysis was not performed in this cohort. Alternatively, the impairment in insulin-induced $Thr^{308}$-phosphorylation may be a consequence of the altered metabolic milieu, as the Type 2 diabetic patients in this study were studied under hyperglycemic conditions. Consistent with this hypothesis, an earlier study revealed insulin-induced $Thr^{308}$-phosphorylation was unchanged between control subjects and Type 2 diabetic patients after an overnight normalization of glycemia (Meyer *et al*, 2002).

The reduction in $Thr^{308}$-phosphorylation of Akt in the Type 2 diabetic cohort coincided with the reduction of phosphorylation of the downstream Akt substrate AS160, thereby implying a causative upstream defect is responsible for reduced phosphorylation on AS160. This is further supported by the observation that two other phospho-proteins that were detected with the PAS antibody, with molecular weights of ~300 kDa and ~46 kDa, showed a tendency for reduced insulin-stimulated

phosphorylation. The identity of these phospho-proteins is currently unknown. However, the ~46 kDa protein is likely to be GSK-3β, since co-migration was observed when membranes were re-probed with a GSK-3β antibody. We hypothesized that the ~300 kDa protein was mTOR, since mTOR is reported to be a direct target of Akt (Nave *et al*, 1999). However, immunoblot analysis of mTOR protein expression indicated that the high molecular weight protein did not correspond to mTOR.

**Hypothesis for the mechanism linking insulin signaling to GLUT4 translocation**

A proposed hypothesis for the involvement of AS160 in mediating translocation of GLUT4 containing vesicles to the plasma membrane is based on evidence from experiments using different mutated forms of AS160 (Sano *et al*, 2003; Zeigerer *et al*, 2004). This hypothesis is based on the fact that AS160 contains a GAP domain for Rab proteins (Kane *et al*, 2002), which are small G proteins involved in GLUT4 membrane trafficking (reviewed in (Cormont and Le Marchand-Brustel, 2001)). Rab proteins are in an activated form when bound to GTP and inactivated when bound to GDP. Rabs have a relatively low intrinsic GTPase activity. GAP domain-containing proteins have the ability to catalyze the conversion of GTP to GDP and thereby keep Rabs in an inactive form. The active AS160 Rab GAP domain may retain the Rab proteins in an inactive form and thereby retain GLUT4 vesicles in an intracellular compartment. When AS160 is phosphorylated in response to insulin, GAP activity may either be inhibited or alternatively AS160 is relocated from the LDM compartment, resulting in release of GLUT4 vesicles that subsequently undergo translocation to the cell surface membrane.

While the exact nature of the Rab target for AS160 is unknown, Rab4 and Rab11 have been linked to GLUT4 traffic in insulin sensitive tissues. Rab4 has been shown to co-precipitate with GLUT4 containing vesicles in intracellular membrane fractions in rat skeletal muscle (Aledo J. C. *et al*, 1995; Sherman *et al*, 1996) and upon insulin stimulation, the abundance of Rab4 was decreased in intracellular membrane compartments and undetected at the plasma membrane (Sherman *et al*, 1996). Rab11 has been suggested to be involved in the endosomal recycling, sorting and exocytotic movement of GLUT4 in rat cardiac muscle (Kessler *et al*, 2000). Clearly more work in this area is required.

In a recent report, a recombinant GAP domain of AS160 was tested for its activity against several Rab proteins in adipocytes (Miinea *et al*, 2005). The study provided evidence that Rab 2A, 8A, 10 and 14 have the highest GTPase activity in response to exposure of the recombinant GAP domain of AS160. Thus, AS160 may constitute a point of convergence between insulin signaling and GLUT4 vesicular trafficking. However, the exact Rabs targeted by AS160 in human skeletal muscle GLUT4 trafficking are still unknown.

## 4.2 INSULIN SIGNALING AFTER ANTI-DIABETIC TREATMENT

In clinical use today, there are several treatment approaches which are administered in order to reduce hyperglycemia in Type 2 diabetic patients. The oral anti-diabetic agent metformin (dimethyl biguanide) has the longest history in diabetes treatment. Metformin was introduced in 1957, where it was reported to have anti-diabetic properties (reviewed in (Bailey and Day, 2004)). Metformin is the most widely prescribed agent for diabetes treatment today. Despite being in clinical use for almost half a century, the identity of its cellular target is still elusive. Metformin primarily acts on the liver, where it inhibits glucose production (Bailey and Turner, 1996), possibly by action via AMP-activated protein kinase (AMPK) (Zhou *et al*, 2001).

A more recent pharmacological agent for Type 2 diabetes treatment is rosiglitazone, a drug that belongs to the TZD family. TZD's act as agonistic ligands of the nuclear hormone receptor transcription factor PPARγ. PPARγ is predominantly expressed in adipocytes, but it is also expressed in skeletal muscle (Zierath *et al*, 1998). Many studies have reported positive effects such as improved glycemic control after rosiglitazone treatment (Patel *et al*, 1999; Fonseca *et al*, 2000; Nolan *et al*, 2000; Raskin *et al*, 2000; Wolffenbuttel *et al*, 2000; Lebovitz *et al*, 2001; Phillips *et al*, 2001; Raskin *et al*, 2001). Even though the insulin sensitization effects of TZD's are well-documented, the molecular mechanisms responsible for these effects in humans are still elusive. Thus, investigations of potential effects on insulin signaling responses and gene expression in human skeletal muscle after treatment with either metformin or rosiglitazone are required. Early treatment of Type 2 diabetic patients in an early phase of the disease progression, before the metabolic derangements become too severe, is of particular importance to facilitate attempts to hinder many of the end-stage complications related to the disease. Moreover, investigations of the treatment effects in moderately obese newly diagnosed Type 2 diabetic patients may have the potential to reveal outcomes that might be masked in obese patients with a longer duration of the disease, where many other factors would have to be taken into account caused by the more severe metabolic derangements.

In Paper II, we investigated *in vivo* insulin signaling in skeletal muscle before and after treatment with either metformin or rosiglitazone. Newly diagnosed Type 2 diabetic patients were recruited and randomized into three groups, one treated with metformin, one with rosiglitazone, and one placebo control group. The treatment period lasted 26 weeks. Before and after the treatment period, patients were subjected to a euglycemic hyperinsulinemic clamp whereby whole-body, as well as skeletal muscle specific glucose uptake was determined. A one-legged intermittent isometric leg extension exercise was performed between 45 min and 150 min of the clamp procedure (Paper II, Figure 1). The intensity of exercise was set to 10% of the maximal isometric force measured individually. Skeletal muscle biopsy samples were obtained at basal (before the clamp) and at insulin-stimulated conditions, with and without exercise.

The clinical and metabolic characteristics of the study participants were assessed before and after treatment and are reported in Paper II, Table 1. BMI, $HbA1_c$ and fasting plasma glucose were decreased after metformin treatment ($P<0.05$), but not after rosiglitazone or placebo treatment. Fasting FFA levels were similar between the groups and unchanged after treatment. However, post-treatment serum FFA levels measured during the hyperinsulinemic clamp were decreased 30% ($P < 0.05$) and 48% ($P < 0.01$) after metformin and rosiglitazone treatment, respectively, and unchanged in the placebo group.

Whole-body and skeletal muscle specific glucose uptake was significantly increased after rosiglitazone treatment (Paper II, Figure 2). In contrast, after treatment with metformin or placebo, insulin-mediated glucose uptake was unaltered. Whole-body glucose uptake was tightly correlated with glucose uptake in skeletal muscle measured with PET scan analysis over a portion of the *vastus lateralis* muscle (Paper II, Figure 6A). This is consistent with earlier work whereby whole body insulin-mediated glucose uptake was positively correlated with skeletal muscle glucose transport (Zierath, 1995). We hypothesized that the increase in insulin sensitivity in skeletal muscle was due to an augmentation in intracellular insulin signaling events. We began analyzing signaling events at the early post-receptor level, namely tyrosine-phosphorylation of IRS-1 and IRS-1-associated PI 3-kinase activity, since these intermediate steps have been reported to be impaired in Type 2 diabetic patients (Björnholm *et al*, 1997; Krook *et al*, 2000). Prior to treatment, insulin infusion increased IRS-1 tyrosine phosphorylation and IRS-1 associated PI 3-kinase 1.7-fold

and 2-fold respectively, with similar effects between the rested and exercised leg. Neither metformin nor rosiglitazone treatment enhanced insulin-stimulated IRS-1 tyrosine phosphorylation nor IRS-1-associated PI 3-kinase activity. We then analyzed the downstream signaling events related to Akt, including $Ser^{473}$-phosphoryaltion of Akt and phosphorylation of the Akt substrate AS160. Before the treatment period, insulin stimulation increased $Ser^{473}$-phosphorylation and AS160 2-3-fold, with similar effects between the rested and exercised leg. The fold-changes in insulin-induced phosphorylation of Akt and AS160 were similar to that observed in Paper I. However, neither metformin nor rosiglitazone treatment enhanced phosphorylation of Akt nor AS160 (Paper II, Figure 4 and 5).

Metformin treatment improved $HbA_{1c}$ and fasting blood glucose levels, but was without effect on insulin-mediated glucose uptake. This is consistent with previous studies of poorly controlled obese type 2 diabetic patients (Stumvoll *et al*, 1995; Hundal *et al*, 2000). Previous studies have provided evidence that improvements in glucose homeostasis after metformin treatment are primarily due to an insulin-sensitizing effect on the liver, leading to a decrease in hepatic glucose production (Stumvoll *et al*, 1995; Inzucchi *et al*, 1998; Hundal *et al*, 2000). Furthermore, increased rates of insulin-stimulated whole-body glucose disposal have been reported (Inzucchi *et al*, 1998; Kim *et al*, 2002). However, the increase in glucose disposal rate was minor and may potentially be explained by an improvement in glucose homeostasis.

In contrast to metformin treatment in the present study, rosiglitazone treatment enhanced insulin-mediated whole body and leg muscle glucose uptake, but was without effect on $HbA_{1c}$ and fasting blood glucose. The improvement in insulin-mediated glucose uptake reported here is consistent with previous studies whereby either troglitazone (Petersen *et al*, 2000; Kim *et al*, 2002) or rosiglitazone (Carey *et al*, 2002; Beeson *et al*, 2003; Miyazaki *et al*, 2003) was administered, further supporting the insulin sensitizing effect on skeletal muscle. However the lack of an improvement in $HbA_{1c}$ and fasting blood glucose is an inconsistent finding, since both improved (Miyazaki *et al*, 2001; Carey *et al*, 2002; Kim *et al*, 2002; Miyazaki *et al*, 2003) and unchanged (Patel *et al*, 1999; Mayerson *et al*, 2002) glycemic control has been observed in Type 2 diabetic patients undergoing TZD treatment.

Apparently, the insulin-sensitizing effect of rosiglitazone on glucose uptake in skeletal muscle is unrelated to changes in insulin signaling at the intermediate steps analyzed in the present study. This is an unexpected finding since similar studies have been performed whereby positive effects of TZD-treatment on insulin signaling intermediates have been reported. For example, similar to results presented in Paper II, a previous study provides evidence that treatment of poorly controlled obese type 2 diabetic patients with metformin for 3-4 months enhanced insulin-mediated glucose uptake independent of improved insulin signaling (Kim *et al*, 2002). However, in contrast to results presented in Paper II, treatment with troglitazone for 3-4 months improved insulin-mediated whole body glucose uptake concomitant with improved insulin action on PI 3-kinase and Akt (Kim *et al*, 2002). Furthermore, treatment of obese type 2 diabetic subjects for one month with rosiglitazone also enhanced insulin-mediated whole body glucose uptake, coincident with increased IRS-1 associated PI 3-kinase and atypical PKC activity (Beeson *et al*, 2003). These studies imply the insulin-sensitizing effect of TZD treatment in obese type 2 diabetic subjects is partly caused by enhanced insulin signaling in skeletal muscle. One common feature between these previous reports (Kim *et al*, 2002; Beeson *et al*, 2003) is that poorly controlled obese Type 2 diabetic subjects were studied, whereas in Paper II, newly diagnosed drug naïve Type 2 diabetic subjects were studied. There are no additional reports as to whether similar effects on insulin signaling occur in moderately obese or even newly diagnosed

Type 2 diabetic subjects, where the metabolic derangements are presumably less severe.

There is a possibility that inconsistencies between the results presented in Paper II and those of studies whereby troglitazone or pioglitazone was administered, could partly be explained by the fact that different members of the TZD family can have different biological effects. Differences in the ligand interaction with PPARγ are dependent on distinctive molecular structures of the ligands. There is evidence that troglitazone and rosiglitazone induce different sets of genes during *in vitro* experiments in various cell types (Camp *et al*, 2000). Furthermore, several studies report differences in lipid profiles after treatment with either rosiglitazone or pioglitazone (King, 2000; Khan *et al*, 2002; Goldberg *et al*, 2005).

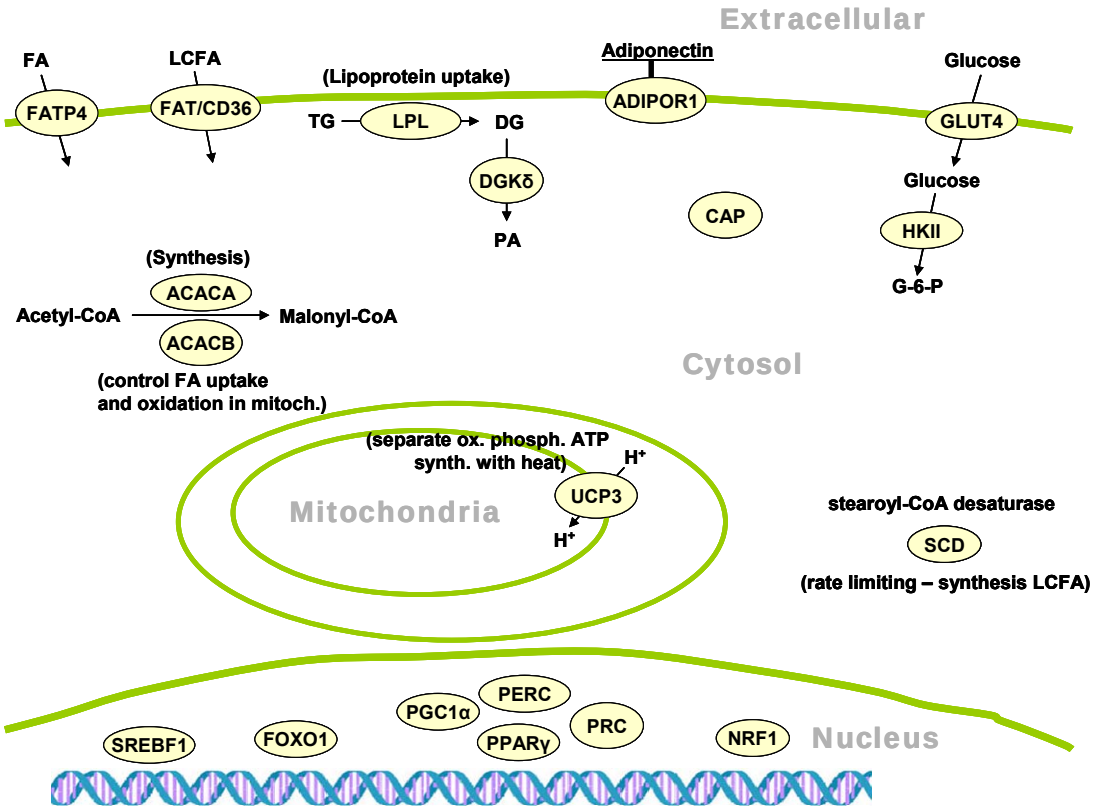


**Figure 5. Target genes involved in glucose- and lipid metabolism and transcriptional regulation.** FATP4, fatty acid transporter 4; FAT/CD36, fatty acid translocase; LPL, lipoprotein lipase; DGKδ, diacylglycerol kinase delta; ADIPOR1, adiponectin receptor 1; CAP, Cbl associated protein; GLUT4, glucose transporter 4; HKII, hexokinase II; ACACA, acetyl-CoA carboxylase alpha; ACACB, acetyl-CoA carboxylase beta; UCP3, uncoupling protein 3; SCD, stearoyl-CoA desaturase; SREBF1, regulatory element binding protein 1; FOXO1, forkhead box O1A; PPARγ, peroxisome proliferative activated receptor gamma; PGC1α, peroxisome proliferative activated receptor gamma coactivator 1 alpha; PERC, peroxisome proliferative activated receptor gamma coactivator 1 beta; PRC, PGC-1 related co-activator; NRF-1, nuclear respiratory factor 1.

### Gene expression analysis

In addition to analysis of the insulin signaling parameters, mRNA expression of genes involved in glucose, and lipid metabolism and transcription regulation was assessed (Figure 5; Quantified data in Paper II, Table 2). In total, expression of 19 genes was measured in skeletal muscle under basal conditions after the treatment period.

Several lines of evidence suggest TZD's achieve a therapeutic effect partly through changes in gene expression (Way *et al*, 2001; Gerhold *et al*, 2002). In diabetic KK/Ta mice, pioglitazone treatment for 2 weeks increased UCP2 and decreased UCP3 mRNA expression (Shimokawa *et al*, 1998), providing evidence TZD treatment promotes changes in gene expression in skeletal muscle. In Zucker diabetic fatty rats, rosiglitazone treatment for 2 weeks increase mRNA expression of SCD and FAT/CD36 in skeletal muscle (Ahuja *et al*, 2001). Thus, improvements in glucose and lipid metabolism after TZD-treatment may occur in concert with changes in gene expression. However, data regarding the effects of TZD-treatment on gene expression in human skeletal muscle is limited.

In cultured skeletal myotubes from Type 2 diabetic patients, rosiglitazone treatment for 4 days increases mRNA expression of FAT/CD36 (Wilmsen *et al*, 2003). However, in a recent study, FAT/CD36 was reported to be decreased in human skeletal muscle after rosiglitazone and troglitazone treatment (Boden *et al*, 2005). Interestingly, several genes involved in FFA uptake, oxidation and oxidative phosphorylation that were up-regulated in adipose tissue were unchanged in skeletal muscle (Boden *et al*, 2005). In our cohort in the present study, the mRNA expression of FAT/CD36 was unchanged. Another report provides evidence that 4 days treatment of troglitazone in skeletal muscle cultures from healthy and Type 2 diabetic patients increased the mRNA expression of PPARγ, mFABP and adipocyte lipid binding protein (Park *et al*, 1998). These results were partly confirmed in a study where human skeletal muscle cell cultures treated with troglitazone for 5 days increased mRNA expression of PPARγ1, PPARγ2, adipocyte lipid binding protein and glycerophosphate dehydrogenase (Kausch *et al*, 2001). Adiponectin receptor 1 was recently reported to be down-regulated in skeletal muscle from Type 2 diabetic patients after rosiglitazone treatment for 12 weeks (Tan *et al*, 2005), although this finding was not reproduced in our cohort neither.

Interestingly in this cohort of newly diagnosed Type 2 diabetics, a tendency for an increase in LPL and SCD mRNA and a reduction in UCP3 mRNA expression was noted in rosiglitazone- versus metformin- or placebo-treated subjects, although this did not reach statistical significance. Thus, changes in expression in these candidate genes or selected insulin signaling proteins in skeletal muscle do not appear to account for improvements in glucose homeostasis. The differences between the effects of TZD's on gene expression between rodents and humans or between human *in vitro* or *in vivo* studies may be related to dosing of the drug or exact TZD studied. Clearly more extensive studies using an unbiased gene array approach may reveal gene expression signatures in skeletal muscle to account for effects of these pharmacological treatments on glucose homeostasis.

**Possible mechanisms for increased glucose uptake after rosiglitazone treatment**

FFA levels were 48% lower after rosiglitazone treatment and 30% lower after metformin treatment during hyperinsulinemic infusion in our cohort of patients (Paper II, Table 1). According to the homeostatic mechanism of the glucose fatty acid cycle (Randle *et al*, 1963), a higher level of FFA would favor FFA oxidation and thereby inhibit glucose uptake and oxidation. The question would then be if the reduction in FFA levels during insulin-stimulation in our cohort could account for the increase in glucose uptake? Indeed, the reduced FFA levels after rosiglitazone treatment, observed during 2 hours of hyperinsulin concentrations, may be explained due to insulin sensitization effects, leading to enhanced insulin-induced inhibition of lipolysis in adipose tissue. Several studies have provided evidence for reduced FFA levels after TZD treatment during fasting condition (Maggs *et al*, 1998; Miyazaki *et al*, 2001; Mayerson *et al*, 2002). Furthermore, in another TZD treatment study evidence for normal fasting FFA levels, but decreased FFA levels during euglycemic

hyperinsulinemic clamp has been provided (Boden *et al*, 2003). This reduction in FFA levels during insulin-stimulation was concurrent with an enhancement of insulin-induced inhibition of lipolysis (Boden *et al*, 2003). Contradictory to this hypothesis, patients treated with metformin also had a marked reduction of FFA levels during insulin-stimulation, although to a lesser extent (30% reduction versus 48% for rosiglitazone group), but had no improvement in glucose uptake. However, the reduction in plasma FFA levels during the hyperinsulinemic clamp condition can partly explain the increased glucose uptake in skeletal muscle in our cohort.

Adiponectin, an adipose tissue specific protein that is abundantly expressed in the circulation, is found to be reduced in relation to BMI, serum triglyceride concentrations and Type 2 diabetes (Hotta *et al*, 2000). Furthermore, plasma adiponectin concentration is elevated due to weight reduction in diabetic and non-diabetic subjects (Hotta *et al*, 2000). Rosiglitazone treatment has been reported to increase plasma adiponectin concentrations concomitant with an improvement in insulin sensitivity and a reduction in $HbA_{1c}$ despite an increase in body weight (Yang *et al*, 2002). A separate study demonstrates similar results in which lean and obese non-diabetics and obese diabetics were treated with troglitazone for 3 months (Yu *et al*, 2002). Interestingly, even the lean non-diabetic subjects treated with troglitazone had increased adiponectin levels, whereas no other effects were observed after treatment (Yu *et al*, 2002). Increased adiponectin levels have also been reported after pioglitazone treatment (Bajaj *et al*, 2004). In our study we did not measure adiponectin levels, although mRNA expression of adiponectin receptor 1 was unaltered after rosiglitazone treatment in our cohort. According to these recent reports, adiponectin is a parameter that would be of interest to analyze in our cohort of newly diagnosed Type 2 diabetic patients.

## 4.3 FIRST-DEGREE RELATIVES OF TYPE 2 DIABETIC PATIENTS

Insulin resistance in skeletal muscle is a characteristic feature in Type 2 diabetes. However, the development of manifest Type 2 diabetes requires both insulin resistance in peripheral tissues and impaired function of compensatory insulin release from the pancreatic β-cells. Thus, impairment only in skeletal muscle is unlikely to lead to Type 2 diabetes. Whether insulin resistance in skeletal muscle is a primarily cause of the initiation of impaired glucose tolerance is unclear. In many cases, Type 2 diabetes manifests in concert with obesity, which makes observations of primary causes related to Type 2 diabetes pathogenesis even more difficult to interpret. Metabolic disturbances in connection with obesity can secondarily induce skeletal muscle insulin resistance, such as dyslipidemia, as well as adipokines released from increased quantity of adipose tissue.

Insulin resistance could possibly represent a primary genetic defect in skeletal muscle. Because subjects with manifest hyperglycemia and well-established Type 2 diabetes usually already display several secondary defects, *e. g.* chronically elevated glucose levels which directly lead to insulin resistance (Rossetti *et al*, 1990; Zierath *et al*, 1994), studies of these subjects are limited when trying to determine inherited from acquired defects. Thus, to overcome this problem, first-degree relative of patients with Type 2 diabetes can be studied. First-degree relatives of Type 2 diabetic patients have a high risk to develop Type 2 diabetes (Meigs *et al*, 2000). Therefore, we decided to investigate insulin signaling and glucose uptake in normal glucose tolerant first-degree relatives of Type 2 diabetic subjects, in an attempt to identify early, possibly inherited, defects that are pre-established in these individuals.

In Paper III, healthy first-degree relatives of Type 2 diabetic patients were recruited together with well-matched control subjects. Muscle biopsy samples were obtained from the subjects after an overnight fast. When muscle strips were incubated

*in vitro* without or with increasing concentrations of insulin (0.6, 1.2 and 120 nM) immunoblotting analysis displayed an increase in phosphorylation of AS160 in a dose-dependent fashion (Figure 6; Quantified data in Paper III, Figure 3).

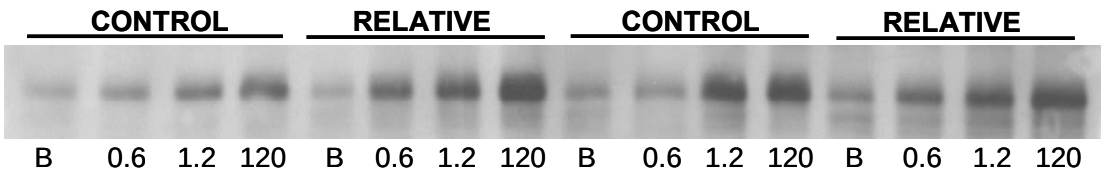


**Figure 6. Immunoblot of AS160 phosphorylation.** A representative portion of an immunoblot with the PAS antibody is shown. Basal and increasing concentrations of insulin (0.6, 1.2 and 120 nM) in skeletal muscle from two control subjects and two first-degree relatives of Type 2 diabetic patients.

The fold-change of insulin-induced AS160 phosphorylation observed in control subjects in the *in vitro* experiment (Paper III) was similar to that observed *in vivo* (Paper I; 2.9-fold *in vivo* versus 2.5-fold *in vitro* at 120 nM of insulin). AS160 phosphorylation increased to a similar degree in first-degree relatives of type 2 diabetic patients and control subjects.

Basal glucose transport in isolated skeletal muscle strips from first-degree relatives and control subjects was identical (Paper III, Figure 2). Insulin-stimulated glucose transport rate tended to be lower at physiological (0.6 nM) and low pharmacological (1.2 nM) insulin concentrations in first-degree relatives; however, this was not statistically significant. At the highest insulin concentration (120 nM), glucose transport rate was reduced 33% (P=0.07) in skeletal muscle strips from first-degree relatives. Interestingly, whereas glucose transport rate measured in the presence of 1.2 nM insulin was significantly higher compared to basal, the glucose transport rate measured at 120 nM insulin tended to be lower than that assessed at 1.2 nM insulin, and was not significantly higher than basal glucose transport. Even though there was a small tendency for reduced insulin-induced AS160 phosphorylation at this concentration, this is unlikely to explain the reduction in glucose transport rate. Moreover, the small tendency for reduced AS160 phosphorylation is within the range of variability, that such interpretation would not be valid. Alternatively, at supra-physiological insulin concentration (120 nM), there may be a degree of inflexibility in the system which may inhibit the GLUT4 trafficking machinery and constitute an early defect in first-degree relatives.

AS160 phosphorylation was positively correlated ($R^2$ = 0.97, $P < 0.05$) with insulin-stimulated glucose transport in healthy control subjects, providing correlative evidence that these two events are linked. In contrast, the relationship between AS160 phosphorylation and glucose transport was disassociated in skeletal muscle from first-degree relatives ($R^2$ = 0.58, NS), providing indicative evidence that at higher insulin concentrations, GLUT4 traffic, and possibly fusion of GLUT4 vesicles with the plasma membrane, may be impaired.

## 4.4 ANALYSIS OF TANIS/SELS mRNA IN MUSCLE AND ADIPOSE TISSUE

During the last few years more and more attention has been directed to the link between inflammation and Type 2 diabetes. SAA is one of the acute phase inflammatory proteins observed to be elevated in Type 2 diabetic patients (Kumon *et al*, 1994; Pickup

*et al*, 1997; Ebeling *et al*, 1999). Recently a novel protein was identified in a diabetic animal model that was implicated to be involved in Type 2 diabetes and inflammation (Walder *et al*, 2002). The protein, named Tanis, was found to be dysregulated in *Psammomys obesus*, a polygenic model for nutritionally induced Type 2 diabetes. The link between Tanis and inflammation is based on the hypothesis that Tanis is the presumed receptor for SAA, and that changes in Tanis expression may be associated with serum SAA levels. A yeast-2 hybrid screening revealed SAA to be the main protein interacting with Tanis and the binding characteristic was further confirmed by surface plasmon resonance analysis (Walder *et al*, 2002).

Previous studies provide evidence that Tanis gene expression is reduced in liver of IGT and diabetic *P. obesus*, and after a 24h fast Tanis gene expression was elevated in diabetic animals compared to normal glucose tolerant animals. Furthermore, both insulin and glucose suppressed Tanis gene expression in 3T3-L1 adipocytes and in C2C12 muscle cell cultures (Walder *et al*, 2002). The human homologue protein of Tanis is a protein designated AD-015. Recently AD-015 has been identified as a membrane selenoprotein and thereby named SelS (Kryukov *et al*, 2003). Selenoproteins are proteins that incorporate the amino acid selenocysteine (Sec) into the peptide sequence coded by the codon UGA (Hatfield and Gladyshev, 2002). The codon UGA is in most cases a stop codon that terminates translation, but can also function as a codon for Sec. In order for the translational process to distinguish the UGA codon as a Sec insertion, the cell requires a complete selenoprotein synthesis machinery, which includes a unique Sec-tRNA, a unique elongation factor, and a selenocystein insertion sequence structural element in the selenoprotein mRNA sequence (reviewed in (Hatfield and Gladyshev, 2002)).

To test the hypothesis that the human homologue of Tanis is dysregulated in human skeletal muscle and adipose tissue in Type 2 diabetes, ten Type 2 diabetic patients and eleven age- and weight matched healthy control subjects were recruited, and skeletal muscle and adipose tissue biopsies were obtained for gene expression analysis. The nucleotide sequence of the human homologue of Tanis, AD-015/SelS, was retrieved by a search of the National Center for Biotechnology Information (NCBI) database and PCR primers were designed to determine mRNA expression of human Tanis/SelS. Tanis/SelS mRNA expression in skeletal muscle was similar between healthy and Type 2 diabetic subjects (Paper IV, Figure 1). Furthermore, Tanis/SelS mRNA expression in adipose tissue biopsies was similar between healthy and Type 2 diabetic subjects (Paper IV, Figure 2).

Insulin action on Tanis/SelS mRNA expression was determined. To correct the hyperglycemia in Type 2 diabetic patients, insulin was infused for ~60 min prior to initiating the glucose infusion of the euglycemic hyperinsulinemic clamp. Plasma free insulin concentrations during the insulin infusion were comparable between Type 2 diabetic and healthy subjects (approximately 60 mU/L). Steady state plasma glucose concentration was maintained at 5.5 mM in Type 2 diabetic and healthy subjects. Following insulin infusion, Tanis/SelS mRNA increased in adipose tissue from Type 2 diabetic subjects (Paper IV, Figure 2), although this observation did not reach statistical significance (P=0.052). In contrast, mRNA levels in healthy subjects were unaltered after insulin stimulation.

Since Tanis has been proposed as a receptor for SAA (Walder *et al*, 2002), fasting plasma concentration of SAA in Type 2 diabetic patients and control subjects was assessed. SAA concentration was slightly higher in Type 2 diabetic subjects compared to control subjects (3.16 ± 0.54 and 2.22 ± 0.31 mg/L respectively, NS). Other studies have reported increased levels of SAA in Type 2 diabetic patients

(Kumon *et al*, 1994; Pickup *et al*, 1997; Ebeling *et al*, 1999). SAA concentrations in the subjects studied in Paper IV were reasonably similar with two previously published studies (2.1 ± 1.3 and 1.2 ± 0.5 mg/L (Kumon *et al*, 1994)) (4.3 ± 1.0 mg/L for male Type 2 diabetics (Ebeling *et al*, 1999)), but considerably lower than a third study (approximately 15 and 25 mg/L (Pickup *et al*, 1997)). This difference can potentially be due to different analytical methods.

When all subjects were analyzed collectively, plasma SAA levels were positively correlated with Tanis/SelS mRNA expression in skeletal muscle ($r = 0.51$, $p<0.05$, Paper IV, Figure 3A), and adipose tissue ($r = 0.60$, $p<0.05$, Paper IV, Figure 3B). Neither skeletal muscle nor adipose Tanis/SelS mRNA was correlated with glucose, insulin, blood lipids levels, or whole body glucose utilization.

The literature regarding the novel protein Tanis is sparse. The studies that have been reported have mostly been performed in cell cultures, particular in liver cells, since Tanis was originally identified to be dysregulated in the liver. Tanis encodes a 189 amino acid protein and contains a putative transmembrane domain (Walder *et al*, 2002). Tanis mRNA is reported to be highly expressed in the three primary insulin target tissues, namely liver, adipose tissue and skeletal muscle (Walder *et al*, 2002). This suggests that Tanis might have a putative role in modulation of insulin action and/or glucose metabolism. When cellular localization was investigated by fractionation analysis in liver cells, Tanis protein was shown to be primarily localized in fractions of plasma membrane and high- and low density microsomal compartments. There was no evidence for expression of Tanis in the soluble protein fraction or in the fraction of nucleus/mitochondria (Gao *et al*, 2003).

In diabetic *P. obesus*, fasting (24 hr) increased hepatic Tanis mRNA expression approximately 3-fold, whereas in control animals only a marginal (1.5-fold) increase was noted (Walder *et al*, 2002). In contrast, Tanis/SelS mRNA expression was unaltered in skeletal muscle and adipose tissue from overnight fasted Type 2 diabetic patients, as compared to control subjetcs. This could reflect tissue, nutritional and/or species specific differences. However, in *P. obesus* rodents, fasting increased liver mRNA expression of Tanis in diabetic, but not in control animals, such that the net effect was reflected as a similar level of Tanis expression between diabetic and healthy rodents under fasting conditions (Walder *et al*, 2002). A limitation in our study design is that skeletal muscle and adipose tissue biopsies were obtained only from fasted subjects. A putative difference under fed conditions may therefore have been masked.

Functional studies reveal that adenoviral-mediated overexpression of Tanis in hepatoma cells is associated with decreased insulin-stimulated glucose uptake and glycogen synthesis (Gao *et al*, 2003). The mechanism by which Tanis interferes with insulin action remains to be determined, as no effect on insulin receptor phosphorylation has been reported (Gao *et al*, 2003). Moreover, *in vitro* studies in cultured cells have provided evidence that insulin and glucose suppress Tanis mRNA expression (Walder *et al*, 2002). However, these results could be misleading since results from these *in vitro* experiments were performed in cells incubated in insulin- and glucose-free media, a situation which is impossible to mimic in humans *in vivo*. In fact, apart from a baseline permissive effect, glucose and insulin does not suppress Tanis mRNA expression in adipose cells (Walder *et al*, 2002). Rather, our data provides evidence to suggest that in the context of Type 2 diabetes, hyperinsulinemia may increase Tanis/SelS mRNA expression in adipose tissue. During the euglycemic hyperinsulinemic clamp procedure, fasting hyperglycemia in the Type 2 diabetic patients was corrected by an insulin infusion for 60 min prior to commencement of the glucose infusion (Koistinen *et al*, 2003). Thus, all subjects were studied under normoglycemic conditions (5.5 mM). Tanis/SelS mRNA expression was increased under insulin-stimulated conditions in Type 2 diabetic subjects, whereas levels were

unchanged in control subjects. Whether increased Tanis/SelS mRNA in adipose tissue in human Type 2 diabetic subjects contributes to reduced insulin action remains to be determined.

Tanis has been indicated to be a member of the glucose-regulated protein family and has been proposed to play a role in the regulation of cellular redox balance in hepatoma HepG2 cells (Gao *et al*, 2004). Tanis is implicated to function as an anti-oxidant, protecting cells from oxidative stress. Since diabetic hyperglycemia is known to induce elevated levels of reactive oxygen species that can aggravate insulin sensitivity (Evans *et al*, 2003), changes in Tanis expression may occur in dysregulated diabetics. While we were unable to provide evidence for a relationship between Tanis mRNA and glycemia, the subjects studied were in fairly good glycemic control. Additional studies in poorly controlled Type 2 diabetic subjects may reveal a positive interaction.

The relationship between Tanis mRNA and the acute phase protein SAA suggests an interaction between innate immune system responses and Tanis expression in skeletal muscle and adipose tissue. The positive correlation between plasma SAA and Tanis/SelS mRNA expression identified in the present study further supports this hypothesis. Thus, the SAA level is a potential surrogate marker for Tanis/SelS gene expression. Future studies are warranted to ascertain the functional role of Tanis/SelS protein in the regulation of glucose homeostasis in humans.

# 5 SUMMARY

- Insulin phosphorylates several Akt substrates in human skeletal muscle during *in vivo* insulin infusion. This thesis work provides evidence that physiological hyperinsulinemia leads to a robust phosphorylation of AS160 in human skeletal muscle.

- In Type 2 diabetic patients, skeletal muscle protein expression of AS160 is unaltered, but insulin-induced phosphorylation of AS160 is attenuated. Furthermore, impaired insulin action on AS160 is associated with reduced Akt $Thr^{308}$-phosphorylation, suggesting that these two events are linked.

- In moderately obese subjects with newly diagnosed Type 2 diabetes, 26 weeks of rosiglitazone treatment improves skeletal muscle glucose uptake through a mechanism(s) independent of insulin signaling at the level of IRS-1/PI 3-kinase/Akt/AS160 or changes in expression of selective candidate genes involved in glucose or lipid metabolism.

- Insulin-induced phosphorylation of AS160 in isolated skeletal muscle from glucose tolerant first-degree relatives of Type 2 diabetic patients with mild insulin resistance is unaltered.

- Insulin-induced glucose uptake in isolated skeletal muscle from glucose tolerant first-degree relatives of Type 2 diabetic patients is unaltered. However, at pharmacological insulin concentration (120 nM) a tendency for a reduction in glucose transport was observed.

- Insulin action on AS160 is positively correlated with glucose uptake in isolated human skeletal muscle. In glucose tolerant first-degree relatives of Type 2 diabetic patients the correlation between insulin action on AS160 and glucose transport is non-significant.

- Tanis/SelS mRNA is expressed to a similar level in skeletal muscle and adipose tissue from Type 2 diabetic patients and age- and weight-matched healthy subjects. Expression of Tanis/SelS mRNA is positively correlated with serum amyloid A in Type 2 diabetic and control subjects, supporting the hypothesis that Tanis/SelS may be the cell-surface receptor for serum amyloid A.

# 6 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, the molecular mechanisms of insulin signal transduction to glucose transport in insulin resistant human skeletal muscle has been addressed. This thesis work provides the first evidence that the recently identified Akt substrate, AS160, is phosphorylated in response to insulin in human skeletal muscle, suggesting a physiological role of this target in insulin action. Furthermore, insulin-induced AS160 phosphorylation is impaired in skeletal muscle of Type 2 diabetic patients, highlighting a proximal defect in the insulin signaling cascade to glucose uptake.

AS160 is the most proximal step identified in the insulin signaling transduction pathway leading to GLUT4 translocation. Experimental evidence from studies in adipocytes reveals that AS160 is required for insulin-induced translocation of GLUT4 to the cell surface plasma membrane in order to facilitate glucose uptake. Given the evidence that AS160 is required for insulin-mediated GLUT4 translocation in adipocytes, our data suggest that aberrant insulin signaling to AS160 contributes to defects in GLUT4 translocation and glucose uptake in skeletal muscle from insulin resistant Type 2 diabetic patients. Furthermore, impaired insulin action on AS160 was associated with reduced Akt $Thr^{308}$-phosphorylation, suggesting that these two events are linked. Since the protein expression of AS160 was unaltered in Type 2 diabetic patients, the reduced phosphorylation of AS160 is likely to be a result of either impaired phosphorylation by upstream kinases or increased Ser/Thr-phosphatase activity, or both. However, the possibility of additional protein interaction, *e. g.* by scaffolding proteins cannot be excluded.

The peptide sequence of AS160 contains a GAP domain for Rab proteins that are believed to be involved in the mechanism of GLUT4 translocation machinery. The identification of the potential specific Rab proteins that are targeted by AS160 in human skeletal muscle is of particular interest. Initial studies in adipocytes have provided evidence for several Rab proteins as candidates to interact with AS160. If these specific Rabs can be identified and proven to be crucial components for insulin-induced GLUT4 translocation, it would illuminate the elusive mechanism of vesicular trafficking of GLUT4.

The impairment in glucose uptake in insulin resistant skeletal muscle is due to an attenuation of GLUT4 transporters on the cell surface of the muscle cells. Several steps in the insulin signal transduction pathway leading to GLUT4 translocation are impaired in skeletal muscle in Type 2 diabetes. Therefore, impairments in insulin signal transduction are responsible for the attenuated glucose uptake in skeletal muscle from Type 2 diabetic patients. However, newly diagnosed Type 2 diabetic patients treated with rosiglitazone had a markedly increase in skeletal muscle glucose uptake, independent of improvement in insulin signaling at the level of IRS-1/PI 3-kinase/Akt/AS160 (Figure 7). This finding raises the possibility that even more distal events in the GLUT4 translocation machinery are augmented after rosiglitazone treatment. Therefore, further studies are necessary to investigate GLUT4 vesicular trafficking and vesicular fusion machinery in skeletal muscle of Type 2 diabetic patients after rosiglitazone treatment. The identification of the molecular mechanism of action by which TZD treatment enhances insulin-sensitivity in tissues is important from at least two perspectives; first, resolution of the mechanism of action will eventually facilitate more specific design of drug compounds, secondly, the identification of the mechanism of action of TZD treatment will also potentially give important clues to the characteristics of the pathophysiology of Type 2 diabetes.

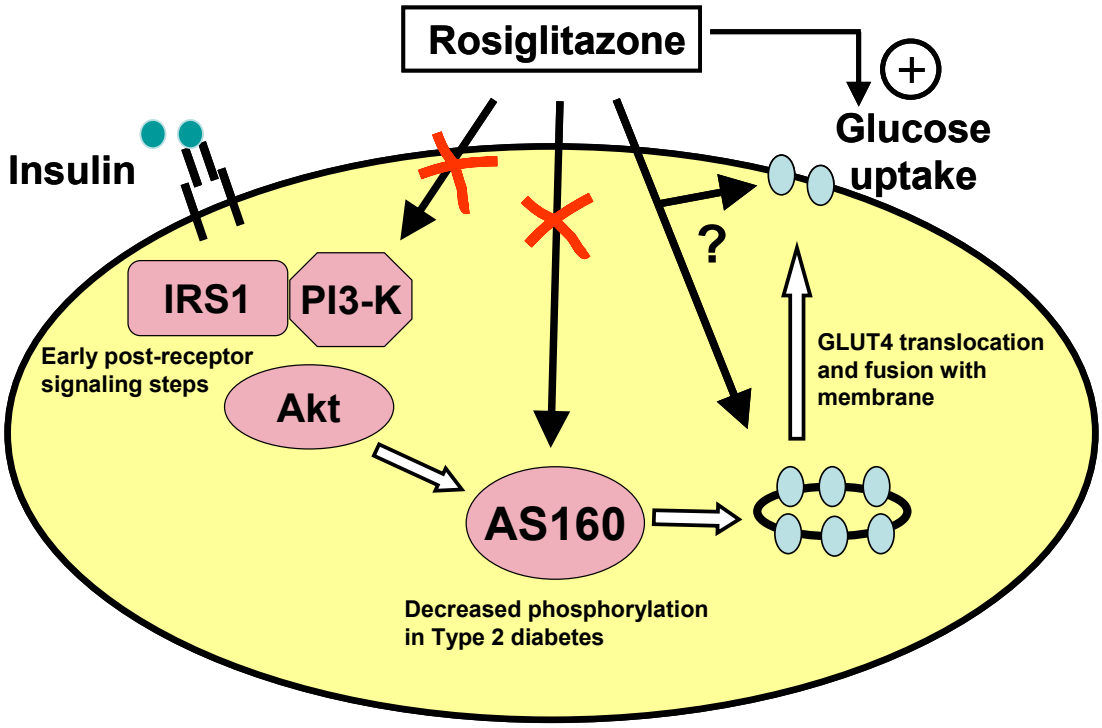


**Figure 7.** Rosiglitazone treatment increased glucose uptake in skeletal muscle independent of enhancement in insulin signaling pathway. This suggests that distal components at the level of GLUT4 translocation and/or fusion with the cell surface membrane are augmented.

The pathological progression of the Type 2 diabetic phenotype is complex and comprises a variety of dependent and independent environmental risk factors in combination with inherited polygenetic traits. In this thesis insulin-induced phosphorylation of AS160 was found to be normal in first-degree relatives of Type 2 diabetic patients. Studying first-degree relatives of Type 2 diabetic patients is of great interest, since this group of individuals is at higher risk of developing Type 2 diabetes. Interestingly, the positive correlation observed between insulin action on AS160 and glucose transport in healthy subjects was not confirmed in the first-degree relatives of Type 2 diabetic patients. Thus, since glucose-intolerant first-degree relatives to Type 2 diabetic patients demonstrate defects in insulin action on glucose metabolism, the presence of a genetic lesion coupled with environmental challenges, such as those presented by a progressive alteration in the metabolic milieu, collectively lead to insulin resistance and the development of type 2 diabetes. The uncoupling of insulin action on AS160 and glucose transport may constitute an early defect in the pathogenesis of Type 2 diabetes. Future longitudinal studies may be of value to clarify the extent of the disease progression in this cohort of glucose tolerant first-degree relatives to Type 2 diabetic patients.

Another contributing cause of insulin resistance in the progression of and in overt Type 2 diabetes is inflammation, through the chronic inflammatory response elements that are characteristic of patients with Type 2 diabetes. We determined the mRNA expression of Tanis/SelS in skeletal muscle and adipose tissue from Type 2 diabetic and control subjects. Expression of Tanis/SelS in skeletal muscle and adipose tissue was similar between control and Type 2 diabetic subjects. However, a positive correlation was observed between the acute-phase serum protein SAA and the mRNA expression of Tanis/SelS in skeletal muscle and adipose tissue, further supporting the hypothesis that Tanis/SelS may be the cell-surface receptor for SAA. Although, the potential role of this receptor in skeletal muscle and adipose tissue is unknown. The

tendency for increased Tanis/SelS mRNA expression in adipose tissue in response to insulin infusion in combination with the knowledge that SAA levels are reduced by TZD treatment, imply that Tanis/SelS might play a role in insulin action. However, further studies are required before any firm conclusion can be drawn regarding a potential interaction.

To achieve the most efficient and manageable treatment of Type 2 diabetes, an understanding of the underlying defects is crucial. However, identification of defects in the insulin-glucose-homeostatic relationship requires deeper knowledge of the molecular mechanisms which elicit specific physiological events. Type 2 diabetes is a heterogeneous and multifactorial disease and thus there are many pieces of the puzzle that need to be identified and put into place before the full picture will be available.

Basic research on insulin signaling in human skeletal muscle has certain limitations by practical reasons, *e. g.* limitations in population and sample size, labor-demanding and time-consuming analysis, thus, a certain measure of generalization is warranted where individual variants of parameters might be overseen. Improvements of future analytical techniques will potentially allow for a high-throughput analysis, whereby large-scale arrays of whole signaling networks of proteins can be assessed, even within larger cohorts of subjects.

The results from this thesis work highlight the importance of designing treatment strategies for insulin resistance in skeletal muscle to target at multiple sites depending on the progression of the disease. In overt Type 2 diabetes, where insulin resistance is attenuated by the derangements in the metabolic milieu, insulin sensitivity in skeletal muscle is important to regain by augmentation of defects in the insulin signaling pathway. Furthermore, the results from newly diagnosed Type 2 diabetic patients with relatively good metabolic control suggest that improvement of glucose uptake by TZD treatment is achieved through improvements distal in the signaling pathway, at the level of GLUT4 translocation. Therefore, more intensified research of the distal mechanism of GLUT4 translocation and trafficking is desirable in the future. Additionally, the uncoupling of insulin signaling and glucose uptake in first-degree relatives of Type 2 diabetic patients, suggests that defects at the level of GLUT4 translocation potentially is one primary defect leading to the manifestation of Type 2 diabetes.

Further studies in human skeletal muscle are necessary to unravel the missing pieces of the puzzle, in an attempt to harness the world-wide epidemic increase of Type 2 diabetes.

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