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Insulin signalling in human adipocytes -mechanisms of insulin resistance in type 2 diabetes

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*”Att veta när man vet något och att veta
när man inte vet något
-det är kunskap”*

Konfucius

*To the most important, my beloved family
David, Linus & Tim*

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Svensk sammanfattning

Prevalensen av fetma ökar drastiskt i stora delar av världen och utgör en stor riskfaktor för att utveckla insulinresistens och typ 2 diabetes. Fettväven kan bli mycket stor om för mycket energi tas upp av kroppen. Vid extrem övervikt är fettväven i kroppen i ett stresstillstånd, vilket gör att risken för att utveckla metabola sjukdomar som t.ex. typ 2 diabetes ökar. Fett lagras i olika fettdepåer i kroppen. Inlagringen i djupare kroppsdelar, runt och i inre organ s.k. visceralt fett, skiljer sig från fettväven som lagras direkt under huden s.k. subkutant fett. Nyare rön visar att mer visceral fettväv ökar risken för att utveckla insulinresistens och typ 2 diabetes.

Fettcellen är tillsammans med muskel- och leverceller de viktigaste för glukosmetabolismen. Fettcellen är en stor cell, som man lätt kan se med blotta ögat. Storleken på cellerna varierar dock kraftigt i en och samma fettvävnad. Upptag av glukos från maten vi äter regleras av hormonet insulin. Insulinresistens är ett tillstånd då cellerna svarar dåligt på insulin, vilket gör att glukoshalten i blodet ökar. Detta förekommer vid typ 2 diabetes, men även vid andra tillstånd där cellerna blir stressade, t.ex. kirurgiska ingrepp. Insulinsignaleringen i fettcellen är komplex och signalöverföringen inne i cellen sker främst via en kaskad av fosforyleringar, där olika proteiner i en signalkedja fosforyleras eller defosforyleras. Slutligen leder denna fosforyleringskaskad till insulinets sluteffekter som t.ex. upptag av glukos, proteinsyntes och celltillväxt. Efter att insulin bundit till och fosforylerat/aktiverat insulinreceptorn delas signalen upp inne i cellen i två huvudvägar; den metabola signalvägen och den mitogena signalvägen. Insulinreceptorsubstrat 1, IRS1, är ett stort protein som insulinreceptorn verkar direkt på. Fosforylering av aminosyran tyrosin på IRS1 är mycket viktigt för fortsatt insulinsignalering i fettcellen. IRS1 fosforyleras även på aminosyran serin som svar på bl.a. insulin. Serinfosforyleringen av IRS1 hämmar eller stimulerar insulinsignaleringen, ofta genom återkoppling av insulinsignalen.

Syftet med den här avhandlingen är att beskriva möjliga cellulära mekanismer i insulinsignaleringen vid insulinresistens som resultat av kirurgisk stress eller vid typ 2 diabetes i fettceller från människa.

Häri har upptaget av glukos analyserats och jämförts i fettceller från olika fettdepåer. Viscerala fettceller har högre basalt och insulinstimulerat glukosupptag och mer

glucostransportörprotein än subkutana fettceller. Däremot är det ingen skillnad i insulinkänslighet angående glukosupptaget i de olika typerna av fettceller.

Vidare fann vi att den kirurgiskt orsakade insulinresistensen hos subkutana fettceller från människa återgår till det normala efter övernattinkubering av cellerna i odlingsmedium. Insulinresistensen vid typ 2 diabetes är däremot permanent och har en annan mekanism än den reversibla, stress-relaterade insulinresistensen. Insulinresistansen vid typ 2 diabetes beror på att signalöverföringen mellan olika proteiner i cellen är defekt. Insulinreceptorns förmåga att fosforylera IRS1 på aminosyran tyrosin är nedsatt hos patienter med typ 2 diabetes. Fosforyleringen av IRS1 på serin 307 (i den humana sekvensen) ökar snabbt hos icke-diabetiska fettceller som svar på insulin. Denna serinfosforylering verkar behövas för att IRS1 effektivt ska tyrosinfosforyleras och därmed leda insulinsignalen vidare inne i cellen. Fosforyleringen av IRS1 på serin 307 är kraftigt nedsatt hos subkutana fettceller från patienter med typ 2 diabetes. Fosforyleringen av IRS1 på serin 312 är däremot liknande i fettceller från icke-diabetiker och diabetiker (Öst *et.al.* (2007) *Faseb.J.* doi: 10.1096/fj.07-8173com). Fosforyleringen av IRS1 på serin 312 är mest involverad i insulinsignaleringens negativa återkoppling. Fosforyleringen av serin 307 sker snabbt och vid låga insulinkoncentrationer, medan fosforyleringen på serin 312 sker först efter lång inkubering och vid höga insulinkoncentrationer.

Detta är en ny mekanism på cellulär nivå som möjligen kan beskriva insulinresistansen i fettceller från människa. Tillsammans styrs återkopplingen via den stimulerande fosforyleringen (serin 307) eller den hämmande fosforyleringen (serin 312) och kontrollerar insulinsignaleringen i cellen. Fosforyleringarna sker möjligen via samma proteinkinas och/eller proteinfosfatas och kan bli mål för terapeutiska läkemedel mot typ 2 diabetes i framtiden.

Abstract

The prevalence of obesity is increasing in most parts of the world and is a strong risk factor for the development of insulin resistance and type 2 diabetes. Adipose tissue is important in whole body energy balance and grows in size with excess energy intake. Adipose tissue in different regions of the body has different characteristics and adipocytes coming from intraabdominal fat depots, are more associated with insulin resistance than adipocytes from subcutaneous fat depots. Insulin signalling is complex and consists of two major signalling pathways in the cell; the metabolic signalling pathway and the mitogenic signalling pathway. After insulin binding to the insulin receptor a cascade of protein phosphorylations and dephosphorylations is started, eventually leading to the target effects of the hormone. Tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), a protein directly downstream of the insulin receptor, is essential for further insulin signalling. Serine phosphorylation of IRS1 also affects insulin signalling through inhibitory or stimulatory effects. Adipocytes are together with muscle cells and liver cells central in the development of type 2 diabetes. The focus of this thesis is to describe mechanisms in insulin signalling in primary human adipocytes in insulin resistant states, surgical stress or type 2 diabetes.

Visceral adipocytes from humans were analysed and compared to subcutaneous adipocytes. Visceral adipocytes were slightly bigger than subcutaneous adipocytes. Furthermore, visceral adipocytes had an increased level of the glucose transporter protein GLUT4 and a higher basal and insulin-stimulated glucose uptake, but the sensitivity to insulin was the same.

Here it was found that surgical insulin resistance is reversible after overnight incubation of the adipocytes and the impaired insulin sensitivity is at the level between IRS1 and PKB/Akt in insulin signalling. In contrast, the insulin resistance in type 2 diabetes is irreversible and the impaired insulin sensitivity is at the level of insulin receptor-mediated tyrosine phosphorylation of IRS1. Adipocytes from patients with type 2 diabetes were investigated and it was found that diabetic adipocytes have an attenuated insulin-stimulated phosphorylation of IRS1 at serine 307 (corresponding to serine 302 in the mouse sequence). In adipocytes from non-diabetic individuals, the phosphorylation of IRS1 at serine 307 occurred rapidly at low concentrations of insulin. This phosphorylation was associated with the tyrosine phosphorylation of IRS1. The phosphorylation of IRS1 at serine 312 (corresponding to serine 307 in the mouse sequence) in response to insulin was similar in adipocytes from non-diabetic individuals and from patients with type 2 diabetes (Öst *et.al.* (2007) *Faseb.J.* doi: 10.1096/fj.07-8173com) and occurred only at high concentrations after prolonged incubation with insulin.

This thesis reports the investigation of mechanisms in insulin signalling at a cellular and molecular level in primary human adipocytes. The insulin resistance resulted from surgical stress is different from that in type 2 diabetes and adipocytes from patients with type 2 diabetes have impaired insulin sensitivity at the level of IRS1. Together, the phosphorylation of IRS1 at serine 307 and serine 312 may control insulin signalling through feedback mechanisms in primary human adipocytes.

Original publications

This thesis is based on the following original scientific papers, which will be referred to in the text by their Roman numerals:

- I. Westergren H., **Danielsson A.**, Nystrom F. H. and Strålfors P. (2005)
Glucose transport is equally sensitive to insulin stimulation, but basal and insulin-stimulated transport is higher, in human omental compared with subcutaneous adipocytes. *Metabolism* 54, 781-785.
- II. **Danielsson A.**, Öst A., Lystedt E., Kjolhede P., Gustavsson J., Nystrom F. H. and Strålfors P. (2005) Insulin resistance in human adipocytes occurs downstream of IRS1 after surgical cell isolation but at the level of phosphorylation of IRS1 in type 2 diabetes. *FEBS J.* 272, 141-151.
- III. **Danielsson A.**, Öst A., Nystrom F. H. and Strålfors P. (2005) Attenuation of insulin-stimulated insulin receptor substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes. *J. Biol. Chem.* 280, 34389-34392.
- IV. **Danielsson A.**, Nystrom F. H. and Strålfors P. (2006) Phosphorylation of IRS1 at serine 307 and serine 312 in response to insulin in human adipocytes. *Biochem. Biophys. Res. Commun.* 342, 1183-1187.

Abbreviations

ERK	extracellular regulated kinase
FAs	fatty acids
GLUT	glucose transporter protein
Grb2	growth factor receptor-binding protein 2
IL-6	interleukine 6
IRS1	insulin receptor substrate 1
IR	insulin receptor
kDa	kilo dalton
MAP kinase	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
nM	nanomolar (millimol/litre)
PKD1/2	phosphoinositide-dependant kinase 1/2
PH	pleckstrin homology
PIP ₂	phosphatidyl-inositol-3,4-bisphosphate
PIP ₃	phosphatidyl-inositol-3,4,5-trisphosphate
PI3K	phosphatidyl-inositol 3 kinase
PKB/Akt	protein kinase B/Akt
PKC	protein kinase C
PPAR- γ	peroxisome proliferator activated receptor- γ
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PTB	phosphotyrosine binding
RBP4	retinol binding protein 4
Shc	src and α 1-collagen homologous protein
SH2	src homology 2
SOS	son of sevenless
TNF- α	tumor necrosis factor alpha

Introduction

The prevalence of obesity and type 2 diabetes is rapidly increasing in all parts of the world and is associated with a lifestyle of excess food consumption and too little physical activity. With excess energy storage, obesity develops, leading to an increased risk of type 2 diabetes. In cells, e.g. fat cells/adipocytes, from patients with type 2 diabetes the insulin signalling is changed and the tissue is insulin resistant. Insulin signalling occurs through a complex network of different signalling pathways. Proteins become phosphorylated and dephosphorylated on different amino acids and thereby more or less active in the insulin signalling cascade. However, the mechanisms of insulin signalling still remain incompletely known. Information on insulin signalling reactions and the underlying cellular mechanisms leading to insulin resistance and type 2 diabetes, especially in human fat cells, is very limited.

This thesis will focus on mechanisms of insulin signalling in some insulin resistant states including type 2 diabetes. The suggested mechanisms and conclusions herein are the results mostly from studies in human subcutaneous adipocytes. The thesis also compares insulin stimulation of glucose transport in subcutaneous and visceral adipocytes. Since the conclusions are based on primary human adipocytes the results are applicable on humans. The findings suggest novel targets for development of new drugs for the treatment of type 2 diabetes in the future.

Obesity and adipose tissue

Obesity is defined by WHO as a body mass index (BMI) > 30 . Obesity develops when energy intake exceeds the energy used. Obesity is associated with several metabolic diseases e.g. insulin resistance, hypertension, cardiovascular diseases, and the development of type 2 diabetes. It is important to treat obesity by food restriction, physical activity or, in cases of extremely fat patients, by surgical treatment [1]. In obesity, adipose tissue becomes chronically inflamed via infiltration of macrophages and together these cells produce inflammatory cytokines, reviewed in [2]. Furthermore, adipose tissue in obesity is exposed to

a variety of stresses, including oxidative stress [3], endoplasmic reticulum (ER) stress [4], inflammatory stress and metabolic stress, which are interconnected and strong inducers of stress signalling and may cause insulin resistance, reviewed in [5].

Adipose tissue is an important regulator of energy balance and overall metabolism of the human body. Adipose tissue is, together with liver and skeletal muscle, central in the development of insulin resistance. Adipose tissue is an organ that consists of fat cells, nerves, connective tissue, blood vessels and immune cells. Adipocytes or fat cells contain a big central oil droplet, mainly consisting of triacylglycerol. This oil droplet occupies about 95 percent of the adipocyte volume. The adipocytes can increase in size by incorporating more triacylglycerol. Thus, these cells differ in size and have a diameter between 20-200 μm . A thin film of $< 0.5 \mu\text{m}$ cytosol is surrounding the lipid droplet. In this cytosol and the plasma membrane much of the insulin signalling occurs. In the plasma membrane of adipocytes there are cave-like structures, called caveolae [6, 7]. In human adipocytes several important proteins involved in insulin signalling are located in caveolae [8, 9].

Insulin and its actions

Insulin is the anabolic hormone secreted from the pancreatic β -cells, with primary actions on adipocytes, muscle and liver cells. The basal concentration of insulin in the blood varies between 0.01 nM and 0.2 nM and the concentration can be raised as much as 10-fold after a meal, making the cells take up more glucose. An important action of insulin is to decrease the glucose concentration in blood by stimulating glucose uptake into muscle and adipose tissue and by suppressing glucose production by the liver. The glucose concentration in the blood is normally 3.5-6.1 mM. After a meal the blood glucose level is raised, which leads to an increased secretion of insulin in the blood. Insulin also stimulates protein synthesis, free fatty acid uptake and synthesis, as well as inhibiting lipolysis in adipocytes [10]. In addition, insulin regulates adipose tissue growth and adipocyte differentiation through control of gene transcription via different adipokines and fat-specific transcription factors, e.g. PPAR γ [11].

Adipocytes and the secretion of adipokines that affect insulin sensitivity

Hormones released from the adipocytes, adipokines, influence the body through effects on for instance appetite, inflammation and insulin sensitivity. Some adipokines such as adiponectin and visfatin have anti-diabetic actions and to some extent improve insulin sensitivity, while others, such as TNF- α , IL-6 and RBP4 are substances that decrease insulin sensitivity, reviewed in [12]. It has also been shown that adipocytes from both mice and humans secrete a newly discovered adipokine, chemerin, which regulates gene-expression in adipocytes, as well as differentiation via the protein kinases ERK1/2 involved in insulin signalling [13]. Recent data indicate that there is a correlation between high levels of RBP4 in plasma and insulin resistance [14]. A study in primary human adipocytes has also shown a link between RBP4 and insulin resistance [15], which strengthens the hypothesis that RBP4 is involved in insulin resistance of type 2 diabetes and obesity [14, 16]. Adipokine secretion may explain how adipocytes are involved in controlling whole body insulin sensitivity and insulin resistance.

Insulin signalling in the adipocyte

The insulin receptor

The insulin receptor (IR) is present in all vertebrate tissues with the highest concentration in the major metabolic organs such as muscle, adipocytes and hepatocytes. In adipocytes the insulin receptor is localised in caveolae invaginations of the plasma membrane (Figure 1) [6, 8, 17]. The IR is composed of two α -subunits (130 kDa) that are localized on the outside of the cell and linked to each other by disulfide bonds. Each are also attached by disulfide bonds to a transmembrane β -subunit dimer (96 kDa), which is a tyrosine protein kinase [18, 19].

Insulin binding to the α -subunits induces trans-autophosphorylation of the β -subunits which become activated. Following this phosphorylation of IR at tyrosine residues intracellular

signalling proteins are recruited and can be phosphorylated by docking on the phosphorylated IR. Among these are members of the IRS protein family and the Shc family (Figure 1). Upon interaction with the IR several tyrosine residues of the IRS proteins are being phosphorylated. This creates docking sites for other intracellular proteins with SH2-domains and the insulin signal is transduced through a cascade of phosphorylations and dephosphorylations that constitute the insulin signalling inside the adipocyte.

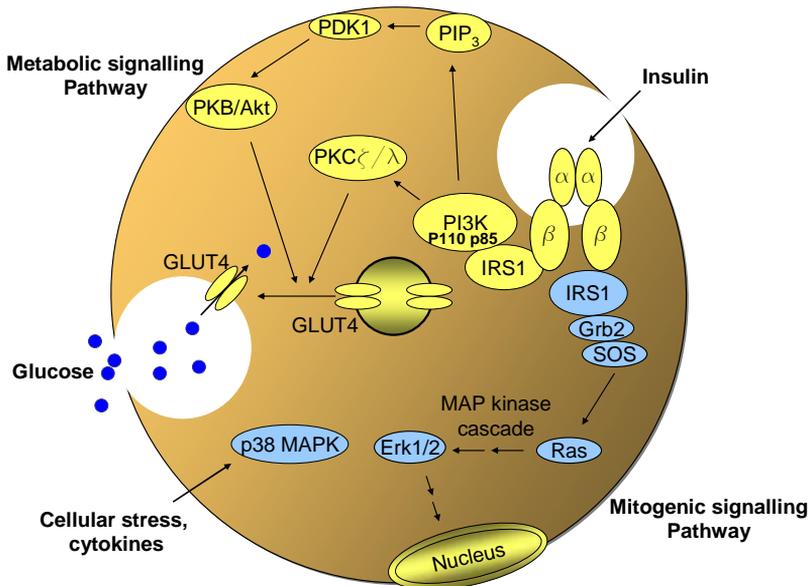


Figure 1. Simplified drawing of the major insulin signalling pathways in an adipocyte: the metabolic signalling pathway and the mitogenic signalling pathway. Insulin binds to the insulin receptor in caveolae, starting cascades of phosphorylations and dephosphorylations inside the cell. Eventually, these phosphorylation reactions affect glucose transport and transcriptional control. Cellular stress and cytokines can affect the cell by an activation of stress protein kinases in the adipocyte.

Protein tyrosine phosphorylation is a balance between the activities of protein kinases and protein tyrosine phosphatases, PTPases. It is important to remember that even in normal insulin signalling PTPases play an important role in insulin action. Upon removal of the hormone, the signal turns off and the effects of insulin are blocked. In addition to PTPases

there are also protein serine/threonine phosphatases that affect insulin signalling by countereacting the serine/threonine-specific protein kinases.

Downstream signalling by insulin occurs through two main kinase cascades: the mitogenic signalling pathway and the metabolic signalling pathway. These signalling pathways are usually depicted as linear, but consist in reality of a complex network of feedbacks and cross-talk with other signalling pathways in the cell [20].

The mitogenic signalling pathway

The mitogenic signalling pathway or the Ras/MAP kinase cascade is in adipocytes mainly activated by insulin through the IRS proteins [21, 22]. The adaptor protein Grb2 is docking to IRS (or Shc) [23], before recruiting Sos, the guanine nucleotide exchange factor for Ras. The activated Ras-GTP recruits and activates the protein kinase Raf, which induces the phosphorylation steps of the MAP kinase cascade, in which the kinases ERK1/2 are being phosphorylated and activated [24]. This signalling pathway is mainly involved in cell growth, survival and differentiation [25] (Figure 1). In human adipocytes IRS proteins mediate signalling by insulin via MAP kinase control of for example the transcription factor ELK-1 [22]. The stress-related MAP kinase p38 MAPK is activated by cellular stress, cytokines [26] and insulin in many cell types [27-29] (Figure 1). It has also been suggested that this protein kinase is involved in control of glucose uptake by insulin [27], but contradicting reports also exist [30]. However, in human adipocytes insulin does not seem to affect the phosphorylation of p38 MAPK (Paper II).

The metabolic signalling pathway

The IR-mediated tyrosine phosphorylation of IRS1 also activates a metabolic signalling pathway by binding to the regulatory subunit (p85) of the PI3K (Figure 1). The catalytic subunit (p110) of PI3K is thereby activated. PI3K phosphorylates phosphatidyl inositol lipids, in the plasma membrane. Thus generated phosphatidyl-inositol-3,4,5-trisphosphate (PIP₃) recruits the serine/threonine protein kinases PDK1 and PKB/Akt to the plasma membrane

[31]. PKB/Akt is phosphorylated at Thr 308 (in the PKB-alpha sequence) by PDK1 and thereby partly activated [32, 33]. PKB/Akt also needs to be phosphorylated at serine 403 (in the PKB-alpha sequence) for maximal activation [34]. Recently the responsible protein kinase for this was identified as mTORC2, a specific form of mTOR in complex with the regulatory subunit rictor [35, 36]. PKB/Akt exists in three closely related isoforms in mammals (alpha, betha and gamma) and controls several biological functions such as cell survival, glycogen metabolism and glucose uptake, reviewed in [37, 38]. Another target of PI3K in adipocytes is the atypical PKC (ζ/λ). Both PKB/Akt [39] and atypical PKC [40] appear to be important for insulin stimulation of GLUT4 translocation to the plasma membrane and enhancement of glucose transport (Figure 1).

Several data demonstrate the need for PI3K-dependent pathways for insulin signalling to glucose uptake, but some data indicate that the activation of this enzyme is not sufficient to enhance glucose transport, as reviewed in [41]. A non-PI3K-dependant pathway was reported to involve tyrosine phosphorylation of the protooncogene Cbl. In response to insulin Cbl associates with the adaptor protein CAP and the complex translocates to the plasma membrane [42]. Together with Crk there is an activation of the G-protein TC10, which seems to be important for the translocation of GLUT4 to the plasma membrane [43], although this has been strongly challenged, reviewed in [44, 45].

The term critical node defines a point in a signalling network that is essential for the biological functions, but also acts in crosstalk between signalling pathways. There are three clearly identified such critical nodes in the insulin signalling pathway. The first is the IR and the associated IRS proteins, reviewed in [44].

Insulin receptor substrate

There are nine members of the IRS protein family and the isoforms IRS1-4 are required for a complete insulin signal transduction in different tissues and species [46]. In the human genome there is no gene coding for IRS3, however [47]. Studies in mice with tissue-specific knockout of IRS1 or IRS2 have found that IRS1 plays a prominent role in insulin resistance in muscle and adipose tissue, while IRS2 is most important in liver [48, 49] and the pancreatic β -

cells [48-50]. Mice lacking IRS4 only exhibit mild effects on glucose homeostasis [51]. The IRS1 protein is found in the cytosol and bound to intracellular membranes, but in human adipocytes it has also been found to co-localize with the IR at the caveolae of the plasma membrane [8, 9], both in the basal state and after insulin stimulation [9].

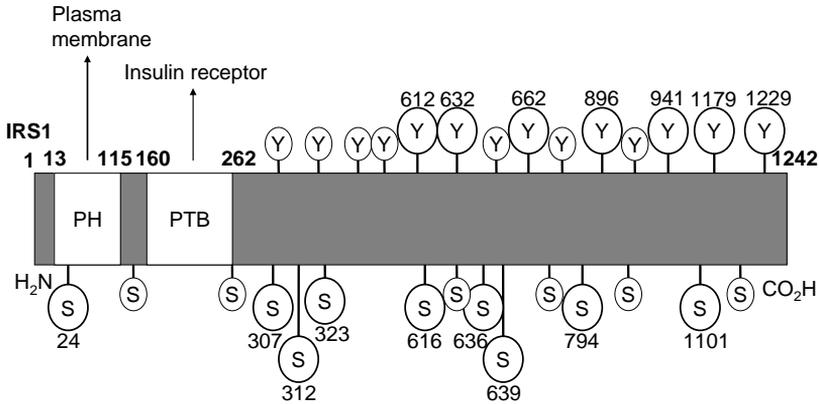


Figure 2. Outline of the human sequence of the IRS1 protein with potential tyrosine, (Y) and serine, (S) phosphorylation sites.

At the amino terminal of IRS1 is a pleckstrin homology (PH) domain that helps to anchor the protein to the membrane close to the IR (Figure 2). Next to the PH domain is a phosphotyrosine binding domain (PTB) that functions as a binding site for the phosphorylated tyrosine in the juxta membrane NPXpY motif of the IR. The sequence of IRS1 is very similar in different species and the motifs potentially implicated in the IRS1 function are conserved in mouse, rat and human IRS1 [52]. The C-termini of the IRS proteins contain multiple tyrosine phosphorylation sites. When phosphorylated these sites act as docking platforms for other signalling proteins that contain SH2 domains such as: p85 α regulatory subunit of the PI3K, Grb2, and SHP-2, which propagate and regulate insulin signalling further inside the cell (Figure 1), [41, 53].

Glucose transport

Glucose is a fundamental source of energy for our cells. Since the cell membrane is impermeable to carbohydrates, glucose transport into the cell is facilitated by glucose carrier proteins. At the cellular level, insulin stimulates glucose transport into the adipocytes by inducing translocation of the glucose transporter 4 (GLUT4) from an intracellular vesicle storage compartment to the plasma membrane (Figure 1). In adipocytes GLUT4 is most abundant, but the GLUT1 isoform may provide for basal uptake of glucose [54]. In the basal state GLUT4 cycles between the plasma membrane and one or more intracellular compartments by different, yet not completely known, mechanisms [55]. Upon insulin stimulation of the cells the intracellular GLUT4 vesicles are translocated to and fused with the plasma membrane [56]. In adipocytes GLUT4 appears to fuse with or move into caveolae where the glucose uptake takes place [7, 57]. When insulin stimulation is terminated GLUT4 is internalised, evidently by endocytosis from caveolae [7] (Figure 1). GLUT4 exocytosis is increased up to 10-20-fold by insulin, while the endocytosis of GLUT4 is slightly reduced [55]. This results in a net increase of GLUT4 at the plasma membrane thereby increasing the rate of glucose uptake. It has been shown that insulin-stimulated glucose uptake as well as GLUT4 expression are reduced in human adipocytes in insulin resistance e.g. type 2 diabetes and/or obesity [58-60]. After a meal muscle accounts for most of the glucose uptake in the body and adipose tissue consumes only about 10 to 15 % [61, 62]. In spite of this, it has been shown in animals that an adipose-selective reduction of GLUT4 leads to an impaired insulin-stimulated glucose transport and a whole-body insulin resistance [63]. Even more compelling is that overexpression of GLUT4, in muscle-specific GLUT4-depleted animals, restored insulin sensitivity [64]. Recently it was reported that mice with specific knock-out of GLUT4 in adipose tissue, caused increased expression of the retinol-binding protein RBP4 in the adipocytes and also increased the secretion of RBP4 by the adipocytes [14]. This made the animals insulin resistant secondarily in the muscle cells [14, 63] as well as in the liver [14]. These animal models indicate the importance of the adipose tissue in causing whole-body insulin resistance.

Insulin resistance

Insulin resistance is a condition in which normal concentrations of insulin are not enough to keep the blood glucose level normal. Defects in the ability of peripheral tissues e.g. muscle and fat to take up glucose are linked to insulin resistance. The pancreatic β -cells secrete more insulin to compensate for the impaired insulin sensitivity, but after some time these insulin producing cells progressively lose their ability to release insulin in response to elevated glucose levels and thereby cause type 2 diabetes, reviewed in [65]. The insulin resistance in adipocytes is a result of impaired signal transduction from the insulin receptor to target metabolic effects, such as stimulation of glucose transport and inhibition of lipolysis [18, 66].

Insulin resistance and different fat depots

In recent years it has become more obvious that not only the amount of adipose tissue is important for the development of insulin resistance, but also the distribution of fat in the body, reviewed in [67]. It has been postulated that excess accumulation of intraabdominal adipose tissue, visceral fat, is associated with an increased risk for insulin resistance and metabolic diseases compared to excess subcutaneous fat depots. The reason for this may be anatomical factors (venous drainage of visceral fat to the liver versus systemic drainage for subcutaneous fat), metabolic and endocrine differences [68]. It has been reported that visceral and subcutaneous fat secrete different amounts of adipokines [69, 70] and recently it was reported that visceral adipocytes secrete more RBP4 than subcutaneous adipocytes [70]. Visceral fat also seems to be more infiltrated by macrophages than subcutaneous fat [71]. A central engine in adipose differentiation is the transcriptional factor PPAR γ . Activation of PPAR γ affects insulin sensitivity and leads to expression of several adipocytes specific genes. It has recently been found that visceral adipocytes from humans exhibit lower activity of PPAR γ than subcutaneous adipocytes do [72]. This is in line with the high lipolytic activity in visceral fat [73], since it is known that low PPAR γ activity increases the lipolysis in adipocytes.

It has also been argued that the risk of insulin resistance is increased not only by the amount and location of the adipose tissue, as described earlier, but also by the size of the fat cells [74,

75]. In line with this visceral adipocytes were found to be slightly bigger than adipocytes from subcutaneous fat in humans (Paper I, [76]).

Several studies have compared insulin signalling and action in subcutaneous versus visceral fat, reviewed in [77]. In vitro studies with human adipocytes showed reduced anti-lipolytic effects of insulin as well as reduced IR autophosphorylation and signal transduction through an IRS1 associated PI3K pathway in visceral adipocytes compared to subcutaneous adipocytes [73]. In contrast, in vivo studies in humans show that visceral fat cells have both higher protein expression as well as greater and earlier activation/phosphorylation in insulin signalling via the IR, PKB/Akt and ERK1/2 signal mediators. The level of IRS1 protein was not significantly different and the activation of the protein was not examined [78]. A difference between subcutaneous and visceral human adipocytes regarding glucose uptake has also been demonstrated (Paper I). The sensitivity to insulin stimulation of glucose uptake was the same, but the amount of GLUT4 as well as the basal and insulin-stimulated rate of glucose transport was higher in visceral than subcutaneous adipocytes from humans (Paper I). This is in agreement with other studies examining the basal glucose transport [76, 79] and the insulin-stimulated glucose transport as well as the amount of GLUT4 in human adipocytes [76]. The elevated level of GLUT4 in visceral fat (Paper I, [76]) might be accounted for by the lower activity of PPAR γ in the visceral compared with subcutaneous fat [72], since it has been reported that PPAR γ represses the transcription of GLUT4 [80]. Furthermore, since it is known that visceral adipocytes have higher lipolytic activity [73] and more β -adrenergic receptors [81] the secretion of fatty acids (FAs) is increased. This may result in higher turnover rate of triacylglycerol in visceral adipocytes compared with subcutaneous adipocytes, which may explain the higher rate of glucose uptake in this fat depot (Paper I). It should be noted that to the increased basal glucose uptake may also a higher amount of GLUT1 contribute, but this was not investigated (Paper I).

Insulin resistance and surgical trauma

As a major contributor to the pathogenesis of type 2 diabetes, insulin resistance is also associated with other conditions such as inflammation, reviewed in [2, 82], polycystic ovarian syndrome, PCOS, reviewed in [83] and stress or trauma, such as surgical trauma [84-86].

Little is known about the insulin resistance at the molecular and cellular levels in these conditions, but some studies have been done on adipocytes from humans regarding the insulin resistance of PCOS [87-89] and of type 2 diabetes (Paper II, Paper III, [15, 90, 91]). The interest in trauma and stress-induced insulin resistance has increased during the past years, but the exact mechanisms behind stress-induced insulin resistance still remain unclarified. Studies in humans indicate that plasma concentrations of glucose are raised after elective abdominal surgery and this is due to stress-induced insulin resistance [84-86]. Also in animals it has been found that quite simple abdominal surgery results in post-operative insulin resistance that affects glucose metabolism [86]. At the cellular and molecular level, especially in human adipocytes, a few studies have been done regarding surgical insulin resistance, but the results are conflicting. It is of interest and importance to analyze surgical insulin resistance in humans to find ways to improve post-operative recovery. It has been reported that avoiding preoperative fasting is related to a reduction in postoperative stress and insulin resistance, reviewed in [92].

The mitogenic signalling pathway of insulin has recently been investigated in primary human subcutaneous adipocytes directly after elective abdominal surgery (Paper II). The MAP kinases ERK1/2 and the stress-related MAP kinase p38 were fully phosphorylated and activated directly after the surgery and cell isolation procedures. Insulin had no further effect on the phosphorylation of ERK1/2, suggesting that they are in a fully phosphorylated state due to cellular stress from the surgery and cell isolation. The MAP kinases ERK1/2 and especially the p38 MAPK are well known to be phosphorylated and activated in response to different types of cellular stress [93, 94]. Carlson *et.al.* have also reported a high basal phosphorylation of both p38 MAPK and ERK1/2 in human adipocytes directly after obtaining the cells [90], which they connected to insulin resistance of type 2 diabetes. Recovery of human adipocytes by incubation for 24 h lowered the basal phosphorylation of both MAP kinases ERK1/2 and p38, and insulin then increased the phosphorylation of ERK1/2 (Paper II). The insulin sensitivity of the downstream target effect – stimulation of glucose uptake – was improved ten fold after overnight recovery of the human adipocytes (Paper II). These results suggest that the MAP kinases were fully activated directly after surgery due to the surgically induced insulin resistance in the adipocytes (Paper II).

The metabolic signalling pathway of insulin was also exhibiting surgically induced insulin resistance in human adipocytes (Paper II). Using cells recovered for 24 h as control cells, the

IR autophosphorylation and IR phosphorylation of IRS1 at tyrosine were not affected by the surgery, as assessed directly after isolation of the cells, which is in agreement with earlier findings of a postreceptor insulin resistance [95]. However, the insulin sensitivity for phosphorylation of PKB/Akt was significantly reduced directly after the surgery and cell isolation compared with control cells (Paper II). Only the sensitivity to insulin was affected by surgical stress. Neither the basal level of phosphorylation nor the maximal effects of insulin were affected. Also the basal glucose uptake, the maximal effect of insulin on glucose uptake, and the amount of GLUT4 were not affected by the surgical stress-induced insulin resistance (Paper II), which is in agreement with an earlier study that examined rat skeletal muscle after abdominal surgery [96]. This shows the importance of examining the phosphorylation/activation of the proteins relaying the insulin signal at different concentrations of insulin to detect differences in insulin sensitivity. Another stress-related insulin resistance, burn injury, has also been found to be associated with attenuation of insulin-stimulated PKB/Akt activation in skeletal muscle [97]. These findings suggest that PKB/Akt is involved in stress-associated insulin resistance, but the results regarding the sensitivity to insulin are different. It has, however, also been reported that signalling via IRS1, PI3K and PKB/Akt in rat skeletal muscle responded even better to insulin after surgery [96], which may be due to species differences. However, the same laboratory found no effect on insulin-stimulated glucose uptake in the adipocytes of the animals [98]. Nordenström *et al.* reported a reduction of basal and insulin-stimulated glucose uptake by 35 percent in human adipocytes after elective surgery, but the sensitivity to insulin was the same [95].

One reason for the earlier conflicting reports in understanding insulin resistance of trauma may be species differences as well as the difficulty to obtain proper control tissue or cells after surgery [96, 99, 100]. When working with primary cells it is important to maintain physiologically relevant conditions during cell recovery after surgery and cell isolation. Using different surgical procedures may make the adipocytes respond differently to the surgical stress. It has, however, been found that the effects on the MAP kinases due to surgery described above was the same in human adipocytes independently of different surgical procedures such as, local or general anesthesia (Paper II).

The impaired insulin sensitivity of adipocytes that apparently results from the surgical procedures was reversible and is manifest at a post insulin receptor level, at the level between IRS1 and PKB/Akt (Figure 3).

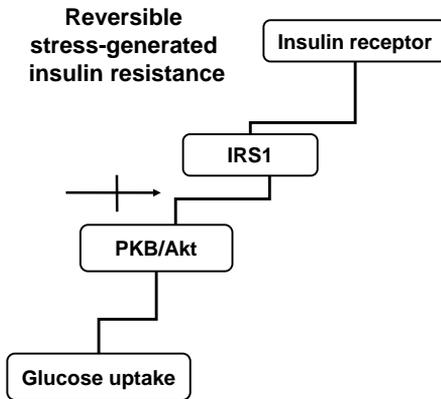


Figure 3. The impaired insulin sensitivity in surgical insulin resistance in subcutaneous human adipocytes is at the level between IRS1 and PKB/Akt.

Insulin resistance and type 2 diabetes

Insulin resistance is a primary characteristic of non-insulin dependent diabetes mellitus (NIDDM) or type 2 diabetes. The incidence of type 2 diabetes is rapidly increasing and at the turn of this century almost 171 million people were estimated to have diabetes and by 2030 an expected 366 million individuals will be affected [101]. About 80% of all type 2 diabetes patients are obese [102], which reflects obesity as a major risk factor of type 2 diabetes as already mentioned. The insulin resistance in type 2 diabetes has been investigated for a long time and both genetic and environmental factors contribute to the etiology. Multiple genes are involved and the genetic component may be very complex, reviewed in [103]. Yet the mechanisms causing this metabolic disease in target cells are not known. Animal models of insulin resistance have shown that the IRS1 has a role in insulin resistance in adipose tissue through both short-term mechanisms, inducing for example serine phosphorylations of the

protein, as well as long term mechanisms, such as degradation of the protein. Both mechanisms inhibit the insulin signal transduction further downstream in the cell, reviewed in [46, 104].

Mutations of IRS1 have been discovered in some patients with type 2 diabetes [105-110] and a lowered expression of the IRS1 protein has also been described in adipocytes from patients with type 2 diabetes [111]. A decreased expression of the IRS1 protein was found even in adipocytes from some obese individuals and relatives of patients with type 2 diabetes [91], as well as a decreased extent of tyrosine phosphorylation of IRS1 in response to insulin, reviewed in [112]. In another study, however, the amount of IRS1 protein was not significantly different in adipocytes from control subjects and from patients with type 2 diabetes (Paper III). It has been suggested that reduced IRS1 levels in human adipocytes is secondary to high serum levels of glucose and insulin [113] and is therefore a consequence rather than a cause of type 2 diabetes. It should be noted that individual human variations are large, which may cause conflicting results when comparing small groups of individuals.

The insulin signalling in subcutaneous adipocytes from patients with type 2 diabetes was recently investigated without interference from surgical insulin resistance (Paper II). The maximal effects of insulin on the state of phosphorylation of the different signal mediators examined were the same in adipocytes from control subjects and from patients with type 2 diabetes (Paper II). There was no difference in the insulin sensitivity for tyrosine autophosphorylation of the IR. Adipocytes from patients with type 2 diabetes exhibited, however, a decreased sensitivity to insulin for the tyrosine phosphorylation of IRS1, compared with adipocytes from control subjects (Paper II). This is in agreement with earlier findings of insulin resistance in human skeletal muscle, where IRS1 was identified to have a decreased tyrosine phosphorylation and thereby decreased downstream signal transduction capacity [114-116]. The insulin resistance in human adipocytes was maintained downstream of IRS1 to glucose uptake (Paper II), as well as to the phosphorylation of ERK1/2 of the mitogenic signalling pathway of insulin [15]. A defect ERK1/2 phosphorylation has also been found in adipocytes from patients with type 2 diabetes, but the sensitivity to insulin was not investigated [90]. In contrast, it has earlier been reported that the MAP kinase pathway, is not altered in skeletal muscle from patients with type 2 diabetes [114, 115].

Conflicting results also exist regarding the PKB/Akt activity in insulin resistance. In human adipocytes a lowered phosphorylation of PKB/Akt at serine 473 and to some extent at threonine 308 has been demonstrated, together with an impaired translocation of PKB/Akt to the plasma membrane in adipocytes from patients with type 2 diabetes [117]. Results from muscle of obese patients with type 2 diabetes indicated that the maximal effect of insulin on PKB/Akt activity was reduced, but not the sensitivity to insulin [118], while an effect on the sensitivity has been reported in human adipocytes (Paper II). Another study in human skeletal muscle from patients with type 2 diabetes showed that PKB/Akt responded normally to insulin [119]. Some of these conflicting findings are based on studies in different cell types. It should also be noted that the PKB/Akt has two phosphorylation sites, threonine 308 and serine 403 (PKB-alpha/Akt1 sequence), that need to be phosphorylated for full activation of the protein kinase [34]. Since the protein kinase can be partially activated by phosphorylation at one of the sites this may be a further reason for conflicting reports.

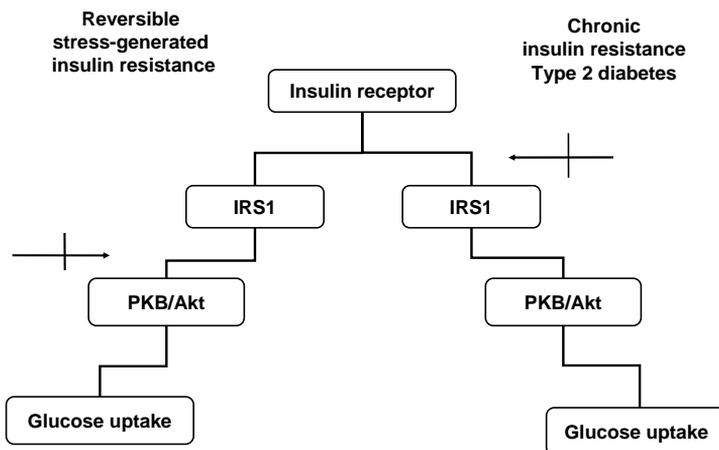


Figure 4. The impaired insulin sensitivity in insulin signalling in subcutaneous human adipocytes in the surgically induced insulin resistance and in patients with type 2 diabetes.

In subcutaneous adipocytes from patients with type 2 diabetes, there is an impaired sensitivity in insulin signalling at the level of IR mediated phosphorylation of IRS1 at tyrosine. Furthermore, the insulin resistance of type 2 diabetes is not reversible, which is in contrast to the insulin resistance due to the surgical stress that is reversed by overnight incubation (Paper II, Figure 4). This indicates that different mechanisms are operating in these two insulin resistant states.

Phosphorylation of IRS1 in diabetes

Tyrosine phosphorylation of IRS1

As shown in Figure 2 the IRS1 protein contains several phosphorylation sites. Since this thesis is about human adipocytes the phosphorylation sites in the text are specified according to the human sequence if not otherwise indicated. IRS1 contains over 20 potential tyrosine phosphorylation sites [120, 121]. At least six are important binding sites for PI3K, while three others are important for downstream signalling through other SH2-containing proteins, reviewed in [122]. It has been shown in 32D cells that phosphorylation of tyrosine residues 612, 632 and 662 of IRS1 mediate the maximal response to insulin via the mitogenic signalling pathway and that the tyrosine residues 896 (Grb2 binding site), 1179 and 1229 (both phosphotyrosine phosphatase SHP-2 binding site) are not important in the mitogenic response to insulin in these cells [123]. The same authors also showed that phosphorylation of tyrosine residues 612, 632 and 662 are important in the association with PI3K and that there may also be biological signals mediated by non-tyrosine phosphorylated IRS1 [123]. Also in rat adipocytes the tyrosine residues 612 and 632 of IRS1 have been found important for PI3K activation and GLUT4 translocation in response to insulin [124].

Serine phosphorylation of IRS1

Besides the tyrosine phosphorylation of IRS1, the protein contains over 200 Ser/Thr residues, of which 70 may be phosphorylated in response to insulin or other stimuli. The phosphorylation of IRS1 at different serine residues affect and control insulin signalling

through either positive or negative effects on the ability of IRS1 to be phosphorylated on tyrosine residues and to transmit the signal downstream. Serine phosphorylation of IRS1 may also provide for crosstalk with other signalling pathways in the cell, reviewed in [125, 126]. Results regarding the functions of IRS1 phosphorylation at serine residues are mostly based on findings from different cell lines and animals. In human cells very few studies have been done and our knowledge about the involvement of serine phosphorylation of IRS1, especially in insulin resistance, in humans is limited. The following discussion will therefore include also studies in animals and animal cells.

Phosphorylation of IRS1 at serine 312

In general serine phosphorylation of IRS1 is coupled to inhibitory effects on insulin signalling. The phosphorylation of IRS1 at serine 312 (corresponding to serine 307 in the mouse sequence) has especially been associated with negative control of insulin signalling. One explanation for the inhibitory effect on signalling may be that this phosphorylation site is located close to the PTB-domain of IRS1 (Figure 2). Both the PH-domain and the PTB-domain of IRS1 are essential for targeting IRS1 to the IR and thus for correct IR-mediated tyrosine phosphorylation of IRS1 and further insulin signalling. Phosphorylation at serine 312 can sterically block and disrupt IR-IRS1 interactions and reduce IR-mediated tyrosine phosphorylation of IRS1 [127]. In addition to the steric blocking by this phosphorylation it has been reported that phosphorylation of serine 312 enhances the proteolytic degradation by insulin in rat H411E hepatoma cells [128] and thereby reduces IR-IRS1 signalling. It has been found in animal models of obesity-related insulin resistance that absence of the protein kinase p70 S6K, involved in integrating nutrients and insulin signals, improves insulin sensitivity through loss of IRS1 phosphorylation at serine 312 [129]. Recently, it was found that both the extent of basal phosphorylation of IRS1 at serine 312 and the insulin-stimulated phosphorylation is similar in human subcutaneous adipocytes from non-diabetic individuals and from patients with type 2 diabetes [15]. Interestingly, the phosphorylation of this site in human subcutaneous adipocytes required prolonged insulin stimulation at high concentrations (Paper IV), a situation found in insulin resistant patients with type 2 diabetes, when the β -cells compensate to secrete more insulin in early stages of type 2 diabetes. This suggests that serine 312 phosphorylation may further impair an already insulin resistant state. Taken together, the

phosphorylation of IRS1 at serine 312 seems to be associated with impaired insulin signalling and insulin resistance, but maybe of less importance in human adipocytes from patients with type 2 diabetes [15].

IRS1 serine 312 has also been reported to be phosphorylated in response to other stimuli than insulin in different cell types e.g. in response to TNF- α [130, 131], anisomycin [131], IL-6 [132], or oxidative stress [133], which all have inhibitory effects on insulin signalling. Also fatty acids (FAs) activate protein kinases leading to IRS1 serine 312 phosphorylation and thereby degradation of IRS1 in 3T3-L1 adipocytes [134] and decreased insulin-stimulated glucose transport in rodent muscle [135].

Inhibitory effects on insulin signalling by phosphorylation of IRS1 at serines other than serine 312

In addition to the phosphorylation of IRS1 at serine 312 there are several other serine residues that affect the function of IRS1 and inhibits insulin signalling. IRS1 is suggested to be a node in the insulin signalling pathway [44], which is essential for connecting other pathways to insulin signalling. Signalling pathways activated by other stimuli than insulin can thus interfere with insulin signalling through serine phosphorylation of IRS1 (Figur 5). I will just discuss a few of all known serine phosphorylation sites of IRS1 that may be involved in the regulation of its function.

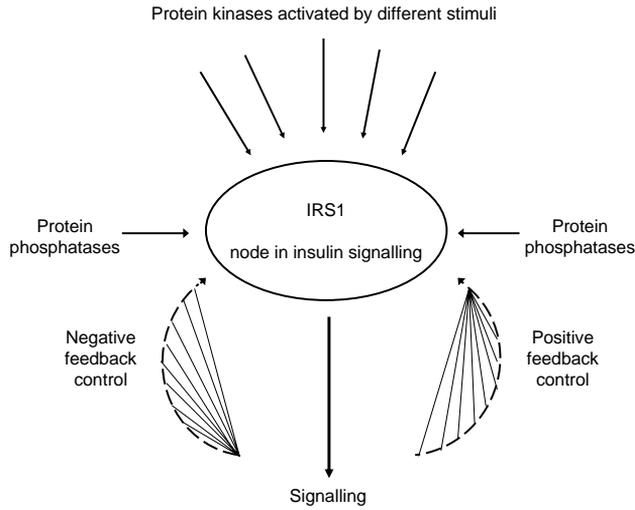


Figure 5. Illustration of the IRS1 protein, as a node of the insulin signalling network. Several different stimuli activate several different protein kinases and protein phosphatases that affect both tyrosine - and serine phosphorylation of IRS1. Upper arrows indicate phosphorylation by protein kinases at both tyrosine and serine residues. Activation of different protein phosphatases dephosphorylate the protein at different phosphorylation sites. Negative or positive feedback control occurs mainly through serine phosphorylation of IRS1 in response to different stimuli, affecting the function of the IRS1 protein.

Seven serine residues have been reported to be phosphorylated in response to insulin or other stimuli [136, 137], which in turn disrupt the connection to the IR, causing reduced downstream signalling and insulin resistance. Phosphorylation of serine 24 in the PH-domain of IRS1 may disrupt the interaction of IRS1 with the membrane and thus inhibit tyrosine phosphorylation by the IR [138, 139]. Serines 636/639 have, together with serine 312, been found to be phosphorylated in response to insulin in different cell lines and animal models [129, 140, 141]. Serines 636/639 are phosphorylated in response to insulin also in in vitro-differentiated human adipocytes, impairing insulin signalling [142]. TNF- α activates the mTOR/PKB/Akt pathway, leading to phosphorylation of IRS1 at serine 636/639 in cell cultures [143] and thereby decreasing the tyrosine phosphorylation of IRS1. It was also suggested that nutrients, such as hyperglycemia, activate (raptor)-mTOR signalling in vitro, leading to phosphorylation of IRS1 at serine 636/639 and downregulation of the PI3K/PKB/Akt pathway in mouse L6 myotubes [144]. Serines 636/639 are located near the

tyrosine residue 632 of IRS1 and may therefore block downstream insulin signalling. IRS1 serine 616 has been shown to be phosphorylated in response to insulin in cell lines [128], muscle and adipose tissue of mice [145], and in rat adipocytes [146]. This residue has been postulated as a negative feedback regulator via ERK1/2 in insulin signalling [147]. FAs induces PKC-mediated phosphorylation of IRS1 at serine 1101, thus blocking IRS1 phosphorylation at tyrosine and downstream activation of the PKB/Akt pathway [148]. Furthermore, the adipokine, leptin, has been reported to increase the phosphorylation of IRS1 at serine 323 and therefore down-regulate the insulin signal in the presence of obesity, both in skeletal muscle of mice and in lymphocytes of human beings [149]. The phosphorylation of IRS1 at serine 323 has also been reported to induce insulin resistance in response to prolonged, chronically elevated levels of IL-6 in skeletal muscle cells of mice [132].

Phosphorylation of IRS1 at serine 307

Positive effects on insulin signalling by phosphorylation of IRS1 at serine residues have also been described in a few cases. Insulin-induced phosphorylation of IRS1 at serine 307 (corresponding to serine 302 in the mouse sequence) has been found to stimulate the tyrosine phosphorylation of IRS1 in 32D myeloid cells [150]. Similarly, the phosphorylation of IRS1 at serine 307 in response to insulin was suggested to be positively related to the tyrosine phosphorylation of IRS1 in primary human adipocytes (Paper III, IV, [15]). It has been shown that phosphorylation at serine 307 may block and inhibit protein phosphotyrosine phosphatases and thereby increase the tyrosine phosphorylations of IRS1 and insulin signalling [151]. Contradictory reports exist though, suggesting a negative effect of phosphorylation at serine 307 on insulin signalling in other cell lines [152, 153]. Likewise, it has been shown that inhibition of (raptor)-mTOR/p70 S6K inhibits insulin-mediated phosphorylation of IRS1 at serine 307 and thereby increases insulin sensitivity through enhanced IRS1 tyrosine phosphorylation and PKB/Akt phosphorylation in mouse C2C12 myotubes [154].

In human adipocytes from non-diabetic individuals insulin increased the phosphorylation at serine 307 two to three fold, while insulin did not affect the state of phosphorylation at this residue in adipocytes from patients with type 2 diabetes (Paper III). The cause-effect

relationship of phosphorylation of IRS1 at serine 307 and the effect on insulin signalling have been examined in primary subcutaneous human adipocytes (Paper III). The insulin-stimulated phosphorylation of IRS1 at serine 307 was blocked by rapamycin, a selective inhibitor of (raptor)-mTOR, in human adipocytes from non-diabetic individuals (Paper III). The attenuated phosphorylation of serine 307 decreased the tyrosine phosphorylation of IRS1 (Paper III), thus mimicking the situation in adipocytes from patients with type 2 diabetes, as recently reported (Paper II). Mutation of IRS1 serine 307 to alanine in 32D cells inhibited the mitogenic, but not the metabolic, signalling pathway in these cells [150]. Similarly, RBP4 blocked the insulin-induced phosphorylation of IRS1 at serine 307 and reduced the tyrosine phosphorylation of IRS1 with downstream effects mainly on the mitogenic signalling in human subcutaneous adipocytes [15]. When instead the phosphorylation of serine 307 was induced by ocadaic acid, a serine/threonine phosphatase inhibitor of PP2A and PP1, in adipocytes from patients with type 2 diabetes the phosphorylation of IRS1 at tyrosine was rendered more insulin sensitive, mimicking adipocytes from non-diabetic individuals (Paper II). Although the effect of ocadaic acid is not specific, it is compatible with other findings indicating that the sensitivity to insulin for IRS1 phosphorylation on tyrosine is positively related to phosphorylation of IRS1 at serine 307 (Paper III, [15, 150, 151]) (Figure 6).

Stimulatory effects on insulin signalling by phosphorylation of IRS1 at serines other than serine 307

Phosphorylations of other serines than serine 307 have been reported to positively affect insulin signalling, but often with discrepancies. It has been reported that the phosphorylation of IRS1 at serines 636/639 in muscle and adipocyte cell lines enhances insulin action on glucose transport [155]. Furthermore, a rapid phosphorylation of IRS1 at serine 323 by physiological concentrations of IL-6 has been shown to stimulate insulin signalling, including glucose uptake in skeletal muscle cells of mice [132]. Similar finding regarding the phosphorylation of IRS1 at serine 323 has also been reported in response to insulin in skeletal muscle cell lines [156]. Another example of positive effects on insulin signalling under certain conditions is the phosphorylation at serine 794. Activation of 5'-AMP-activated protein kinase, AMPK, results in the phosphorylation of IRS1 at serine 794, thus enhancing effects via PI3K on the insulin metabolic signalling pathway in mouse C2C12 myotubes

[157], although this site has also been reported to attenuate insulin signalling in animal models of insulin resistance [158].

Positive and negative feedback control in insulin signalling through phosphorylation of IRS1 at serine 307 and at serine 312

In primary subcutaneous adipocytes from humans the phosphorylation of IRS1 at serine 307 (corresponding to serine 302 in the mouse sequence) and serine 312 (corresponding to serine 307 in the mouse sequence) was recently compared in response to varying concentrations of insulin and with regard to the time-course after addition of insulin (Paper IV, Figure 6). It is interesting to see how the phosphorylation of these two sites is mirrored in the phosphorylation of IRS1 at tyrosine (Paper IV).

The insulin-induced tyrosine phosphorylation of IRS1 is a rapid event and reaches its maximum within 1 minute. The phosphorylation of serine 307 is being maximally phosphorylated a little later, after about 5 minutes. In contrast to these rapid phosphorylation events, the phosphorylation of IRS1 at serine 312 is much slower and reaches a maximum after about 30 minutes and also with a second peak (75 % of max) at 90 minutes (Paper IV). The delayed maximal effect of insulin on serine phosphorylation at 312 has also been described in rat [146] and 3T3-L1 adipocytes [131, 145]. During this time-course the degree of tyrosine phosphorylation of IRS1 varied before attaining a steady-state level after about 60 minutes (Paper IV). The tyrosine phosphorylation of IRS1 rapidly increased and was elevated for about 15 minutes, corresponding to the elevated phosphorylation of serine 307. At about 30 minutes the tyrosine phosphorylation declined to a minimum, corresponding to a mirror image of serine 312 phosphorylation, which reached its maximal phosphorylation after 30 minutes. At this time point the phosphorylation of serine 307 was declined and returned to basal after about 1 hour. Before attaining a steady-state level the phosphorylation at tyrosine again reaches a peak (about 50% of max) after about 50 minutes, corresponding to a dip in the serine 312 phosphorylation, again mirroring each other (Paper IV).

Stimulation of 3T3-L1 adipocytes with insulin for one hour or longer has been shown to stimulate serine/threonine phosphorylation of IRS1 and at the same time increase the proteasome catalyzed degradation of the IRS1 protein [159]. Interestingly, the amount of IRS1 protein was not decreasing in primary human adipocytes for at least three hours of insulin stimulation (Paper IV).

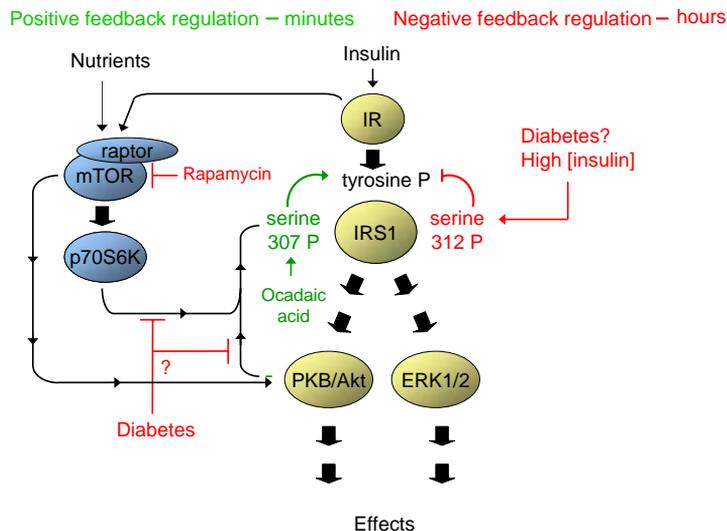


Figure 6. Suggested potential feedback mechanisms in the control of IRS1 in human subcutaneous adipocytes. Possible protein kinases involved in the phosphorylation of IRS1 at serine 307 are mTOR/p70 S6 kinase or PKB/Akt. Rapamycin inhibits (raptor)-mTOR, leading to attenuated phosphorylation of IRS1 at serine 307, while ocaidaic acid inhibits phosphoserine/threonine phosphatases, PP1 and PP2A, leading to an enhanced phosphorylation at serine 307. Phosphorylation of IRS1 at serine 307 may function in a positive feedback control and phosphorylation of IRS1 at serine 312 in a negative feedback control in insulin signalling. The phosphorylation of IRS1 at serine 307 is attenuated in adipocytes from patients with type 2 diabetes.

The phosphorylation of IRS1 at serine 307 occurs at physiological concentrations of insulin (Paper III and IV) indicating signal amplification that occurs when insulin signalling proceeds inside the cell. The rapid phosphorylation of IRS1 at serine 307 together with the high sensitivity to insulin is similar to the positive feedback regulation through phosphorylation of

IRS1 at serine 323 in rodents and cell lines [132, 156]. However, the phosphorylation of IRS1 at serine 323 inhibits insulin signalling after prolonged incubation with IL-6 at high concentrations [132]. Interestingly, a similar mechanism exists in human adipocytes regarding the combined phosphorylation of IRS1 at serine 307 and serine 312. At first, the phosphorylation of IRS1 at serine 307 stimulates insulin signalling to an enhanced tyrosine phosphorylation of IRS1 and secondary, after prolonged incubation with insulin at high concentrations, an inhibition of the insulin signalling occurs through phosphorylation of IRS1 at serine 312 (Paper IV). Taken together, phosphorylation at serine 307 and serine 312 may cooperate to control insulin signalling (Figure 6).

Protein kinases and protein phosphatases involved in the feedback in insulin signalling

Several protein kinases, activated by different stimuli, have been reported to be involved in the phosphorylation of IRS1 serine at 312 in vitro, in cell lines and in animals, e.g. PKC [135], ERK1/2 [146], JNK [130, 160], IKK β [161], p70 S6K [129, 141], and mTOR [128, 131, 145] (Figure 5). Interestingly, it was recently reported that despite of higher expression of stress protein kinases, such as p38 MAPK and JNK, and a greater amount of the IRS1 protein in human visceral fat from obese subjects, there was no increased phosphorylation of IRS1 at serine 312 in response to insulin in adipocytes [162]. It should be noted that these authors used 10 minutes incubation with insulin at high concentration, which need to be extended to 30 minutes for maximal phosphorylation at serine 312, as reported earlier in subcutaneous adipocytes from humans (Paper IV).

Similarity to insulin-stimulated phosphorylation of IRS1 at serine 312, the phosphorylation of serine 307 is rapamycin sensitive, which indicates the involvement of (raptor)-mTOR/p70 S6 kinase in the insulin-stimulated phosphorylation of both these serine sites (Paper III, [131, 141, 150, 153]). However, serine 312 appears to be indirectly phosphorylated by (raptor)-mTOR/p70 S6 kinase in HEK293T celler [141]. In human subcutaneous adipocytes the protein kinases directly responsible for phosphorylation of IRS1 at serine 307 and at serine 312 have not been determined. Since mTOR is activated by insulin and nutrients it is possible

that both serine 307 and serine 312 play a role in integrating insulin signalling with the nutritional status of the cell, as earlier reported [150]. Another possible protein kinase in insulin signalling for the phosphorylation of IRS1 at serine 307 is PKB/Akt, as serine 307 of IRS1 is located in a consensus sequence for phosphorylation by PKB/Akt [163]. Furthermore, it has been reported that PKB/Akt and IRS1 form a stable complex in vivo [151]. That several protein kinases are involved in the insulin-stimulated phosphorylation at serine 307 is not probable, since treatment with rapamycin inhibits the phosphorylation of IRS1 in human adipocytes (Paper III). If more than one protein kinase would be involved, rapamycin would have had to affect both protein kinases.

The different time-courses for phosphorylation of IRS1 at serine 307 and 312 and the very different sensitivities to insulin for their phosphorylation (Paper IV) can be a sign of the involvement of two different protein kinases in the phosphorylation of these two sites. It could also indicate sequential phosphorylation of the two sites, one serine phosphorylation depending on the other. However, loss of insulin-stimulated phosphorylation at serine 307 in diabetes does not affect phosphorylation at serine 312 [15], indicating that the one is not dependent on the other.

Again, it should be mentioned that the physiological mechanisms involving protein phosphorylations in insulin signalling are the result of a balance between protein kinase and protein phosphatase activities in the cell. It is known that PKB/Akt is a target for PP2A in adipocytes [164]. The improved insulin signalling via IRS1 in response to increased serine 307 phosphorylation in adipocytes from patients with type 2 diabetes after treatment with ocadaic acid (Paper III), could indicate the involvement of protein kinase PKB/Akt via the ocadaic acid-sensitive protein phosphatase (PP2A). Furthermore, it has been reported that phosphorylation at serine 312 is indirectly increased by ocadaic acid via mTOR [131], which suggests an ocadaic acid effect via activation of mTOR also regarding the phosphorylation at serine 307 (Paper III). Interestingly, it has also been reported that (raptor)-mTOR interacts with and regulates the activity of the PP2A in TAg Jurkat cells (Jurkat cells expressing simian virus 40 tumor antigen) in vitro [165]. The authors suggest that inhibition of (raptor)-mTOR by rapamycin-treatment interferes with p70 S6 kinase function by activating PP2A. This suggests that the effect of rapamycin to decrease the phosphorylation of IRS1 at serine 307 in human adipocytes (Paper III) can be explained by directly or indirectly, via activation of PP2A through the rapamycin-inhibition of mTOR/p70 S6 kinase. From the discussion above

it is possible that the effects of both rapamycin and ocadaic acid on the phosphorylation of serine 307 (Paper III) and maybe of serine 312 converge at the same protein kinase, e.g. mTOR/p70 S6 kinase or PKB/Akt, or protein phosphatase, e.g. PP1 or PP2A. Identification of the protein kinases and protein phosphatases that phosphorylate respectively dephosphorylate the different phosphoserine residues of IRS1 is of great interest as they are potential targets for novel pharmaceutical agents.

Methodology and future directions

The results in this thesis are largely based on comparison of the insulin sensitivity to phosphorylation of different proteins of insulin signalling. When investigating the extent of phosphorylation at different concentrations of insulin and at different time points it is important to use a method that has a fast termination step. Protein phosphorylations, as well as other protein modifications, happen within minutes as also found in this thesis. The cell incubations herein have been terminated by separation of the cells from the medium very rapidly, within 3 seconds, and within 10 seconds the cells are frozen [166]. Cells are then thawed by boiling in sodium dodecyl sulphate salt (SDS) with a mix of protein phosphatase and protease inhibitors. This procedure minimizes intracellular reactions continuing after termination, but also postlysis modification of proteins, which may happen during, e.g. immunoprecipitation. Furthermore, the immunoblotting is very sensitive and specific, but it should be remembered that it is a semiquantitative method. We have therefore in most cases performed all experiments several times and present averages. Incubation of adipocytes with radioactive phosphorous (^{32}P) and analysis of the signal mediators by two-dimensional electrophoresis is another method to examine protein phosphorylations. In practice this is problematic, however, since no more than one sample can be run on the same two-dimensional gel. This makes it difficult to compare samples coming from the same dose-response curve or time-course, unless an internal standard is used.

Herein a monoclonal general anti-phosphotyrosine antibody has been used to detect the tyrosine phosphorylation of IR and IRS1. This antibody does not discriminate between different phosphorylation sites in IR and IRS1, which may be important for transmission of different signals in the cell. It is an important remaining issue to examine insulin effects on

the state of phosphorylation of IRS1 at different tyrosine phosphorylation sites by phosphorylation-site-specific antibodies or for example mass spectrometry.

A further important issue that remains concerns the subcellular location of the signalling proteins. Since the immunoblotting herein was made on whole cell lysates the results reflect over all phosphorylation states, which may indeed vary at different locations in the cell. IRS1 in different compartments of the adipocytes may be phosphorylated at different tyrosine and serine residues and thus for example function in either positive or negative feedback in insulin signalling. These pools of IRS1 may be associated with metabolic or mitogenic signalling and can be phosphorylated or degraded to different extents and thus regulate insulin signalling in different ways. Carlsson *et.al.* have reported subcellular fractionation of IRS1 and found that IRS1 phosphorylated at tyrosine is mostly in the intracellular membrane fraction, while IRS1 phosphorylated at serine 312 is located in the cytosolic fraction of 3T3-L1 adipocytes [131].

To fully examine the cause-effect relationship between insulin resistance and the phosphorylation of IRS1 at serine 307 or 312 transfection of IRS1 mutated at serine 307 and/or at serine 312 in adipocytes from healthy individuals and from patients with type 2 diabetes will be an important future project. This could be done, in combination with knock-down of wildtype IRS1, by site-specific mutagenesis of for instance IRS1 serine307alanine in order to exclude phosphorylation at serine 307 or by IRS1 serine307glutamine in order to mimic permanent phosphorylation. This has earlier been reported in mouse 32D myeloid cells [150]. It would also be interesting to investigate this in other cell types from humans such as liver cells, muscle cells and β -cells of the pancreas.

Conclusions

This thesis focuses on different molecular and cellular mechanisms of insulin signalling in insulin resistant states in primary human adipocytes. Most reported studies of insulin resistance are based on other cell lines and animal models. The focus on primary human cells herein has provided novel findings and insights in insulin signalling in humans.

The findings and conclusions herein can be summarized as follows:

- ❖ The amount of GLUT4 and the basal and insulin-stimulated glucose uptake is higher in adipocytes from visceral compared to subcutaneous fat. However, the sensitivity to insulin for stimulation of glucose uptake is the same in visceral and subcutaneous adipocytes (Paper I)
- ❖ Surgical stress induces a reversible insulin resistance and the mechanism is different from the chronic insulin resistance in type 2 diabetes (Paper II)
- ❖ The impaired insulin signalling in adipocytes from patients with type 2 diabetes is at the level of insulin receptor-catalyzed phosphorylation of IRS1 (Paper II)
- ❖ Insulin-stimulated phosphorylation of IRS1 at serine 307 is attenuated in adipocytes in patients with type 2 diabetes.
- ❖ The phosphorylation of IRS1 at serine 307 may be a positive feedback signal, associated to the tyrosine phosphorylation of IRS1. Insulin-stimulated phosphorylation of serine 307 is rapamycin sensitive, which may indicate a role for (raptor)-mTOR/p70 S6 kinase in this phosphorylation (Paper III)
- ❖ The phosphorylation of IRS1 at serine 307 is a rapid event occurring at physiological concentrations of insulin and it is positively correlated with the steady-state level of tyrosine phosphorylation of IRS1 (Paper IV)
- ❖ Phosphorylation of IRS1 at serine 312 is associated with a negative feedback control in insulin signaling that occurs after prolonged incubation with insulin at high concentrations (Paper IV)

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