Fat Cell Insulin Resistance

an experimental study focusing on molecular mechanisms in type 2 diabetes

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Klokheten har vi från våra erfarenheter.
Erfarenheterna kommer från våra dumheter.
Sacha Guitry
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ABSTRACT

The aim of the present thesis was to further increase our understanding of mechanisms contributing to and maintaining cellular insulin resistance in type 2 diabetes (T2D). For this reason, the effects of high glucose and insulin levels on glucose transport capacity and insulin signaling, with emphasis on insulin receptor substrate 1 (IRS-1) were assessed in fat cells. Altered levels of IRS-1 have previously been observed in adipose tissue from insulin-resistant and T2D subjects.

A high glucose level (≥15 mM) for 24 h exerted only a minor impairment on glucose transport capacity in human adipocytes, as opposed to rat adipocytes. However, when combined with a high insulin level (10^4 µU/ml), basal and insulin-stimulated glucose transport was significantly impaired in both human and rat adipocytes. This was associated with a depletion of IRS-1 and IRS-2 protein levels in rat adipocytes, as a result of post-translational changes and altered gene transcription, respectively. In human adipocytes was only IRS-1 protein levels reduced. The high glucose/high insulin setting achieved maximal impairment of glucose transport within 6 h. Subsequent incubations of rat adipocytes under physiological conditions could partially restore insulin sensitivity. Interestingly, in both human and rat fat cells, decreased levels of IRSs occurred after the establishment of impaired glucose transport, suggesting that the observed depletion of IRSs is a consequence rather than a cause of insulin resistance. Nonetheless, IRS depletion is likely to further aggravate insulin resistance.

Tyrosine phosphorylation of IRS-1 upon insulin stimulation activates the signaling pathway that mediates glucose transport. Pre-treatment of human adipocytes with high glucose and insulin levels was not associated with any alterations in the total IRS-1 Tyr612 phosphorylation following 10 min insulin stimulation. However, a significant increase in basal Tyr612 phosphorylation was observed. Furthermore, a rise in basal IRS-1 Ser312 phosphorylation was found. This is associated with reduced IRS-1 function and is considered to target IRS-1 to degradation pathways, and thus could potentially explain the observed decrease in IRS-1 protein levels. Our results imply an enhanced activation of insulin’s negative-feedback control mechanism that inhibit IRS-1 function. This could potentially have contributed to the observed impairment of insulin action on glucose transport in these cells. Accordingly, we have also shown that the downstream activation of protein kinase B upon insulin-stimulation is significantly impaired in human adipocytes exposed to the high glucose/high insulin setting, indicating a defect in the signaling pathway mediating glucose transport.

We also investigated whether there are humoral factors in the circulation of T2D patients that contribute to peripheral insulin resistance. Human adipocytes cultured for 24 h in medium supplemented with 25% serum from T2D subjects, as compared to serum from non-diabetic subjects, displayed significantly reduced insulin-stimulated glucose uptake capacity. The effect could neither be attributed to glucose, insulin, FFA, TNF-α or IL-6 levels in the serum, but other circulating factor(s) seem to be of importance.

In conclusion, chronic conditions of elevated glucose and/or insulin levels all impair insulin action on glucose turnover, but to different extents. A clear distinction between rat and human fat cells in the response to these different milieus was also observed. Alterations in the function of the key insulin signaling protein IRS-1 might be involved in the mechanisms underlying the impaired glucose uptake capacity. IRS-1 reduction however, occurs after but probably aggravates the existing insulin resistance. The effects of high glucose and/or insulin levels may be of importance in T2D, but additional novel factors present in the circulation of T2D patients seem to contribute to cellular insulin resistance.

Keyword: adipocyte; insulin signaling; insulin; glucose; IRS-1; protein kinase B; glucose uptake; insulin resistance; typ 2 diabetes; serum; tyrosine phosphorylation, serine phosphorylation
LIST OF PAPERS

This thesis is based on the following papers which will be referred to by their Roman numerals:

I) **Renström F, Burén J, Eriksson JW** 2005 Insulin receptor substrates-1 and -2 are both depleted but via different mechanisms after down-regulation of glucose transport in rat adipocytes. Endocrinology 146(7):3044-3051

II) **Renström F, Burén J, Svensson M, Eriksson JW** 2007 Insulin resistance induced by high glucose and high insulin precedes insulin receptor substrate 1 protein depletion in human adipocytes. Metabolism 56(2):190-198

III) **Renström F, Lundgren M, Lindmark S, Eriksson JW** 2007 In human adipocytes exposed to high glucose and insulin levels, IRS-1 displays altered phosphorylation and may be targeted to degradation. Manuscript


Insulin resistens kan definieras som ett tillstånd med nedsatt biologisk effekt av en viss mängd insulin. Insulin har många biologiska funktioner i kroppen, men förenklat gör frisättning av detta hormon från bukspottkörteln i samband med måltid att upptaget av glukos i muskler ökar, att kroppens glukosproduktion i levern hämmas och att glukosupptaget ökar och energifrisättning i form av fettsyror från fettväven hämmas. Glukosnivån i blodet står normalt under strikt kontroll eftersom både för höga och för låga nivåer leder till allvarliga rubbningar och skador på kroppen. Vid insulin resistens störs regleringen av ämnesomsättningen i lever, muskel och fettväv vilket leder till ökade nivåer av både glukos och fett i blodet, vilket i sin tur ytterligare försämrar insulinets förmåga att påverka ämnesomsättningen i muskler och i lever. Bukspottkörteln kommer till en början att öka insulinfrisättningen i ett försök att kompensera för insulin resistensen. Om typ 2-diabetes utvecklas så kommer frisättningen av insulin att minska, varför är oklart. Detta leder till de höga glukosnivåer i blodet som är karaktäristiskt för typ 2-diabetes och som kommer att stiga successivt om inte behandling sker.

De bakomliggande orsakerna till att målecceorns förmåga att svara på insulin försämras är långt ifrån fullständigt klarlagda. I fettceller från individer med insulin resistens och typ 2-diabetes har man funnit en minskad mängd av proteinet IRS-1. IRS-1 är ett nyckelprotein som ingår i den komplexa signaleringskedja som aktiveras inne i cellen när insulin binder till sina måceller, vilket bland annat leder till att cellen tar upp glukos från omgivningen. Man har även funnit att insulinets förmåga att aktivera detta

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protein (genom specifik fosforylering) är försämrad i både fett- och muskelceller från individer med typ 2-diabetes.

Tidigare försök i vår forskargrupp har visat att när fettceller från råtta odlas tillsammans med höga glukos och/eller insulinivåer för att efterlikna situationen vid typ 2-diabetes och insulinresistens påverkas mängden av IRS-1 och IRS-2. I delarbete I ville vi studera vilka mekanismer som ligger bakom de förändrade proteinnivåerna av IRS-1 och IRS-2 i fettceller från rätta. Vi kunde visa att förändringarna av IRS-2 berodde på ett förändrat uttryck (transkription) av IRS-2 genen, medan den minskade mängden IRS-1 verkade beror på orsaker som inte är kopplade till genuttrycket. I delarbete III kunde vi se att en minskad mängd av IRS-1 i fettceller från människa var kopplad till en ökad fosforylering av aminosyra 312 på IRS-1 molekyl (se nedan). Resultat från andra forskargrupper tyder på att denna ökade fosforylering kan leda till en ökad nedbrytning av IRS-1 och därmed förklara den minskade mängd IRS-1 som ses.

I delarbete II ville vi undersöka hur fettceller från friska individer påverkas av höga glukos och/eller insulinnivåer. Till skillnad från våra tidigare fynd med rättfettceller verkar glukosupptagsförmågan hos människans fettceller inte påverkas nämnvärt av en hög glukoskonzentration i omgivningen under odling. En liten försämring i den maximala insulinstimulerade glukosupptagsförmågan kunde ses först efter 24 timmars odling, med oförändrade nivåer av IRS-1 och IRS-2. Fettceller som odlats med höga insulinivåer uppvisade däremot ett försämrat insulinstimulerat glukosupptag redan efter 2 timmar. Ett försämrat basalt d v s icke-insulinstimulerat glukosupptag kunde ses efter 6 timmar. Inga förändringar i nivåerna av IRS-1 eller IRS-2 kunde ses i dessa celler. Ingen ytterligare försämring av fettcellernas glukosupptagsförmåga kunde ses efter odling med både hög glukos och hög insulinivå. Däremot halverades mängden av IRS-1 under dessa förhållanden vilket vi sedan i delarbete III kunde koppla till en ökad fosförylering av en specifik aminosyra av typen serin på IRS-1 molekyl (se ovan).

Ett intressant fynd i fettceller från både rätta och människa var att minskningen av IRS-proteiner uppkommer först efter det att insulinresistens med cellens glukosupptagsförmåga har uppstått. Detta fynd tyder på att den minskade mängden av
IRS-proteiner är en följd av, och inte en orsak till insulinresistensen, men bidrar troligen till att ytterligare försämra cellernas förmåga att ta upp glukos.

**I delarbete III** ville vi undersöka om framkallandet av insulinresistens med höga insulinnivåer, ensamt eller i kombination med höga glukosnivåer, påverkar regleringen av funktionen hos IRS-1. Vi fann att fettceller från människa som utsatts för höga insulinnivåer under 2 timmar uppvisade en försämrad insulinstimulerad glukosupptagsförmåga, vilket stämmer med resultaten i delarbete II. Samtidigt sågs en störning i det känsliga system som styr funktionen av IRS-1 genom fosforylering av specifika aminosyror av typen tyrosiner och seriner på IRS-1 molekylen. Ingen ytterligare effekt kunde ses efter odling med höga insulinnivåer i kombination med höga glukosnivåer, vilket talar för att höga insulinkoncentrationer ensamt är orsak till försämringen i fettcellernas glukosupptagsförmåga i våra modeller, vilket i sin tur kan vara kopplat till en störd funktion hos IRS-1.

Idag vet vi att fettväven inte bara fungerar som ett energilager utan även producerar många proteiner, s k adipokiner, och inflammationssubstanter s k cytokiner (t ex TNF-α och IL-6) vilka medverkar till regleringen av kroppens energibalans, t ex så påverkar adipokinen leptin mättnadskänslan. Vid övervikt rubbas balansen av dessa adipokiner och cytokiner och mycket tyder på att denna balansrubblning kan bidra till utvecklingen av insulinresistens. **I delarbete IV** fann vi att det finns ytterligare faktorer i blodet hos individer med typ 2-diabetes, förutom de tidigare kända faktorerna glukos, insulin, fettsyror, TNF-α och IL-6 som kan minska fettcellernas insulinstimulerade glukosupptagsförmåga. Dessa faktorer kunde dock inte definieras i vår undersökning. Effekten av dessa faktorer var inte beroende av den omgivande glukosnivån. Fynden från denna undersökning kan ha klinisk betydelse eftersom resultaten tyder på att även om individer med typ 2-diabetes har en normaliserad blodglukosnivå så verkar det finnas andra faktorer i blodet som kan påverka fettcellernas insulinkänslighet negativt.

Sammanfattningsvis visar resultaten i denna avhandling att även fast det är väl dokumenterat att höga glukosnivåer i blodet försämrar insulinets förmåga att stimulera glukosupptag så verkar den bakomliggande orsaken inte vara en direkt effekt på den enskilda fettcellen. Vare sig defe克ter i cellernas förmåga att ta upp glukos är en grundläggande orsak till insulinresistens eller inte så förväntas det tillstånd med
kroniskt höga insulinnivåer i blodet som är kopplat till insulinresistens att ha en ytterligare negativ påverkan. De minskade nivåerna av IRS-1 som observerats i fettceller från individer med insulinresistens verkar vara en konsekvens av, snarare än en grundläggande orsak till insulinresistensen. Våra resultat tyder också på att det finns ytterligare faktorer, bortsett från socker, insulin, fettsyror, TNF-α och IL-6, i blodet hos individer med typ 2-diabetes som direkt kan hämma fettcellernas insulinkänslighet. Dessa försök visar också att studier på fettceller på djur har begränsningar eftersom försök med fettceller från råtta och människa delvis uppvisar olika resultat.
ABBREVIATIONS

A-SAA  acute-phase serum amyloid A
ADA  adenosine deaminase
ANOVA analysis of variance
BCA  bicinchoninic acid
BSA  bovine serum albumin
CAP  Cbl-associated protein
CHX  Cyclohexamide
DMEM  Dulbecco’s modified Eagle’s medium
FCS  foetal calf serum
FFA  free fatty acid
GLUT  glucose transporter
Grb2  Growth factor receptor-binding protein 2
IL  interleukin
IR  insulin receptor
IRS  insulin receptor substrate
JNK  c-Jun N\textsuperscript{H2}-terminal kinase
MCP-1  monocyte chemoattractant protein 1
mTOR  mammalian target of rapamycin
PBS  phosphate buffered saline
PDGF  platelet-derived growth factor
PDK1  phosphoinositide-dependent kinase-1
PI3K  phosphatidylinositol 3-kinase
PIA  N\textsuperscript{6}-(R-phenylisopropyl) adenosine
PIP  phosphoinositide-phosphate
PKB  protein kinase B
RBP4  retinol binding protein 4
RT-PCR  reverse transcriptase polymerase chain reaction
S6K1  S6 kinase 1
SDS-PAGE  sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM  standard error of the mean
SH2  Src homology 2
SHP2  SH2-containing protein tyrosine phosphatase
TNF-\textalpha  tumor necrosis factor alpha
WHO  World Health Organisation
INTRODUCTION

Diabetes mellitus

Diabetes mellitus is a disease recognized more than 2000 years ago and is characterized by chronic hyperglycemia (1). Excessive urine production with a compensatory thirst is characteristics signs of diabetes mellitus. The word “mellitus” means sweet and refers to the elevated blood glucose that will spill over into the urine. There are two major forms of diabetes mellitus, characterized by an absolute and a relative insulin deficiency, respectively. Type 1 is characterized by an autoimmune destruction of insulin producing β-cells and patients are thus absolutely dependent on insulin therapy (2). Type 2 is the most common form, accounts for 85-90% of all diabetes cases, and results from a combination of insulin resistance and impaired insulin secretion. With proper diet and exercise these individuals can manage without medication for some time, but eventually they usually need oral medication and finally insulin injections.

Insulin resistance and type 2 diabetes

Insulin resistance is today recognized as an important characteristic of several disorders like obesity, hypertension and cardiovascular disease (3, 4) and it is a central defect in the development of type 2 diabetes (5), i.e. disorders that are the most common causes of death in the industrialized world. The prevalence of type 2 diabetes is rapidly increasing world wide, and more than 300 million people will be affected by 2030 according to estimations (6). This highlights the importance of unravelling the mechanisms involved in development of insulin resistance.

Insulin resistance is an inability of insulin sensitive tissues (e.g. muscle, liver and fat) to respond to circulating levels of insulin and was first described in the 1930s (7). Little is known about early events that initiate its progression, which is slow and span over several years. Gathered knowledge regarding the pathogenesis indicate that environmental factors, dominated by obesity, stress and a sedentary lifestyle (1, 8) interact with unfavourable genetic predispositions (9), e.g. a greater propensity for obesity has been found in subjects with a genetic predisposition for diabetes (10), where a net positive energy balance lead to disordered fat storage and metabolism (11).
The relatively recent finding that adipose tissue, unlike what was previously believed, is an active endocrine tissue producing and releasing proteins, also referred to as adipokines, that regulate whole body energy homeostasis has made us realize that the pathogenesis of insulin resistance and type 2 diabetes is very complex, and that it involves interplay between many tissues, hormones and metabolic substrates (12, 13).

**Insulin action and glucose homeostasis**

The body is dependent on continuous supply of energy to support metabolic processes and the main sources under normal circumstances are glucose and free fatty acids (FFA). After a meal, carbohydrates are digested and glucose is absorbed by the gut and enters the circulation where they will be utilized by energy demanding tissues. However, prolonged elevated blood glucose levels can be harmful and lead to organ damage, e.g. cardiovascular disease, nephropathy, neuropathy and retinopathy. Too low blood glucose levels (< 2.5 mM) cause brain energy deficiency which can result in seizures, unconsciousness and death. Glucose turnover is, for this reason, highly regulated by insulin, a hormone whose action under normal circumstances maintains blood glucose levels within a narrow range (approximately 4-7 mM). Elevated blood glucose levels after a meal trigger β-cells in the pancreas to secrete insulin into the circulation. Insulin’s action inhibit glucose production via glycogenolys and gluconeogenesis in the liver, which maintains normal blood glucose levels inbetween meals, and stimulate glucose uptake in insulin-sensitive tissues e.g. skeletal muscle, liver and fat (Fig 1).

![Figure 1. Schematic picture of insulin action on glucose homeostasis](image)
Role of adipose tissue in glucose homeostasis

Although the quantitative contribution of adipose tissue to glucose disposal is minor, with ~60% of dietary glucose being utilized by muscles (14, 15), it significantly affects glucose homeostasis, for example through its regulation of circulating FFA levels. This becomes evident in the insulin resistant state when insulin’s action to inhibit hydrolysis of stored triglycerides to glycerol and FFA, i.e lipolysis in the adipose tissue is impaired, leading to a continuous release of FFA into the circulation. Elevated FFA levels decrease glucose uptake and glycogen synthesis in muscle (16-18), negatively affect whole-body glucose disposal (19) and impair insulin’s ability to suppress liver glucose production. They also decrease insulin clearance by the liver (20-22) and impair insulin secretion by the β-cells (23, 24). Defects in the insulin-regulated FFA turnover in adipose tissue therefore markedly affect glucose homeostasis and promote insulin resistance. Since fat is a well-established model of insulin’s target tissues, fat cells are often used and they are suitable for experimental studies on carbohydrate and lipid metabolism.

Cellular glucose uptake in response to insulin

Insulin’s action to stimulate glucose uptake is mediated through insulin receptors (IR) on the surface of target cells (Fig 2). Upon ligand binding the intracellular domain of IR is autophosphorylated at tyrosine residues (25) and within seconds, will bind and tyrosine phosphorylate different adaptor proteins (26). The key adaptor protein involved in regulating growth and carbohydrate metabolism in response to insulin is the insulin receptor substrate (IRS) family. Four IRS isoforms (IRS-1, IRS-2, IRS-3 and IRS-4) have been identified that are expressed in a tissue-specific manner (27, 28). Where IRS-1 is the major tyrosine-phosphorylated IRS in response to insulin in adipocytes (29, 30). IRS proteins contain several potential tyrosine phosphorylation motifs that, upon phosphorylation create binding sites for different Src homology 2 (SH2) domain-containing effector proteins e.g. PI3K, SHP2 and Grb2. The insulin signal will amplify through these effectors and propagate through various downstream signaling pathways, providing a possible explanation for the diversity of insulin signaling (27, 31).
Glucose transport is mediated by the activation of the effector protein PI3K and its downstream signaling pathways. The critical role of PI3K has been established in several studies where inhibitors of PI3K, e.g. wortmannin or LY294002, completely block insulin-stimulated glucose transport (32-34). A stimulatory effect is observed upon expression of constitutively active PI3K (35, 36). Mice lacking the catalytic subunit of PI3K are insulin resistant and glucose intolerant (37). When IRS-1 becomes tyrosine phosphorylated by the IR it binds the regulatory p85 subunit of PI3K. This will position the catalytic subunit p110 close to the cell membrane where it catalyzes phosphorylation of the 3’ position in the inositol ring of phosphoinositide (PI) lipids, preferentially the formation of PI(3,4,5)-phosphate (PIP₃) from PI(4,5)-bisphosphate (PIP₂) (38). Phosphorylation at the 3’ position recruits 3’phosphoinositide-dependent kinase-1 (PDK-1) and protein kinase B (PKB) (39, 40) to the plasma membrane. Subsequently activated PDK-1 in turn, phosphorylate and activate downstream effectors like PKB also known as Akt, and the atypical protein kinase C (PKC) (41, 42).

PKB is clearly activated in response to insulin stimulation in different cell types (43) and full activation requires phosphorylation on both threonine 308 and serine 473 (44).
PKB exists in three isoforms (α, β and γ) and PKB β is predominantly activated in response to insulin in adipocytes (45). Expression of constitutively active PKB stimulates glucose transport in 3T3-L1 adipocytes (45-48). However, there is some controversy whether or not PKB activation is a critical step in insulin-mediated glucose transport. For example, dominant negative mutants of PKB do not completely block the effect of insulin on glucose transport (49). Besides its yet not fully established role in glucose uptake, PKB also phosphorylate downstream effectors involved in regulation of lipid synthesis (50), glycogen synthesis (51), cell survival (52) and protein synthesis (53).

Members of the PKC kinase family are also involved in mediating glucose transport. Insulin has been shown to activate PKC ζ/λ in 3T3-L1 and rat adipocytes (54, 55) and an overexpression of constitutively active atypical PKC increases GLUT4 translocation and glucose uptake. In contrast, dominant-negative mutants inhibit insulin stimulated glucose transport by 50% (54, 56). There are also indications that PKC ζ is able to stimulate glucose transport through an insulin-independent pathway (57). PKC α and β on the other hand, act as negative regulators of insulin signaling (58, 59). The function and relevance of each PKC isoform in insulin signaling and their potential role in the pathogenesis of insulin resistance and type 2 diabetes remain to be determined.

Glucose transport

Glucose is a polar molecule and thus is unable to cross the cell membrane through simple diffusion. For this reason the final step in insulin action leading to cellular glucose uptake, involves translocation of glucose transporter proteins (GLUTs) from intracellular compartments to the cellular membrane where they facilitate glucose uptake (60) (Fig 2). There are currently 13 known members of the GLUT family (61), of which GLUT4 is associated with insulin-stimulated glucose transport in adipocytes and muscle (60, 62). Translocation of GLUT4 from intracellular compartments to the cell membrane is the main rate limiting step of insulin-stimulated glucose transport in these tissues (63). The adipocytes, as most cell-types, also express GLUT1 that is relatively insensitive to insulin and controls the basal rate of glucose uptake (64).
Additional pathways involved in glucose transport

Platelet-derived growth factor (PDGF) can stimulate PI3K activity without eliciting translocation of GLUT4 and glucose transport (65). This indicates that the activated PI3K pathway, although necessary, is not sufficient to stimulate glucose transport. Overexpression of active PI3K increases GLUT4 translocation and glucose uptake with 50-100% of maximal insulin-stimulated glucose transport, still insulin is able to further increase glucose uptake (36, 66). These findings indicate that at least one additional signaling pathway is involved in the regulation of glucose transport. In line with this, the CAP/Cbl/TC10 pathway was recently discovered. This pathway also seems to emanate from activated IRSs and originates from lipid rafts located on the cellular membrane. This pathway is thought to participate in GLUT4 translocation by modulating actin structures (67). The importance of this pathway for cellular glucose transport in response to insulin remains to be determined, and was not investigated in the present study.

IRSs and insulin resistance

With the growing insight that IRSs are key enablers of insulin’s pleiotropic effects in insulin-sensitive tissues, a potential role for these proteins in the development of insulin resistance and type 2 diabetes emerged. This was supported by numerous studies in cell and animal models of insulin resistance (68-70), e.g. knock-out mice lacking IRS-1 display retarded growth and insulin resistance but do not develop diabetes due to a compensatory increase in insulin secretion (71, 72) whereas mice lacking IRS-2 display impaired β-cell insulin secretion and type 2 diabetes (73).

In humans, mutations in IRS-1 have been observed in some patients with severe insulin resistance and type 2 diabetes. However, these mutations are rare and can not explain the impaired insulin action in the majority of patients (74-77). Furthermore, a decreased protein level and gene expression of IRS-1 have been observed in adipose tissue, but not skeletal muscle, from insulin-resistant, type 2 diabetic patients and in a cohort of subjects with first-degree relatives with type 2 diabetes (30, 78-81).
Reduced tyrosine phosphorylation of IRS-1 and downstream activation of PI3K have been found in both adipose tissue and skeletal muscle in subjects with insulin resistance and type 2 diabetes as well as in animal models of insulin resistance (81, 82). Taken together, these findings support a pivotal role of IRS-1 in these disorders, although the underlying mechanisms and the role of the observed alterations in IRS-1 in the development of insulin resistance and type 2 diabetes are yet to be determined.

Regulation of IRS-1 function

The biological function of IRS-1 is partly regulated through tyrosine and serine/threonine phosphorylation. Tyrosine phosphorylation of IRS-1 occurs upon insulin-stimulation and is essential for activation of the downstream effector proteins that mediate glucose transport. Some of these are serine kinases that mediates insulin action, but also phosphorylate IRS proteins on specific serine/threonine residues under physiological conditions. This uncouples IRSs from the IR and downstream effectors, thereby terminating insulin action. The action of these serine kinases is part of insulin’s negative-feedback control mechanism (83, 84). PKB has also been recognized as an IRS kinase and is an exception since serine phosphorylation of IRS-1 by PKB protects IRS-1 from the action of phosphatases and maintains IRS-1 in an active form (85).

Inhibitors of the PI3K pathway block serine/threonine phosphorylation of IRS-1 (85, 86). This implicate that downstream effectors of PI3K are involved, and PKCζ (86, 87), mTOR (88, 89), JNK (90, 91) and S6K1 (92) have been identified as potential IRS kinases. Interestingly, there are implications that agents known to induce insulin resistance such as obesity, FFA and cellular stress take advantage of these IRS kinases. (92-96) Interestingly, treatment with insulin-sensitizing, anti-inflammatory salicylates reverse serine/threonine phosphorylation of IRSs in insulin-sensitive tissues in diet-, obesity- and free fatty acid-induced models of insulin resistance (93, 97). These findings have further shown the importance of identifying the IRS kinases, their target residue and activators.

Insulin signaling is also attenuated by tyrosine dephosphorylation of IRS-1 by the action of phosphatases (98, 99) and degradation of IRS-1 through the proteasome
pathway (100, 101). Additional pathways through proteasome-independent processes have also been implicated (102) (103).

When insulin fails…

For as yet unknown reasons the action of insulin is impaired, and insulin resistance develops. Insulin resistance can manifest itself as either unresponsiveness or insensitivity to insulin. In the majority of cases a combination of both is observed. Unresponsiveness results in an impaired maximal effect of insulin, whereas insensitivity means that a higher concentration of insulin than normal is needed to produce a certain effect (104). Insulin resistance eventually leads to elevated blood glucose levels since glucose uptake by insulin-sensitive tissues is impaired and insulin is no longer able to inhibit the endogenous glucose production by the liver (Fig 3). Initially the β-cells will manage to preserve glucose homeostasis by increasing the production and secretion of insulin. This gives rise to the characteristic hyperinsulinemic state associated with the insulin resistance. Eventually, in cases that progress to type 2 diabetes the insulin production by β-cells diminishes, and as the insulin levels in the circulation decline an increase in blood glucose level is observed. The state of hyperglycemia is characteristic for type 2 diabetes and if not controlled it will eventually cause macrovascular (coronary heart disease, cerebrovascular and peripheral artery disease) and microvascular (retinopathy, nephropathy and neuropathy) complications and thereby lead to disabling conditions and increased mortality.

Figure 3. Hyperglycemia as a result of impaired insulin action.
Hyperinsulinemia-induced insulin resistance

Chronic high insulin levels negatively affects glucose transport both in vivo (105-108) and in vitro (69, 109, 110) but the underlying mechanisms are not fully understood. Although prolonged insulin-stimulation has been shown to result in insulin receptor degradation in vitro, this phenomenon has been reported to occur secondary to a decrease in glucose transport capacity both in vitro and in vivo (105, 106, 109, 111, 112). Since only 10% of receptors appear to be needed for maximal stimulation of glucose transport this further supports that insulin receptor degradation is not a critical primary mechanism (113). Thus, the negative effect of high insulin levels on cellular glucose transport appears to be associated with post-receptor defects. Quite recently, a negative-feedback control mechanism of insulin action was discovered that potentially could be responsible for the previously observed post-receptor defects (83, 84, 114).

Hyperglycaemia-induced insulin resistance

There is overwhelming evidence that chronic hyperglycemia in vivo is associated with impaired glucose disposal in insulin-sensitive tissues and disturbance in glucose-induced insulin release from β-cells (115-118). Interventions that correct hyperglycemia also restore insulin sensitivity which suggests that hyperglycemia is a direct cause of insulin resistance (119) (119, 120). The underlying mechanisms are not fully understood and contradictory data have been reported in different in vitro studies. Rat and 3T3-L1 adipocytes exposed to elevated glucose levels show either a down-regulatory or no effect on glucose uptake capacity (70, 121, 122). Results obtained with muscle in vitro are more conclusive (123-125) although contradicting results have been reported (126). Although the cellular mechanisms involved in glucose toxicity are largely unknown, an increased flux through the hexosamine biosynthesis pathway has been suggested (127, 128). This is accompanied by the activation of PKC that reduces glucose transport by interfering with IR activity and IRS-1 function (81, 129, 130). Hyperglycemia has also been associated with increased production of reactive oxygen species in adipocytes which can trigger an inflammatory response, including a rise in TNF-α, that is known to negatively affect glucose uptake (131, 132).
Several in vitro studies have shown that high glucose and insulin levels per se have direct effects on glucose transport capacity. Additional studies have also revealed synergistic effects of high concentrations of glucose and insulin (70, 121, 133).

**Obesity-induced insulin resistance**

*Adipose tissue as an endocrine organ*

From being considered as merely a storage place for energy, the adipose tissue today is recognised as a main regulator of energy homeostasis, producing and releasing adipokines and cytokines with endocrine, paracrine and autocrine effects. As a consequence of increased fat mass the level of these adipokines are altered in the obese state and are likely to contribute to the obesity-related insulin resistance. Some of these adipokines, e.g. adiponectin and leptin evoke insulin-sensitising effects by increasing FFA oxidation in muscle and inhibiting glucose output from the liver (134) and by regulating energy intake (135), respectively. Obesity and insulin resistance are associated with decreased adiponectin levels (136) and with leptin resistance (137). The adipose tissue also secrete molecules with the ability to negatively regulate insulin sensitivity, e.g. acute-phase serum amyloid A (A-SAA) (138), resistin (95) and serum retinol binding protein 4 (RBP4) (139) for which elevated levels in the circulation is associated with obesity and insulin resistance, and normalisation improves insulin sensitivity. The mechanisms of action remain to be understood, and although the action of resistin in rodents is well established its role and function in humans is under debate (140). Adipocytes also express monocyte chemoattractant protein 1 (MCP-1) in an insulin-sensitive manner. In states of hyperinsulinaemia like obesity and insulin resistance, elevated levels of MCP-1 are observed. MCP-1 has been shown to impair glucose uptake and GLUT4 expression in 3T3-L1 adipocytes (141).

*Low grade inflammation*

Obesity, in particular visceral obesity, is closely related to insulin resistance and is considered to be a powerful risk marker for the development of type 2 diabetes (142-144). One contributing factor is that obesity and in particularly visceral obesity has been recognized as a chronic low grade inflammatory condition (145, 146) that greatly
affects insulin sensitivity. This effect is probably in part moderated by enhanced production of the pro-inflammatory cytokines IL-6 and TNF-α by the increased number of infiltrated macrophages (147) and also by adipocytes (148, 149). The expression of IL-6 and TNF-α is positively correlated with degree of adiposity and insulin resistance (148, 150) and decrease upon weight loss (151, 152). Both cytokines negatively affect glucose transport in adipocytes in vitro, and the effect is associated with decreased GLUT4 and IRS-1 gene expression and IRS-1 function (132, 148, 153, 154).

TNF-α is known to stimulate lipolysis in adipocytes (132, 155) and inhibit lipoprotein lipase (LPL) activity (156). The resulting elevation in circulating FFA impairs whole-body glucose disposal in vivo (19) and glycogen synthesis and glucose transport in muscle in vitro. However, data regarding direct effects of elevated FFA on insulin action in adipocytes are contradictory, showing enhanced, decreased or no effect on cellular glucose uptake capacity (157-160).
AIMS

The overall aim of this present thesis was to further increase our understanding of the mechanisms that contribute to the development and maintenance of cellular insulin resistance. In particular, the effects of high glucose and insulin levels were addressed to mimic the situation in type 2 diabetes.

Specific aims:

• To investigate the intracellular mechanisms involved in insulin resistance and downregulation of IRS-1 and IRS-2 protein levels in primary epididymal rat adipocytes, upon exposure to high glucose and insulin levels (study I).

• To investigate the effect of long-term exposure to a high glucose and/or insulin environment on glucose transport capacity and protein levels of IRS-1 and IRS-2 in primary human adipocytes (study II).

• To study the phosphorylation-based regulation of IRS-1 function in human adipocytes upon induction of insulin resistance with high insulin levels alone or in combination with high glucose levels (study III).

• To elucidate whether there are additional factors, apart from insulin, glucose and FFA, present in serum from type 2 diabetic subjects with the ability to produce insulin resistance in primary human adipocytes (study IV).
METHODS

Animals (paper I)

Male Sprague Dawley rats were caged in groups of four and housed under a 12/12 h light/dark cycle. Animals were given free access to standard pelleted rat chow and water and were allowed to acclimatize to their new surroundings for at least 5 days before they were used for study purposes. Rats weighing 150-200 g were killed by decapitation and the epididymal fat pads were immediately removed and transported to the laboratory. The Umeå Ethics Committee for animal research approved the study protocol.

Type 2 diabetic patients and healthy volunteers (paper II-IV)

The healthy volunteers in study II-IV were recruited by advertisement in a local newspaper and the subjects with type 2 diabetes in study IV were recruited among patients at the Diabetes Unit of Umeå University Hospital. Individuals with stroke, cancer, angina pectoris or myocardial infarction within the last 12 months were excluded. Subjects arrived at the Metabolic Unit at Umeå University Hospital at 08.00 after an over-night fast (study IV) or, alternatively, at 14.00 (study II and III) after a 6-hour fast. The timing in study II and III was chosen to avoid experimental procedures on cell cultures during night. In either case a venous blood sample was obtained for analysis of different blood parameters and one subcutaneous needle biopsy was taken after local dermal anaesthesia from one, or if necessary, from both sides of the lower part of the abdomen. The Umeå University Ethics Committee approved the study protocols and each participant gave their informed consent.

The two study groups in paper IV consisted of 10 healthy non-diabetic subjects and 11 subjects with type 2 diabetes classified in accordance with the 1998 WHO criteria (161). Three healthy non-diabetic, and three type 2 diabetic subjects were recruited as serum donors. One of the type 2 diabetic adipocyte donors had no antidiabetic medication, seven were treated with oral antidiabetic drugs alone and two in combination with insulin. One subject was treated with insulin alone. Eight of the type 2 diabetic subjects and one non-diabetic control subject were treated with
antihypertensive agents. Five of the type 2 diabetic subjects were statin users. Of the three type 2 diabetic serum donors one had no antidiabetic medication and the remaining two was treated with insulin, one in combination with an oral antidiabetic drug. No medication was taken before investigations on the study days. For a more detailed description of medications readers are referred to paper IV.

**Adipocyte isolation and determination of cell size**

Regardless of source the obtained adipose tissue was transported to the laboratory in warm (37 °C) medium 199 supplemented with 5.6 mM glucose and 40 mg/mL BSA. The adipose tissue was washed with pre-warmed medium (same as above) and blood clots were removed. Epididymal fat pads were also minced. Isolated adipocytes were prepared by treatment with collagenase (0.6 mg/ml in medium same as above) in polypropylene containers at 37 °C for ~1 h in a shaking (~150 rpm) water bath. The collagenase-treated tissue was filtered through a nylon mesh (Ø 0.3 mm) and washed four times in fresh medium. Collagenase has been shown to affect membrane function (113) and could thus potentially affect adipocyte metabolism. For this reason several batches of collagenase were tested and the one that displayed the least interference with the insulin-stimulated glucose transport in freshly isolated rat adipocytes was purchased in a larger quantity, allowing the same batch to be used throughout an experimental study.

Fat cell size was measured according to the method of Smith et al. (162). In brief, the cell diameter of 100 consecutive cells was determined by examination with a light-microscope equipped with an inbuilt ruler.

**Adipocyte culture**

Isolated adipocytes were cultured in Teflon or polypropylene flasks containing DMEM, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% FCS (study I-III) or 25% serum from healthy non-diabetic or type 2 diabetic subjects (study III). D-glucose and insulin were present at the indicated concentrations and time-periods. Cells were incubated at 37 °C with gentle agitation (25 rpm) under a gas phase of 95% O₂ and 5% CO₂ for 24 h, unless otherwise specified.
Glucose uptake assay

Following culture or collagenase treatment cells were washed four times and glucose uptake capacity was determined essentially as previously described (163). In brief, cells were resuspended (lipocrit 3-5%) in medium 199 supplemented with BSA (4%), ADA and PIA but without glucose and pre-incubated in vials for 15 min at 37°C in a shaking water bath with different insulin concentrations, as indicated. $[^{14}\text{C}]$-U-D-glucose was added (0.21 mCi/L, 0.7-1.0 μM) and glucose uptake was subsequently assessed during the following 45 min. The cell suspension was transferred to pre-chilled tubes and separated from the incubation medium by centrifugation through silicone oil. The fat pellet was collected and cell-associated radioactivity was measured by scintillation counting. Glucose uptake is under these experimental conditions mainly determined by the rate of transmembrane glucose transport (164). The cellular clearance of glucose from the medium was calculated according to the following formula and taken as an index of the rate of glucose uptake:

\[
\text{Cellular clearance of medium glucose} = \frac{\text{Cell-associated radioactivity} \times \text{Volume}}{\text{Radioactivity in medium} \times \text{Cell number} \times \text{Time}}
\]

Western blot analysis of proteins in total cellular lysates

After the incubation period, cells were washed four times with PBS and lysed. In order to study insulin stimulated phosphorylations, cells were washed four times in fresh medium and incubated 10 min at 37°C in a shaking water bath in vials containing medium 199, BSA (4%), ADA, PIA and glucose with or without a maximal insulin concentration (1000 μU/mL). Cells were washed three times in PBS, centrifuged, collected and lysed. After being rocked 30 min at 4°C detergent insoluble material was sedimented by centrifugation and supernatants were collected and frozen in aliquots at −80°C. Protein content was measured with the BCA protein kit (Pierce Chemical Co, Rockford, IL) with bovine serum albumin as standard.
Separation of proteins was performed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The total amount of protein added per lane was the same within each set of experiments. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane that was subsequently blocked with 5% dry milk dissolved in PBS, or in accordance with instructions from the manufacturer of the antibody in question. Detection of various proteins (IRS-1 pTyr612-IRS-1, pSer307-IRS-1, pSer312-IRS-1, IRS-2, PKB, pSer473-PKB, and GLUT4) was performed using polyclonal antibodies and detection of β-actin using a monoclonal antibody. ECL Western blotting kit was used to visualize immunoreactive bands. Bands were quantified by densitometry (Molecular Analyst (study 1, II and IV) and Quantity One (study III), Bio-Rad Laboratories). Films from each set of experiments were analysed at the same time and the background signal from each film was subtracted. Protein quantification was adjusted for the corresponding β-actin level or as otherwise specified. The different culture conditions did not consistently change the protein level of β-actin.

**cDNA synthesis and semiquantitative PCR**

This section will only be briefly described since details are reported in paper I. After the 24 h incubation period, total RNA from each culture condition was extracted using TRIzol® and isolated in accordance with the manufacturer’s instructions. Four micrograms of total RNA was reverse transcribed using SuperScript™ First Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies, Inc (Paisley, UK). cDNA was stored at −20°C until further use.

Semiquantitative RT-PCR was conducted according to a method where cDNA from each culture condition was divided in three equal parts and PCR was performed for different increasing numbers of cycles. The time course of appearance of different PCR products will give an estimate of their relative abundance, and their levels can thus be compared between different culture conditions (165). β-actin was used as an internal control and the relative mRNA expression was not affected by the different culture conditions. The PCR products were separated on a 1.2% agarose gel and analyzed by
ethidium bromide staining. Gels were photographed under UV light illumination using Flour-S MultiImager (Bio-Rad Laboratories, Hercules, CA).

**Blood chemistry**

Commercial kits were used in accordance with the manufacturer’s instructions to analyse adiponectin and leptin (Linco Research Inc., St Charles, MO), FFA (Wako Chemical USA Inc., Richmond, VA), interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α (R&D Systems Inc., Minneapolis, MN). Serum insulin concentrations were measured by micro particle enzyme immunoassay (Immulite 2000, DPC, Los Angeles, CA) and high sensitive C-reactive protein (hs-CRP) by CRP (Latex) HS Tina-quant kit (Roche Diagnostics Co., Indianapolis, IN). All other measurements were done according to the routine methods at the Department of Clinical Chemistry, Umeå University Hospital.

**Statistical analysis**

Statistical analyses were performed using the SPSS package (SPSS Inc., Chicago, IL, USA). Results are given as means ± S.E.M. ANOVA, t-test, Wilcoxon signed ranks and Mann-Whitney were used as indicated. Variables are logarithmically transformed where appropriate (paper IV) due to skewed distributions. P-values less than 0.05 were considered as statistically significant.
RESULTS

Paper I

Insulin receptor substrates (IRSs) are key mediators of insulin’s action to enhance cellular glucose uptake. The aim was to investigate the underlying mechanisms behind the previously observed reduction in protein levels of IRS-1 and IRS-2, evoked by a high glucose/high insulin environment for 24 h (70), and their significance in the associated impairment of insulin-stimulated glucose uptake capacity in primary rat adipocytes. The aim was also to more extensively investigate the time course of development as well as the reversibility of insulin resistance.

The relative mRNA levels of IRS-1 and IRS-2 were assessed upon 24 h pre-treatment with high concentrations of glucose (15 mM) and/or insulin (10^4 μU/mL). A high insulin concentration, regardless of the surrounding glucose level, consistently decreased IRS-2 mRNA levels, compared to the control treatment. A high glucose concentration alone slightly increased IRS-2 mRNA levels. No alteration in the mRNA expression of IRS-1, IR, PI3K or PKB was observed. However, pre-treatment with high insulin levels alone increased GLUT4 mRNA expression, and when combined with high glucose levels a further increase was observed.

Presence of the protein synthesis inhibitor Cyclohexamide (CHX) during a 16 h culture period did not affect the ability of the high glucose/high insulin setting to significantly decrease basal and insulin-stimulated glucose uptake capacity, and depletion of IRS-1 was still observed. On the other hand, the presence of CHX alone during the culture period induced a marked depletion in the protein level of IRS-2 (by ~80%) and reduced basal glucose transport capacity (by ~70%).

A high glucose/high insulin environment during 2 h was sufficient to induce a marked cellular insulin resistance with respect to glucose transport, and a maximal impairment was seen after 3 h. No reduction in the protein levels of IRS-1 and IRS-2 were evident after a 6 h treatment but were significantly decreased at 16 h. After 24 h no further
reduction of IRS-1, but a slight additional suppression of the IRS-2 protein level was observed.

After maximal impairment of cellular glucose transport capacity was achieved by 6 h pre-treatment in high glucose and high insulin levels, cells were transferred to a low glucose concentration (5 mM) without insulin and subsequent incubation for up to 16 h. This could partially restore basal (by ∼50%) and insulin-stimulated glucose uptake capacity (by ∼90%). No further improvement was obtained with 24 h culture.

Main conclusions:
The reductions in IRS-1 and IRS-2 protein levels observed upon long-term treatment with high glucose and insulin levels occur via different mechanisms and are the result of post-translational mechanisms and altered gene expression, respectively. Depletion of both IRS-1 and IRS-2 protein is a secondary event, which occurs after the development of impaired glucose transport in this cellular model of insulin resistance. These results suggest that depletion of cellular IRSs in adipose tissue is a consequence rather than a cause of insulin resistance and type 2 diabetes.

Paper II

The aim of this study was to investigate the ability of high glucose and/or insulin levels to elicit cellular insulin resistance in primary human adipocytes and, if so, to evaluate the time course and content of key proteins in the insulin signaling cascade mediating glucose uptake.

Pre-treatment with high glucose levels (20 mM) alone was only associated with a minor impairment (by ∼20%) in the subsequently assessed maximally insulin-stimulated glucose uptake capacity. The impaired insulin responsiveness was not observed until after a 24 h pre-treatment and was not associated with any alterations in the protein levels of IRS-1 or IRS-2.

The maximally insulin-stimulated glucose uptake capacity was significantly reduced already after 2 h in a high insulin (10⁴ μU/mL) environment and a maximal reduction
(by ~55%) was evoked after 6 h. Basal glucose transport was reduced by ~50% after 24 h, and no significant alteration in the protein level of IRS-1 and IRS-2 was observed. Pre-treatment with high glucose and high insulin levels in combination impaired basal and maximally insulin-stimulated glucose uptake capacity in a similar fashion as pre-treatment with high insulin levels alone. However, after 6 h a ~45% reduction in the protein level of IRS-1 was observed that was further suppressed (by ~65%) after the 24 h pre-treatment.

Pre-treatment with high glucose alone did not affect the protein level of PKB or its ability to become serine phosphorylated (i.e. pPKB/PKB ratio) upon a subsequent 10 min acute insulin stimulation. In the high glucose/high insulin setting the cellular content of PKB was unaffected but insulin’s action to acutely stimulate phosphorylation of PKB was reduced by ~50% after the 24 h culture period. This was associated with a 3 to 7 fold increase in the unstimulated pPKB/PKB ratio. Due to limited amount of retrieved adipose tissue pPKB/PKB ratio was not assessed in the pre-treatments with high insulin levels alone.

Main conclusions:
An environment with a high insulin level significantly impairs glucose uptake capacity in human adipocytes. High glucose and insulin levels synergistically decrease IRS-1 protein content, but this does not appear until after an established impairment in glucose uptake capacity. A high glucose environment has only a minor negative impact on cellular glucose transport. Importantly, these results highlight significant differences in the cellular response to elevated glucose and/or insulin levels between human and rat adipocytes see study I and ref (70).

Paper III

The function of IRS-1 is enhanced by tyrosine phosphorylation, as is seen following acute insulin stimulation. Serine/threonine phosphorylation of IRS-1 inhibits its function, and this can also be enhanced by insulin itself through a negative-feedback control mechanism that terminates insulin action under physiological conditions. The aim of this study was to investigate whether cellular insulin resistance and reduced IRS-
content were associated with alterations in phosphorylation of IRS-1 at Tyr\(^{612}\), Ser\(^{307}\) and Ser\(^{312}\) in primary human adipocytes. Phosphorylation of both serine residues are implicated in attenuation of insulin action and Ser\(^{312}\) is furthermore believed to target IRS-1 to degradation through the proteasome pathway.

The synergistic effect of high glucose and high insulin levels to reduce cellular levels of IRS-1 was also associated with a significant increase in basal IRS-1 Ser\(^{312}\) phosphorylation. The significant increase in basal IRS-1 Ser\(^{312}\) phosphorylation observed in the high glucose/high insulin setting was not associated with any further impairment of the insulin-stimulated glucose uptake capacity that was evoked by high insulin levels alone.

The observed impairment in insulin-stimulated glucose uptake capacity, initiated already after 2 h exposure to high insulin levels, was not associated with any reduction in the total IRS-1 Tyr\(^{612}\) phosphorylation, assessed by 10 min acute insulin stimulation subsequent to the incubation period. However, markedly elevated basal IRS-1 Tyr\(^{612}\) phosphorylation was observed (>300%). This was also associated with increased basal IRS-1 Ser\(^{307}\) phosphorylation.

Main conclusions:

The synergistic effect of high glucose and insulin levels to reduce protein levels of IRS-1 is probably mediated through enhanced Ser\(^{312}\) phosphorylation that is considered to target IRS-1 to the proteasome degradation pathway. The observed impairment in insulin-stimulated glucose transport coincided in time with, and may be the result of a disturbed regulation of IRS-1 function due to enhanced serine/threonine phosphorylation. However, more extensive work is needed to establish the exact role of IRS-1 Ser\(^{307}\) and Ser\(^{312}\) in this model of insulin resistance.

Paper IV

The aim of this pilot study was to explore whether there are factors other than glucose, insulin and FFAs in serum from type 2 diabetic subjects that have the ability to negatively affect insulin action in primary human adipocytes.
In adipocytes isolated from non-diabetic subjects the maximally insulin-stimulated glucose uptake capacity was significantly impaired (by ∼30%) following a 24 h culture period in medium supplemented with 25% serum from type 2 diabetic subjects, as compared to serum from non-diabetic subjects. This was seen even though glucose was adjusted to a physiological level (5 mM). This was not associated with any differences in the EC\textsubscript{50} value for insulin’s effect on glucose transport. Glucose uptake capacity in adipocytes isolated from type 2 diabetic subjects was similar regardless of pre-treatment condition. No significant alterations in the levels of key proteins in the intracellular insulin signaling cascade (IRS-1, IRS-2 and GLUT4) were observed that could explain the impaired insulin-stimulated glucose uptake capacity in adipocytes from non-diabetic control subjects incubated with serum from type 2 diabetic donors.

Comparison of blood chemistry between non-diabetic control and type 2 diabetic serum donors revealed no evident factor that could be responsible for the observed reduction in insulin responsiveness. Insulin, FFA, IL-6 and TNF-α could be discarded as potential candidates. Despite individual differences in anthropometric and biochemical characteristics between subjects, each serum from the different type 2 diabetic donors displayed similar degree of impairment on insulin-stimulated glucose transport.

**Main conclusions:**

The results from this pilot study indicate that in the circulation of type 2 diabetic subjects there are novel factors beside glucose, insulin, FFA, IL-6 and TNF-α that negatively effect insulin action on glucose transport capacity.
SUMMARY OF RESULTS

- Alterations in levels of IRS-1 and IRS-2 in rat adipocytes following exposure to high glucose and/or insulin levels are the result of post-translational mechanisms and altered gene expression, respectively.

- In human adipocytes, cellular content of IRS-1 is reduced by a synergistic effect of high glucose and insulin levels. This appears to be mediated by enhanced IRS-1 Ser\(^{312}\) phosphorylation, which has been reported to target IRS-1 to a proteasome degradation pathway.

- In both human and rat adipocytes the observed reductions in IRSs appear after an established impairment in glucose transport and are thus likely to be an effect rather than a cause of insulin resistance in these models.

- High insulin but not glucose levels produce a marked impairment in insulin-stimulated glucose uptake capacity in human adipocytes. This can be partly explained by an altered regulation of IRS-1 function, assessed by tyrosine and serine phosphorylations.

- A significant difference between rat and human adipocytes was observed regarding the cellular response to altered levels of glucose and insulin levels, respectively, with respect to insulin action on glucose transport and IRS content.

- Besides factors like glucose, insulin, FFA, TNF-α, and IL-6, additional humoral factors appear to be able to directly promote cellular insulin resistance.
DISCUSSION

The development of type 2 diabetes is associated with different stages displaying high insulin and/or glucose levels in the circulation. The ability of β-cells to initially compensate for insulin resistance by increasing the insulin secretion will result in elevated circulating blood insulin levels. When insulin resistance progresses to type 2 diabetes, β-cell failure occurs. As a result insulin levels start to decline with a parallel increase in glucose levels resulting in chronic hyperglycemia unless proper treatment is initiated. In order to investigate mechanisms involved in the development of cellular insulin resistance we have performed experimental studies with isolated fat cells, a well established model of insulin’s target cells that is very suitable for experimental studies on both lipid and carbohydrate metabolism. To mimic the in vivo characteristic milieus in the insulin-resistant and type 2 diabetic states, fat cells were cultured in environments with high glucose and/or high insulin levels.

Direct effects of high glucose and/or insulin levels on cellular glucose transport

The majority of studies performed on murine animals and cells indicate that elevated glucose and/or insulin levels to different degrees directly affect basal and insulin-stimulated glucose uptake capacity in adipocytes (69, 70, 109, 127). Hyperglycemia has consistently been found to decrease glucose utilization in peripheral tissues in vivo (116). Previous work presented by this group show that primary rat adipocytes display significantly impaired basal and insulin-stimulated glucose uptake capacity upon 24 h treatment with ≥15 mM glucose compared to cells cultured in a low glucose environment (≤ 10 mM) (70). It was thus a quite unexpected finding in study II that a high glucose level had no effect on the basal, and only induced a minor impairment in the maximally insulin-stimulated glucose uptake capacity in human adipocytes. The effect was furthermore not evident until after a 24 h treatment. The minor ability of high glucose to impair glucose transport in human adipocytes is supported by a previous study by Smith et al. where a 16 h culture period with 25 mM glucose did not induce any consistent impairment in cellular glucose transport (80).
A high insulin environment significantly decreased both basal and insulin-stimulated glucose uptake capacity in human adipocytes, and no additive effect of combined high glucose levels was observed. These results are quite opposite to our previous findings with primary rat adipocytes where elevated insulin concentrations during 24 h treatments elicited no effect on the subsequently assessed cellular glucose uptake capacity, unless combined with high glucose levels. The combination further impaired both basal and insulin-stimulated glucose transport capacity compared to high glucose levels alone. Interestingly, long-term treatment of rat adipocytes with the high glucose/high insulin setting and human adipocytes with high insulin levels, respectively displayed similar time-course of impairment of the insulin-stimulated glucose transport. Already after a 2 h pre-treatment a significant decrease in the subsequently assessed insulin-stimulated glucose uptake capacity was observed and maximal impairment was reached after 6 h (70). In a more extensive investigation of the time-course of induction we were able to show that a maximal reduction of both basal and insulin-stimulated glucose transport capacity was established after 3 h in primary rat adipocytes. This was however not assessed in human adipocytes. These findings indicate a very rapid cellular response to the present alterations in the environment, quite opposite to that observed in human adipocytes exposed to a high glucose environment. Taken together these results indicate differences between rat and human primary adipocytes in the sensitivity to elevated insulin and glucose levels in the surroundings, but similar underlying mechanisms of impaired insulin-mediated glucose transport might to be present.

**Can cellular insulin resistance be reversed?**

In study I primary rat adipocytes exposed to the high glucose/high insulin setting for 6 h to ensure the induction of an impaired glucose transport, a subsequent incubation at low glucose concentrations without insulin for up to 16 h was shown to partially restore basal and insulin-stimulated glucose uptake capacity. In study IV however, the impaired glucose transport observed in freshly isolated adipocytes from type 2 diabetic subjects was not clearly improved upon 24 h incubation under more physiological conditions. This finding is supported by some, but not all (166), previous studies where 6-24 h incubations at a physiological glucose level were not associated with any improvement in insulin action with respect to glucose uptake in adipocytes from type 2 diabetic subjects (80, 167). Reviewing together these results indicate that the
underlying cellular mechanisms involved in the development of insulin resistance, due to elevated insulin and glucose levels, can at least initially be partly reversed. This process, however, appears to be much more time-consuming than the relatively rapid induction of insulin resistance. In contrast, adipocytes from type 2 diabetic patients appear to have lost their ability to fully restore their glucose transport capacity, at least during the currently tested time-period and this could potentially be due to many years of exposure to a diabetic milieu.

In this context it is important to remember that type 2 diabetes is a multifactorial and polygenic disorder where it’s likely that many factors affect glucose transport capacity (see below). The effects exerted by these factors could potentially impair adipocyte glucose metabolism in an irreversible manner. Interestingly, the results from study IV indicate the presence of a factor (or factors) in the circulation of type 2 diabetic subjects, in addition to previously known biomolecules like glucose (117), insulin (106), FFA (19), TNF-α (154) and IL-6 (148), with the ability to negatively affect insulin action with respect to glucose uptake capacity. Resistin and endothelin-1 are two potential candidates since both proteins have been reported to impair IRS-1 function and glucose uptake in 3T3-L1 adipocytes (95, 168), and their plasma levels have been shown to be elevated in different insulin-resistant states (169-171). However, there still remain to be determined whether resistin is involved in the development of insulin resistance in humans and not only in rodents (140). Other likely candidates are the adipocyte expressed proteins A-SAA, RBP4 and MCP-1. Elevated serum levels of these proteins have been associated with impaired insulin sensitivity in obesity and insulin resistance (138, 139, 141). Identification of these biomolecules would be of great interest since our results imply that even though normoglycemia is achieved these factors can still negatively affect insulin action in subjects with type 2 diabetes. Another interesting question is whether these factors appear in the circulation as a primary or secondary phenomenon in type 2 diabetes. Thus, studies on serum from insulin-resistant pre-diabetic subjects might be included in future experimental work to further address this question.

The inability to fully restore insulin sensitivity in human adipocytes from type 2 diabetic subjects in cell culture is at variance with results reported with muscle strips where incubation under physiological conditions restored insulin action (172). A high
glucose concentration has been established to negatively affect insulin action in skeletal muscle (123, 124) and in accordance with this the reversed insulin resistance in muscle strips from type 2 diabetic patients was not observed if the subsequent incubation was conducted at a high glucose concentration. Since our results indicate that high glucose levels only have a minor ability to impair insulin-stimulated glucose uptake capacity in human adipocytes, one possible explanation for the observed differences in reversibility could be that the mechanisms underlying the observed impairment in glucose transport in the two different cell types are slightly different. This is supported by the findings that in insulin-resistant and type 2 diabetic subjects the protein level of the key insulin signaling protein IRS-1 are significantly reduced in adipose tissue (30, 78, 173) but appear to be unaffected in skeletal muscle (79, 174).

**The role of IRS-1 and IRS-2 in insulin resistance**

Different in vivo and in vitro studies on human and animal models of insulin resistance and type 2 diabetes suggest tissue-specific alterations in gene expression and protein levels of IRS-1 and IRS-2 (30, 68, 70, 71, 73, 78, 175-180). Our group has previously shown that a 24 h treatment with high insulin levels alone had no effect on the protein level of IRS-1 but decreased IRS-2 content in primary rat adipocytes (70). On the other hand, a high glucose treatment decreased IRS-1, but increased IRS-2 protein content. When high insulin and glucose levels were combined both IRSs were significantly reduced. With the results obtained in study I we were able to show, for the first time to our knowledge, that high insulin concentrations were accompanied by reduced IRS-2 mRNA levels compared to the control treatment, regardless of surrounding glucose concentrations. High glucose levels alone on the other hand appeared to slightly increase IRS-2 mRNA levels. These results indicate that the previously observed alterations in cellular IRS-2 protein content seen with high glucose and/or insulin levels are the result of an altered gene expression in rat adipocytes. Similar results regarding the effect of insulin on IRS-2 gene transcription have been reported in liver and muscle cells (181-183) and were shown to occur in a PI3K/PKB-dependent manner (182).

When protein synthesis was inhibited, glucose transport capacity and decreased protein level of IRS-1 was still observed following treatment in a high insulin/high glucose environment in rat adipocytes. These results indicate that the underlying cause of the
observed reduction in IRS-1 protein content is the result of post-translational mechanisms. This is most likely the result of increased degradation since several studies have shown that following chronic insulin stimulation of cell lines or isolated primary cells, the level of IRS-1 protein was reduced through enhanced degradation (176, 177, 184-187). Since impairment of glucose transport elicited by the high glucose/high insulin setting was still observed in the presence of protein synthesis inhibition these results further indicate that the underlying defect was not dependent on a functioning protein synthesis machinery. Similar observations have also been reported by others (188). Another interesting observation was that while protein synthesis inhibition in the control culture had no effect on the subsequently assessed cellular glucose uptake capacity it significantly depleted protein levels of IRS-2. This suggests a very rapid turnover rate of IRS-2 but it also implies that IRS-2 is not an essential component of the signaling cascade involved in GLUT4 translocation and glucose uptake in rat adipocytes. However, upon IRS-1 depletion found in adipocytes from type 2 diabetic subjects, IRS-2 has been reported to become the main docking protein for PI3K (30). Characterisation of the IRSs also shows that the IRS-2 molecule shares many structural and functional characteristics with IRS-1 (189), although it requires higher insulin concentrations to be activated (30).

As in rat, a high insulin level had no effect on the protein level of IRS-1 in human adipocytes. High insulin levels have been shown to reduce IRS-1 protein content in 3T3-L1 adipocytes (69, 176). However, 3T3-L1 cells have traditionally been cultured in the presence of high glucose levels and in some of these studies it was reported that the effect observed upon chronic insulin treatment required the addition of glucose (177, 190, 191). A similar observation was made in study II since high insulin levels in combination with high glucose concentrations significantly reduced protein levels of IRS-1 in human adipocytes. The synergistic effect of high glucose and insulin levels that led to reduced IRS-1 protein content was shown to be associated with enhanced serine phosphorylation of IRS-1 at residue 312 in study III. This was an interesting observation since increased Ser\(^{312}\) phosphorylation of IRS-1 has been associated with IRS-1 degradation through the 26S proteasome (81, 192). The present experiments should be repeated in the presence of inhibitors of the proteasome degradation pathway, e.g. lactacystin or rapamycin, in order to confirm that the observed reduction in IRS-1 is the result of enhanced degradation.
There was a slight but non-significant reduction of IRS-1 amount, and increase in Ser$^{312}$ phosphorylation when human adipocytes were treated with high insulin levels alone. This could potentially indicate that elevated insulin levels alone were indeed able to reduce the cellular content of IRS-1, however, not as profound as when combined with high glucose levels. Further work is needed to clarify the effect of elevated glucose and insulin levels in the mechanisms underlying IRS-1 depletion in human adipocytes upon induction of insulin resistance. However, the effects elicited by the high glucose/high insulin setting on the protein level of IRS-1 agree with the observed level of IRS-1 in adipose tissue from insulin-resistant and type 2 diabetic subjects (30). The protein level of IRS-2 appeared to be unaffected by the different pre-treatments in human, whereas they were significantly affected in rat adipocytes. The result obtained in primary human adipocytes is in agreement with the observation that the IRS-2 protein level in the adipose tissue appear to be unaffected in type 2 diabetic subjects (30).

Importantly, in both rat and human adipocytes we could show that protein depletion of the IRSs appeared after impaired glucose uptake capacity was established. Thus, these results indicate that the observed reductions in IRSs in both species are a consequence rather than a cause of insulin resistance with respect to glucose transport, although they are likely to further aggravate the impairment of insulin action.

It could be argued that the finding that elevated insulin levels reduce the protein level of IRS-1 only when combined with high glucose levels, is in contradiction with the current knowledge regarding the development of type 2 diabetes. Thus, reduced adipocyte content of IRS-1 has been observed already in pre-diabetic insulin-resistant subjects. On the other hand, this could potentially be the result of genetic predispositions in these subjects. This is supported by the fact that decreased gene and protein expression of IRS-1 have been observed in ~30% of healthy individuals with a family history of type 2 diabetes (78). It should also be emphasised that insulin resistance and type 2 diabetes are multifactorial and polygenic disorders (1, 8, 9). Additional factors, beside glucose and insulin, associated with the development of insulin resistance could obviously affect IRS-1 protein levels. Insulin resistance is strongly associated with obesity, which in turn is associated with a chronic low grade inflammation in the adipose tissue (145, 146). This has been shown to increase the level of TNF-α and IL-6 (148) (150) which in turn has been shown to decrease gene
transcription of IRS-1 in the adipocytes (148, 154). Taken together, different factors, during different stages of insulin resistance and type 2 diabetes development could be responsible for the observed reduction in protein levels of IRS-1. However, the results obtained in study II and III suggest that the decreased protein level of IRS-1 observed in adipose tissue in conditions with insulin resistance is not caused by the hyperinsulinemic state per se. It can however not be excluded that elevated insulin levels during more extensive time-periods might exert additional effect.

The results obtained in study III indicate that chronically elevated insulin levels seem to disturb the finely tuned phosphorylation-based regulation of IRS-1 function in human adipocytes. Tyrosine phosphorylation of IRS-1 is necessary to fully activate PI3K and ensure the subsequent translocation of GLUT4 to the cell membrane in response to insulin-stimulation (31, 32). Although no significant alteration in the ability of maximal IRS-1 tyrosine phosphorylation, following acute insulin stimulation, was observed upon induction of insulin resistance a significant rise in basal IRS-1 tyrosine phosphorylation was observed following pre-treatment with high insulin levels. Insulin, under normal circumstances, has been shown to regulate its own action through a serine/threonine phosphorylation-based negative-feedback control mechanism (83, 114). Effector proteins downstream of PI3K mediate cellular glucose transport in response to insulin stimulation. In addition, some of these effectors also enhance IRS-1 phosphorylation at serine and threonine residues. This uncouples IRS-1 from the IR and downstream effectors, thus terminating insulin action (84, 114). The effector proteins involved are still largely unidentified but PKCζ (86, 87), mTOR (88, 89), JNK (90, 91) and S6K1 (92) have been implicated. Hypothetically; in accordance with insulin’s negative-feedback mechanism the major rise in basal tyrosine phosphorylation of IRS-1 observed upon pre-treatment in a chronic high insulin environment indicates that enhanced serine phosphorylation of IRS-1 also is present. This in turn, would suggest that the function is impaired in a proportion of the cellular pool of IRS-1 molecules. This would explain why no increase in basal glucose transport was evident in adipocytes in response to the elevated basal IRS-1 tyrosine phosphorylation. Interestingly, the rise in basal tyrosine phosphorylation of IRS-1 coincided in time with a significant reduction in the insulin-stimulated glucose uptake capacity. This suggests that an impaired function of IRS-1, at least in part, may account for the observed reduction in glucose uptake capacity. However, direct measurement of IRS-1 function,
e.g. measurement of IRS-1/PI3K interaction, is required to establish the involvement of impaired IRS-1 function in the present model of cellular insulin resistance.

Phosphorylation of IRS-1 at Ser\textsuperscript{312} and Ser\textsuperscript{307} was assessed in study III since enhanced phosphorylation of these residues has been associated with impaired glucose transport. Enhanced Ser\textsuperscript{307} phosphorylation has been observed in animal models of insulin resistance, including diet-induced obesity and upon induction of hyperinsulinaemia (193). Phosphorylation at Ser\textsuperscript{307} together with Ser\textsuperscript{312} has also been shown to be necessary to reduce IRS-1 association with the IR, thus terminating insulin action (193, 194). Enhanced basal IRS-1 Ser\textsuperscript{307} phosphorylation was observed upon treatment with high insulin and coincided with the observed decrease in insulin-stimulated glucose uptake. A significant increase in IRS-1 Ser\textsuperscript{312} phosphorylation on the other hand, was only observed in the high insulin/high glucose setting. Although this was associated with a reduced protein level of IRS-1, as previously discussed, it was not associated with any further deterioration in the insulin-stimulated glucose uptake capacity compared to pre-treatment with high insulin levels alone. However, pre-treatment with high insulin levels alone was associated with a slight increase in IRS-1 Ser\textsuperscript{312} phosphorylation, although non-significant. The present experimental setting should be repeated and the IRS-1/IR and IRS1/PI3K interactions assessed to more extensively...
determine the role Ser\textsuperscript{307} and Ser\textsuperscript{312} phosphorylation in the present model of insulin resistance in human adipocytes. Taken together, the results in study III indicate alterations in the regulation of IRS-1 function that may account for the observed impairment in insulin-stimulated glucose uptake capacity in the present models of cellular insulin resistance.

In study II, a considerably reduced Ser\textsuperscript{473} phosphorylation of PKB in response to acute insulin stimulation was observed, but PKB content was left intact following treatment with the high glucose/high insulin setting. A similar observation has been made in adipocytes from patients with type 2 diabetes (195). This further supports that perturbations in the intracellular signaling cascade of insulin leading to glucose transport activation is affected and contribute to the development of insulin resistance in peripheral tissues.

**Human vs rat adipocytes**

One important conclusion to be drawn from the present work is that there are major species differences in the cellular response to high glucose and/or insulin levels. For example, glucose transport in rat adipocytes is unaffected by high insulin levels, whereas both basal and insulin-stimulated glucose transport is significantly reduced in human adipocytes. Differences in the regulation of IRS-1 and IRS-2 are also clear. This emphasises the importance of conducting experiments in cells that are relevant for physiology or pathophysiology. A summary of the results in study I-III and previously obtained results (70) in this group regarding rat adipocytes are shown in table 1.

**Table 1. Summary of results in human vs rat adipocytes. Effects of long-term exposure to elevated levels of glucose and/or insulin.**

<table>
<thead>
<tr>
<th></th>
<th>High glucose</th>
<th>High insulin</th>
<th>High glucose &amp; insulin</th>
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<tbody>
<tr>
<td><strong>Insulin-stimulated glucose uptake</strong></td>
<td>Human</td>
<td>Rat</td>
<td>Human</td>
</tr>
<tr>
<td>IRS-1</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
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<tr>
<td>IRS-2</td>
<td>↔</td>
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</tr>
</tbody>
</table>

Arrows indicate alterations in glucose uptake or cellular protein content in human and rat adipocytes with the indicated pre-treatment condition. Results are from paper I, II and III and (70).
CONCLUSION

Although our in vitro results can not be directly transferred to the in vivo situation, cellular metabolism is obviously modulated by the interplay between different cells and tissues, the results on isolated adipocytes presented in this thesis indicate that…

1)…although elevated glucose levels are known to significantly decrease glucose utilization in peripheral tissues in vivo, this does not appear to involve direct effect on adipocyte glucose transport.

2)…the chronic hyperinsulinemic state that is seen in the development and early progression of type 2 diabetes, most likely contributes to the impairment in insulin action on glucose transport in adipose tissue. The results further imply that the impairment, at least partly, is mediated by insulin’s negative-feedback control mechanism exerted on IRS-1 function.

3)…the reduction in IRS-1 protein content previously observed in adipocytes from insulin-resistant and type 2 diabetic subjects is most likely a result of mechanisms triggered by these conditions per se, and it is not a primary mechanism leading to impaired cellular glucose transport.

4)…besides previously known factors like glucose, insulin, FFA, TNF-α, and IL-6 additional factors appear to be present in the circulation of type 2 diabetic subjects, that are able to directly produce cellular insulin resistance. Thus, such mediators probably can contribute to tissue insulin resistance in type 2 diabetes.

5)…the different effects observed on glucose uptake capacity and levels of IRSs between rat and human adipocytes in response to treatment with high glucose and/or insulin levels highlight the importance of conducting research in relevant cell types.
CONCLUDING REMARKS

Insulin resistance is the first measurable defect in the development of type 2 diabetes, a disease with rapidly increasing prevalence. The complications associated with this disorder are the common causes of morbidity and mortality in the developed world. The initial defects that trigger the development of insulin resistance are so far unknown but environmental factors like stress, physical inactivity and obesity are thought to interact with unfavourable genetic pre-dispositions. Obesity per se is strongly associated with insulin resistance. The obese state is also characterized by low grade inflammation and altered levels of adipokines that could contribute to insulin resistance in different tissues. This study indicates that the extended time-period with hyperinsulinaemia that is associated with insulin resistance and precedes type 2 diabetes can contribute to the impaired insulin action on glucose transport observed in adipose tissue. As insulin resistance and type 2 diabetes progress, factors appear in the circulation that further promote insulin resistance. Our results further suggest that there is a reduction in the insulin signaling protein IRS-1 protein following hyperglycemia and hyperinsulinaemia. However, this is not a primary mechanism of cellular insulin resistance in adipose tissue, but probably is a cellular response mechanisms induced by obesity, insulin resistance and type 2 diabetes.

Figure 5. Summary of development of insulin resistance and type 2 diabetes.
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