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Insulin signaling in primary adipocytes in insulin sensitive and insulin resistant states

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During the course of the research underlying this thesis, Siri Aili Fagerholm was enrolled in Forum Scientium, a multidisciplinary graduate school at Linköping University, Sweden.

Den mätta dagen, den är aldrig störst.
Den bästa dagen är en dag av törst.

Nog finns det mål och mening i vår färd -
men det är vägen, som är mödan värd.

Karin Boye. ”I rörelse”. Härdarna, 1927.

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Abstract

Increasing numbers of people world-wide develops the disease type 2 diabetes. Development of type 2 diabetes is characterized by a shift from an insulin sensitive state to an insulin resistant state in peripheral insulin responding organs, which originates from the development of insulin resistance in the adipose tissue. Insulin resistance in combination with reduced pancreatic insulin secretion lead to overt type 2 diabetes.

In this thesis, the insulin signaling network in primary adipocytes was analyzed. Key proteins and mechanisms were studied to gain deeper knowledge of signaling both in the insulin sensitive state and in the insulin resistant state produced by rapid weight gain as well as in type 2 diabetes.

The surface of the adipocyte is dotted with invaginations in the cell membrane called caveolae that act as important metabolic and signaling platforms in adipocytes, and also harbor the insulin receptor. In paper I we show that insulin stimulation of primary adipocytes results in a rapid phosphorylation of the insulin receptor and caveolin-1, and that internalization of the proteins is mediated by endocytosis of caveolae.

Weight gain due to overfeeding and obesity has been associated with the development of insulin resistance in insulin sensitive tissues such as the adipose tissue. In paper II we show that short-term overfeeding for one month of lean subjects results in an insulin resistant state. At the end of study, the subjects had developed mild systemic insulin resistance. Moreover, in isolated subcutaneous adipocytes we found several alterations of the insulin signaling pathway that mimicked alterations found in isolated subcutaneous adipocytes from subjects with type 2 diabetes.

In paper III we present a first dynamic mathematical model of the insulin signaling network in human adipocytes that are based on experimental data acquired in a consistent fashion. The model takes account of insulin signaling in both the healthy, insulin sensitive state and in the insulin resistant state of type 2 diabetes. We show that attenuated mTORC1-mediated positive feedback to control of phosphorylation of IRS1 at Ser307 is an essential component of the insulin resistant state of type 2 diabetes. A future application of the model is the identification and evaluation of drug targets for the treatment of insulin resistance and type 2 diabetes.

In paper IV we examine the protein kinase that catalyzes the insulin stimulated mTORC1-mediated feedback to IRS1. We find that the phosphorylation of IRS1 at Ser307 is not likely to be catalyzed by the kinases S6K1, mTOR or PKB. However, a catalyzing protein kinase for the *in vitro* phosphorylation of IRS1 at Ser307 was found to be associated with the complex mTORC1.

In conclusion, this thesis provide new insights and characterize mechanisms of the intrinsically complex insulin signaling network of primary adipocytes, both in insulin sensitive and insulin resistant states.

Populärvetenskaplig sammanfattning

Flera hundra miljoner människor lever idag med sjukdomen typ 2 diabetes och antalet personer med sjukdomen förväntas öka. Ökningen beror delvis på att allt fler människor blir överviktiga eller feta och utvecklar insulinresistens. Insulinresistens innebär att vävnader som normalt är känsliga för insulin såsom fett- och muskelvävnad inte svarar fullt ut på insulinets stimulerande verkan för att förmå vävnaderna att till exempel ta upp socker ur blodet efter en måltid. Vid typ 2 diabetes utsöndras dessutom otillräckliga mängder insulin till blodet av bukspottskörteln. Insulinresistens i kombination med mindre insulinfrisättning leder till höga sockernivåer i blodet som är skadligt för kroppen på lång sikt.

Målet med min forskning har varit att öka kunskapen om insulinets påverkan på samspelande proteiner i fettceller. Specifikt, har jag analyserat proteiner i insulin-signalöverföringsvägen i enskilda fettceller vid insulinkänsliga tillstånd och vid insulinresistens till följd av snabb viktuppgång och vid typ 2 diabetes.

Insulin binder till insulinreceptorer på cellytan. Insulinreceptorerna hittas i små inbuktningar i fettcellens yta som kallas ”caveolae”. Vi har funnit att när fettcellerna stimuleras med insulin så snörps caveolae av från cellytan och proteinet caveolin-1 tas in i cellen tillsammans med insulinreceptorn.

Vi har även studerat framrenade fettceller från smala personer som under en månad kraftigt ökade sitt energiintag. Under studien åt personerna mer än dubbelt så många kalorier som innan studien påbörjades, vilket gav en markant viktuppgång. Personerna utvecklade samtidigt en måttlig insulinresistens på helkroppsnivå och i fettcellerna fann vi en förändrad aktivitet hos flera proteiner i insulinsignalvägen som svar på insulin.

En fettcell utgörs till största del av en droppe olja (fett) där energi från mat som vi äter lagras för framtida behov. Ju mer olja som lagras in i oljedroppen desto mer växer fettcellerna. Vid fetma är fettcellerna i vissa fall sprängfyllda av olja. Vi föreslår en mekanism för hur fettceller kan begränsa inlagringen av mer fett för att inte spricka. I denna mekanism har proteinkomplexet mTORC1 en avgörande roll genom att kontrollera ett protein som är associerat till komplexet. Det associerade proteinet påverkar aktiviteten hos ett annat mycket viktigt protein i insulin signaleringen, proteinet IRS1.

Vi har också utvecklat en omfattande matematisk modell som beskriver insulinsignalering i fettceller i insulinkänsligt tillstånd, och vid insulinresistens och typ 2 diabetes. Med hjälp av denna modell kan vi visa att insulinresistensen i fettceller från personer med typ 2 diabetes kan förklaras med en nedsatt aktivitet hos mTORC1 mot IRS1, i kombination med minskade nivåer av insulinreceptorn och sockertransportören GLUT4. Modellen kan också användas för att identifiera och testa nya potentiella mål för läkemedel avsedda att användas i behandlingen av insulinresistens och typ 2 diabetes.

List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Rapid insulin-dependent endocytosis of the insulin receptor by caveolae in primary adipocytes
Fagerholm S, Örtegren U, Karlsson M, Ruishalme I, Strålfors P (2009) PLoS ONE 4(6):e5985.

II Short-term overeating induces insulin resistance in fat cells in lean human subjects
Danielsson A, Fagerholm S, Öst A, Franck N, Kjølhede P, Nystrom FH, Strålfors P (2009) Mol Med 15(7-8):228-234.

III Insulin signaling in type 2 diabetes - experimental and modeling analyses reveal mechanisms of insulin resistance in human adipocytes
Brännmark C*, Nyman E*, Fagerholm S, Bergenholm L, Ekstrand E-M, Cedersund G, Strålfors P (2013) J Biol Chem 288(14):9867-9880.

IV Phosphorylation of IRS1 at serine 307 in response to insulin in human adipocytes is not likely to be catalyzed by p70 ribosomal S6 kinase
Fagerholm S*, Rajan R M*, Jönsson C*, Kjølhede P, Turkina V M, Strålfors P (2013) PLOS ONE 8(4):e59725.

* authors contributed equally to the work

Abbreviations

AS160	Akt substrate of 160 kDa
BMI	body mass index
CME	clathrin-mediated endocytosis
EGF	epidermal growth factor
4E-BP1/2	eukaryotic translation initiation factor 4E-binding protein 1 and 2
ERK1/2	extracellular signal-regulated kinase 1 and 2
GLUT4	glucose transporter 4
Grb2	growth factor receptor-bound protein 2
HOMA	homeostasis model assessment
IGF1	insulin-like growth factor 1
IKK	IκB kinase
IL-6	interleukin-6
IR	insulin receptor
IRS1	insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
OED	ordinary differential equation
OGTT	oral glucose tolerance test
PDK1	3-phosphoinositide-dependant protein kinase 1
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PPARγ	peroxisome proliferator-activated receptor γ
PRAS40	proline-rich Akt substrate
PTEN	phosphatase and tensin homolog
PTRF	polymerase I and transcript release factor
RBP4	retinol binding protein 4
S6K	S6 kinase
Shc	SH2-domain containing protein
SH2	Src-homology 2
SREBP1	sterol regulatory element-binding protein 1
TNF-α	tumor necrosis factor alpha
QUICKI	quantitative insulin sensitivity check index

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Introduction

Increased urbanization, changed transportation habits and mechanization of work tasks have seemingly improved the quality of many human lives. The downside of our sedentary lifestyle is the resulting limited daily physical activities that alongside changed dietary habits and longevity, increases the global number of people that are overweight and/or obese. Obesity often goes hand in hand with the development of insulin resistance in peripheral organs such as the adipose tissue. Insulin resistance encompasses a reduced response to insulin as well as defects in insulin signaling and precedes the development of type 2 diabetes. More than half a billion people worldwide may have type 2 diabetes in 2030 as estimated by the International Diabetes Federation [1]. The ever increasing numbers of individuals with type 2 diabetes has been called a "global diabetes epidemic" [1] or a "tsunami of diabetes" [2].

Much research has been done on insulin signaling trying to elucidate the mechanisms behind insulin resistance and type 2 diabetes. Insulin signaling is intrinsically complex and is constantly being redefined as new pieces of knowledge about the signaling system are added to the puzzle.

The aim of this thesis is to provide new insight into insulin signaling in primary adipocytes in insulin sensitive as well as insulin resistant states. In paper I, we show that insulin stimulation of adipocytes results in a rapid internalization of the insulin receptor (IR) via caveolae. In paper II, we show that short-term overfeeding of lean individuals results in an insulin resistant state manifested by systemic insulin resistance and alterations in the insulin signaling pathway in adipocytes, which are changes that mimic alterations found in type 2 diabetes. In paper III, we use mathematical modeling to take network wide data from the insulin signaling network into account and analyze signaling in both the healthy, insulin sensitive state and the insulin resistant state of type 2 diabetes. An mTORC1-mediated positive feedback that controls phosphorylation of IRS1 at Ser307 is an essential component of the insulin resistant state of type 2 diabetes. In paper IV, we examine the protein kinase that catalyzes the mTORC1-mediated feedback.

Adipose tissue and adipocytes

In mammals adipose tissue is classically characterized as either brown or white adipose tissue. In humans the white adipose tissue can primarily be found as upper (abdominal) and lower (including femoral) subcutaneous fat or as intra-abdominal fat (visceral). Subcutaneous adipose tissue consists of a number of interacting cell types including mature adipocytes, mesenchymal stem cells and preadipocytes, fibroblasts, vascular cells and macrophages.

Human adipocytes isolated from the subcutaneous white adipose tissue range in size from 20 μm to over 200 μm in diameter [3-5]. Mature adipocytes contain a large central lipid droplet that constitutes most of the cell volume and leaves room only for a very thin film of cytosol that ranges in width between 50 nm to about 500 nm.

Adipose tissue in glucose homeostasis and lipid metabolism

Adipocytes play an important role in maintenance of whole body glucose homeostasis and as an energy reserve between meals and during starvation. After a meal, the glucose level in the blood is elevated, which is sensed by β-cells in the pancreatic Islets of Langerhans resulting in an enhanced release of insulin from storage vesicles in the β-cells. The released insulin affects insulin target tissues including liver, skeletal muscle and adipose tissues. In skeletal muscle the elevated concentration of insulin leads to an increase in glucose uptake and an increased synthesis of glycogen. Insulin also mediates a net decrease in liver glucose output through increased glycogen synthesis and decreased gluconeogenesis and decreased glycogen breakdown. Elevated concentrations of insulin also increase glucose uptake in the adipose tissue. Insulin action is balanced by the counter-acting hormone glucagon that is secreted by pancreatic α-cells, and together they control glucose homeostasis.

In adipocytes, insulin promotes decreased lipolysis and increased storage of lipids in the form of triacylglycerol in the lipid droplets. This energy reserve can be mobilized via lipolysis stimulated by noradrenaline and adrenaline through hydrolysis of triacylglycerols into fatty acids in fasting states, when energy is needed in other tissues.

Besides being an energy reserve, the adipose tissue is also an important endocrine organ that produces and secretes factors named adipokines. Adipokines communicate with other tissues and organs, as well as function in an autocrine/paracrine manner. A number of adipokines have been characterized during the past 20 years. These include leptin, adiponectin, and retinol binding protein 4 (RBP4) that are all secreted from adipocytes, as well as interleukin-6 (IL-6), resistin and tumor necrosis factor-α (TNF-α) that are secreted from macrophages in the adipose tissue. The levels of adiponectin decrease with obesity, insulin resistance and type 2 diabetes while levels of leptin increase [6,7]. Reviewed in ref. [8,9].

Insulin signaling

Caveolae, caveolin-1 and insulin signaling

Small bulb- or flask-like invaginations of the cell membrane called caveolae (Latin for little caves) are scattered throughout the surface of the adipocyte (Fig 1). Caveolae are usually between 25-150 nm in diameter, and their abundance greatly increases the cell membrane surface area [10]. In general, they are structures rich in cholesterol and sphingomyelin, although the specific lipid composition varies between different subclasses of caveolae [11].

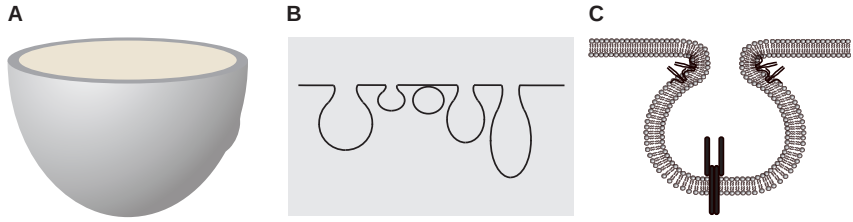

Figure 1. Adipocyte, caveolae in the plasma membrane and a single caveolae.
Cross section of a schematic human adipocyte (A) that contains a large lipid droplet and a thin rim of cytosol, the nucleus is protruding on the right hand side of the cell. Caveolae of different sizes and functions are formed by the plasma membrane of an adipocyte (B). Schematic picture of a single caveola (C), containing caveolin-1 oligomers at the neck and IR.

Caveolin-1 is the most widely used protein marker for caveolae. Caveolin-1 is an integral membrane protein that forms a hairpin-like loop structure as its hydrophobic region is inserted in the plasma membrane bilayer while the amino- and carboxyl-terminal regions extend into the cytoplasm. It binds cholesterol [12] in the plasma membrane and is modified with palmitoyl-groups at the C-terminal domain [13].

Caveolin-1 is necessary for the formation of caveolae [14]. In primary adipocytes, caveolin-1 was found to primarily locate to the neck of caveolae [10] although in 3T3-L1 adipocytes another group reported (with the use of another preparation technique of micrographs) caveolin-1 to cover not only the neck but also the entire caveolae invagination [15].

In addition to caveolin-1 there are two other proteins in the caveolin protein family: caveolin-2 and caveolin-3. Caveolin-1 and caveolin-2 are ubiquitously expressed while caveolin-3 is muscle-tissue specific. Caveolin-2 forms stable high-mass hetero-oligomers with caveolin-1, and co-expression with caveolin-1 is necessary for caveolin-2 to transport to the plasma membrane although there are contradicting reports on whether caveolin-2 is dispensible in caveolae biogenesis [16,17].

Another protein that is localized to the cytoplasmic side of caveolae in human adipocytes is the protein Polymerase I and transcript release factor, PTRF/cavin-1 [18]. PTRF/cavin-1 is necessary for the formation and stability of the caveolae structure through interaction with caveolin-1 [19,20]. Other PTRF related proteins named cavin-2-4, have also been found to localize to caveolae [18,21]. In particular cavin-2 seems to play an important role as a regulator of the expression levels of caveolin-1 and PTRF as well as participate in forming the membrane curvature of caveolae [22].

Caveolae are distinct domains in the plasma membrane that can be utilized for a number of different cellular events such as endocytosis, uptake of nutrients or control of intracellular signaling pathways. In adipocytes, caveolae have been suggested to act as “metabolic platforms” involved in insulin stimulated glucose uptake, uptake of fatty acids and cholesterol as well as *de novo* triacylglycerol synthesis and lipid droplet biogenesis [23].

Caveolae are of interest in the present context because in primary mature adipocytes the IR is localized in caveolae (Fig. 1)[24-28] and, as discussed below, is endocytosed in a caveolae mediated process.

The insulin receptor

IR belongs to the Receptor Tyrosine Kinase family, which includes many plasma membrane receptors that bind various growth factors, e.g. the insulin-like growth factor 1 (IGF1) receptor and the epidermal growth factor (EGF) receptor. The IR is composed of two extracellular α-subunits (135kDa) and two transmembrane β-subunits (95kDa) that are bound to each other with disulfide bonds forming a hetero-tetramer [29] (Fig. 2). Binding of insulin to the α-subunits trigger a conformational change and results in auto-phosphorylation of tyrosine residues in the cytoplasmic part of the β-subunit, which allows the signal to propagate further downstream in the signaling pathway [29-31].

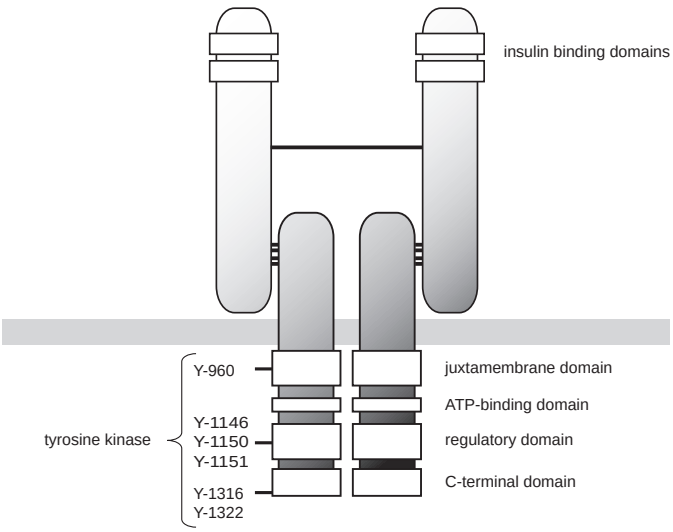

Figure 2. Structure of the IR.

IR is composed of two extracellular α-subunits and two membrane-spanning β-subunits that are linked with disulfide bonds. Insulin binds to the α-subunits which lead to conformational changes and trans auto-phosphorylation of tyrosine sites positioned in the juxtamembrane region, in the regulatory kinase domain and in the C-terminal part of the receptor [29,30]. Subsequently, e.g. IRS1 binds to phosphorylated tyrosine sites in the juxtamembrane region. Tyrosine residues that are auto-phosphorylated and have been shown to be important for the tyrosine kinase activity of the receptor are indicated. Adapted from [29].

Several groups have shown that the IR is localized to caveolae in adipocytes [24-28]. Knockdown of caveolin-1 in 3T3-L1 adipocytes reduced caveolae structures in the plasma membrane and correlated with increased degradation of IR protein, indicative of a stabilizing role of caveolae on IR protein [32]. Moreover, caveolin-1 as well as PTRF knockout mice show loss of caveolae and display a large reduction in the protein levels of IR [33,34]. Also, caveolae integrity affects insulin signaling downstream of the receptor as shown by cholesterol depletion and flattening of caveolae structures after cholesterol extraction with β-cyclodextrin treatment of rat and human adipocytes [35,36]. In human adipocytes, β-cyclodextrin treatment did not affect insulin-stimulated phosphorylation of IR or IRS1 while both metabolic and mitogenic downstream signaling were impaired [35]. Further, inhibition of cholesterol biosynthesis in 3T3-L1 adipocytes affected the membrane localization of caveolin-1 and the insulin stimulated IR activation as well as downstream metabolic effects [37].

The insulin signaling network

Insulin stimulation of an adipocyte results in a cellular response that for simplicity can be divided into a metabolic and a mitogenic response. The metabolic response (Fig. 3) is mediated by the master regulator mammalian target of rapamycin (mTOR) as well as proteins in the AGC-protein kinase family including protein kinase B (PKB), protein kinase C (PKC) and ribosomal protein S6 kinase (S6K). The mitogenic response to insulin stimulation is mainly mediated by the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) pathway (Fig. 4). However the insulin signaling pathways cross-talk, and cross-talk in combination with positive or negative feedbacks, localization and regulation in a time-dependent manner results in a mind-boggling complexity of the insulin signaling network.

IR binds several substrates, including the insulin receptor substrates (IRSs), Src-homology 2 (SH2)-domain containing protein (Shc) and the growth factor receptor-bound protein 2 (Grb2). IR and IRS have been described as a “critical node” in the insulin signaling pathway due to its established and crucial role in insulin signaling [38]. Tyrosine phosphorylation of IRS1 by IR creates binding sites for SH2-domain proteins, thus IRS1 acts as a docking protein linking the activated receptor to specific downstream targets. These targets include the regulatory p85-subunit of phosphatidylinositol 3-kinase (PI3K), which binds to phosphorylated YMXM motifs in IRS1, and Grb2 that binds to a YVNI motif in IRS1 [39].

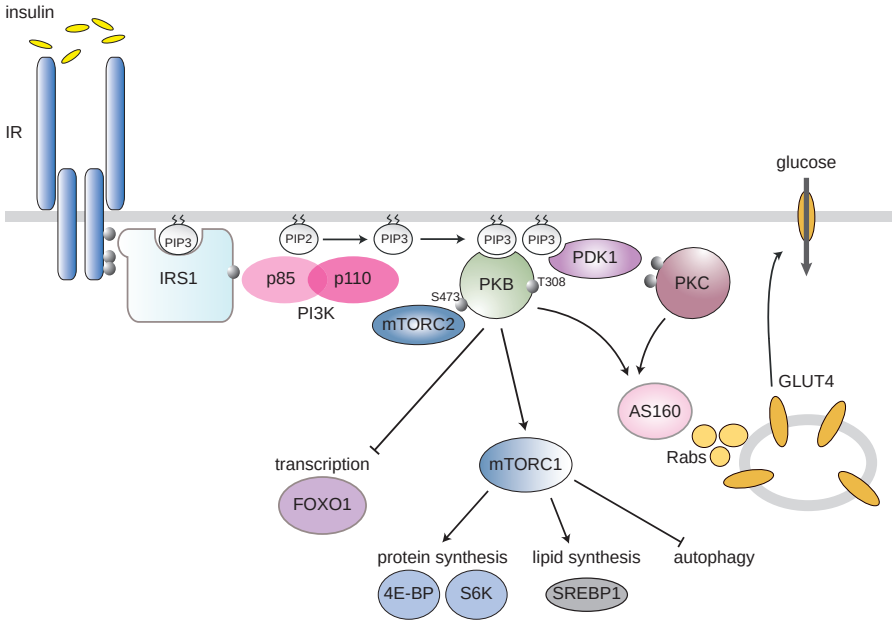

Figure 3. Insulin signaling through the PI3K/PKB pathway.

Insulin binding to the IR initiates intracellular signaling resulting in e.g. enhanced glucose uptake via GLUT4, increased protein and lipid synthesis and reduced autophagy.

PI3K consists of a p85-regulatory and a p110-catalytic subunit, where the regulatory subunit binds IRS1 and in turn activates the p110-catalytical subunit [39]. PI3K phosphorylates the lipid phosphatidylinositol-4,5-diphosphate (PIP2) and thus increases the amount of phosphatidylinositol-3,4,5-triphosphate (PIP3) in the plasma membrane, the protein phosphatase and tensin homolog (PTEN) mediates the opposite reaction. Through binding to PIP3 via their pleckstrin homology (PH) domains the 3-phosphoinositide-dependant protein kinase 1 (PDK1) and PKB are positioned in the plasma membrane, which enables PDK1 to phosphorylate PKB at Thr308. PKB is also phosphorylated at Ser473 by mTOR in complex 2 (mTORC2). Phosphorylation of PKB at both Ser473 and Thr308 leads to full activity of the kinase [40].

Activated PKB in turn affects the activity of mTOR in complex 1 (mTORC1) through inhibitory serine phosphorylation of tuberous scelorosis complex 1/2

(TSC1/2). TSC1/2 inhibition increases the amount of Rheb bound to GTP, which inhibits the protein FKBP38 and thus relieves its inhibitory effect on mTOR. mTORC1 activation results in increased protein synthesis through phosphorylation of S6K and the eukaryotic translation initiation factor 4E-binding protein 1 and 2 (4E-BP1/2). S6K affects translation elongation through the ribosomal protein S6 and 4E-BP1/2 affects translation initiation through elF4E/A/G. S6K and 4E-BP also control the expression of transcription factors that are involved in adipogenesis [41,42]. Also, mTORC1 controls fatty acid synthesis via the sterol regulatory element-binding protein 1 (SREBP1) possibly involving S6K [43-45].

One of many substrates of PKB is the Akt substrate of 160kDa (AS160). AS160 has been suggested as a node for integrating signaling from PKC, PKB and AMPK [46]. Phosphorylation of AS160 relieves an inhibitory effect on GLUT4 translocation to the plasma membrane. GLUT4 in the plasma membrane mediates facilitated diffusion of glucose over the plasma membrane [47,48]. Basal glucose uptake is mediated by GLUT1 whereas only a small portion of GLUT4 is located at the plasma membrane. Insulin stimulates a rapid increase in exocytosis of GLUT4-containing vesicles to the plasma membrane in adipocytes and muscle. Levels of GLUT4 at the plasma membrane are determined by the net rates of exocytosis and endocytosis. The rate of insulin-controlled endocytosis has been shown to be reduced but there are also recent reports of unaltered rates of endocytosis in response to insulin, leaving an open question as reviewed in ref. [49].

PKB also regulates members of the transcription factor family named FOXO, including the isoform FOXO1 that is abundant in adipocytes [50]. PKB phosphorylates and deactivates FOXO1, which is translocated from the nucleus to the cytosol. At low concentrations of insulin, nuclear localized FOXO1 increases gene expression of for example IR, 4E-BP and ATGL [50,51]. FOXO1 also plays a regulatory role in preadipocyte differentiation in mice [52].

Insulin stimulation of adipocytes also results in a mitogenic cellular response mainly mediated by the MAP kinases ERK1/2 pathway (Fig. 4). ERK1/2 phosphorylates and regulates a large number of substrates including transcription factors such as Elk-1 and c-Fos [48,53]. It has also been shown that ERK1/2 positively regulates mRNA translation in response to insulin through the phosphorylation and inactivation of the TSC1/2 complex [54] and through phosphorylation of raptor in mTORC1 [55,56]. Moreover, in response to stimulation with growth factors, also

phosphorylation of S6 was shown to be regulated by the ERK1/2 pathway acting in parallel to mTORC1 regulation of S6 [57]. Thus, translation is regulated both by the PI3K/PKB/mTORC1 and the ERK1/2 pathway.

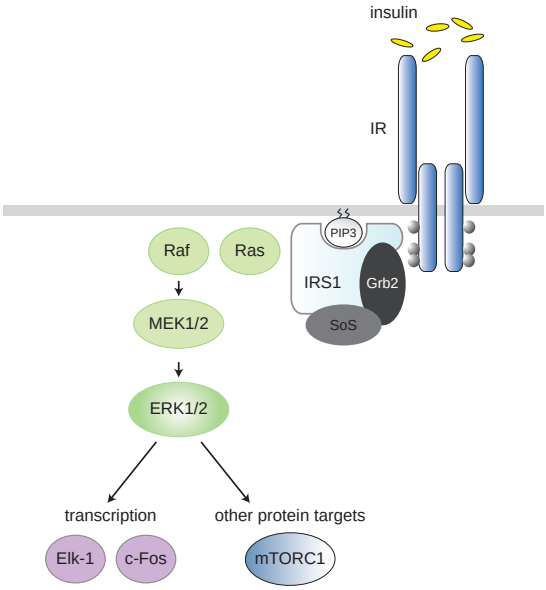

Figure 4. Insulin signaling through the ERK1/2 pathway.

Insulin binding to the IR initiates signaling pathways to control mitogenic processes by the ERK1/2 pathway. ERK1/2 has a wide number of substrates, for example it regulates transcriptional activity via the transcription factors Elk-1 and c-Fos [48,53]. ERK1/2 also regulates other protein targets including TSC1/2, raptor in mTORC1 and S6 [54-57]. Thus, insulin signaling through ERK1/2 can affect both transcription and translation through cross-talk with the PI3K/PKB insulin signaling pathway.

Endocytosis of the insulin receptor

Mechanisms of endocytosis and the endosomal network

Endocytosis is a process whereby extracellular substances are taken into the cell interior through internalization of a re-shaped plasma membrane. In later years, the complexity of the endocytic system in terms of its diverse functions and mechanisms have gained appraisal. For example, endocytosis takes part in the temporal and spatial regulation of insulin signaling and in the control of the lipid composition of the plasma membrane. There are a number of different endocytic pathways employed in the mammalian cell that include classical clathrin-mediated endocytosis (CME), endocytosis via caveolae, macropinocytosis and phagocytosis, reviewed in ref. [58,59].

In general, internalization of an endocytic vesicle from the plasma membrane is followed by fusion with and sorting of cargoes in early endosomes and subsequent recycling to the plasma membrane or degradation [60]. The concept of early endosomes comprises vesicles of many sizes and with varying functions. With time, early endosomes can either fuse with or directly convert into late endosomes [61]. Cargo (such as the IR) can be recycled back to the plasma membrane while cargo destined for degradation is shuttled through the endocytic system of vesicles/endosomes with increasing acidity to late endosomes and finally to lysosomes where degradation takes place.

Rab GTPases play an important role in the endocytic system as regulators of endosome fusion events and are used as markers for endosomes in different stages of the system. Rab5 is often used as a marker for early endosomes, Rab7 for late endosomes and Rab11 for recycling endosomes [60]. The adaptor protein APPL1/ 2 binds Rab5 at newly formed endosomes, endosomes that in turn convert into early endosomes that are marked by the phospholipid phosphatidylinositol 3-phosphate and the protein early endosome antigen 1 [60,61].

Endocytosis through classical CME has been studied most intensively. Mechanisms behind CME show great variation depending on cell type and the type of cargo that is to be transported. The formation process of the clathrin coated pit is highly complex and involves sequential recruitment of different accessory proteins and

adaptor proteins [62]. Dynamin GTPases act as scissor molecules that detach the coated vesicle from the plasma membrane, the coat of polymerized clathrin is then released and the vesicle and cargo join the endocytic system of multiple vesicles [59]. Depending on the adaptor protein the destiny of the internalized coated pit can be regulated [63]. A classic example of CME is the internalization of transferrin and its receptor [60].

Endocytosis mediated by caveolae is a less studied endocytic pathway, but it is recognized as being involved in the internalization of lipids, proteins and pathogens. These include glycosyl-phosphatidylinositol (GPI)-linked proteins and pathogens such as cholera toxin B [64] and SV40 [65], as well as receptors including TGF-β receptors [66] and EGF receptors [67]. Some consider caveolae as highly immobile structures that are not involved in constitutive endocytosis but need a stimuli to become internalized [68], while on the other hand e.g. Mundy et al. [69] suggested that there are different pools of caveolae, and showed that in CHO cells one pool contains caveolae that are involved in constitutive trafficking. Recently, in TIRF microscopy studies of Cav1-GFP expressing HeLa cells, a majority of caveolae structures were found to be constitutively dynamic, and cycled between the plasma membrane and the cytoplasm with a turnover time ranging from less than two seconds to minutes [70].

Upon stimulation of HeLa and CV-1 cells with cholera toxin B, SV40 or vanadate, caveolae were endocytosed and traveled through the endosomal pathways as stable units [71,72]. Endocytosis of caveolae is dynamin-dependant and dynamin-2 interacts directly with caveolin-1 at the necks of caveolae [73]. Rab5 as well as a number of kinases are also involved in fission of caveolae from the plasma membrane [59]. Rab5 likewise regulates the internalization of the IR [74].

A type of endosomes originating from internalized caveolae was identified in 2001 by Helenius et al. [65] and named *caveosomes*. Caveosomes were initially defined as large endocytic organelles rich in caveolin-1 that are pH-neutral and lack classical markers for early endosomes, lysosomes and ER/Golgi. However, the authors re-defined the concept of caveosomes as merely modified late endosomes or lysosomes with a high number of caveolin-1 due to the experimental procedure of caveolin protein overexpression [75].

Endocytosis of the insulin receptor

Internalization of IR has been suggested to occur through CME mechanisms as well as through caveolae, possibly reflecting the different cell types studied. Studies of hepatocytes [76], CHO cells [77] and 3T3-L1 adipocytes [78] suggested that IR is internalized through CME. On the other hand, using gold- or ^{125}I-labeled insulin in order to study endocytosis of the IR in hepatocytes [79,80] and primary rat adipocytes [81] internalization of IR through non-coated invaginations was suggested. Also, the use of both CME and CME-independent internalization of the IR in CHO cells has been shown [82].

In paper I we studied the mechanism of IR internalization in primary rat adipocytes. Insulin induced tyrosine auto-phosphorylation of IR and caused a rapid ($t_{1/2}$<3 min) internalization of the IR into an intracellular endosomal fraction. Concomitantly, caveolin-1 was phosphorylated at Tyr14 and appeared in the same endosomal fraction.

Phosphorylation of caveolin-1 at Tyr14 has been suggested to be a regulatory mechanism for internalization of caveolae in endothelial cells [83]. Also, the phosphotyrosine protein phosphatase inhibitors pervanadate and sodium ortho-vanadate increased the phosphorylation of caveolin-1 at Tyr14 as well as intracellular caveolae vesicles [83]. Sodium ortho-vanadate treatment of primary rat adipocytes increased the phosphorylation of caveolin-1 at Tyr14 both at the plasma membrane and in the endosomal fraction. However, vanadate treatment did not affect phosphorylation or endocytosis of the IR (paper I).

Caveolin-1 can through its cytosolic N-terminal part bind to an amino acid sequence motif that is present in several caveolae localized proteins including in the tyrosine kinase domain of IR [84]. Indeed, in 3T3-L1 cells IR associated with caveolin-1 and phosphorylated caveolin-1 at Tyr14 [24]. Interestingly, binding of a peptide corresponding to the scaffolding domain of caveolin-1 increased the tyrosine kinase activity of IR towards IRS1 *in vitro* [25].

In paper I we demonstrated the co-localization of the IR and caveolin-1 by immunocapture of endosomal vesicles using SDS-PAGE and immunoblotting, and also by immunogold labeling of endosomal vesicles and transmission electron microscopy. Clathrin was not endocytosed with the IR and an inhibitor of clathrin-

coated pit-mediated endocytosis, chlorpromazine, did not inhibit internalization of the IR, while transferrin receptor internalization (a marker for CME) was inhibited. Taken together, in paper I we show that insulin stimulated internalization of the IR was mediated by caveolae in primary rat adipocytes on the basis of: (i) insulin stimulation resulted in tyrosine phosphorylation and internalization of the IR and caveolin-1 in the same timeframe. (ii) IR and caveolin-1 was detected in the same endosomal vesicles after insulin stimulation as shown by co-immunocapture with anti-IR or anti-caveolin-1 antibodies. (iii) IR and caveolin-1 was co-detected in vesicles from the endosomal fraction with immunogold labeling and transmission electron microscopy. Further, (iv) chlorpromazine, which inhibit clathrin-coated pit mediated endocytosis, had no effect on IR internalization.

In rat adipocytes, internalization of IR was not affected by chlorpromazine treatment neither at low (2nM) nor at high (100nM) insulin concentration, which implies that endocytosis via caveolae predominate as the stimulated internalization route (paper I). However, in the literature there are examples of receptors that appear to internalize through more than one endocytic route. One example is the EGF receptor, which in response to low concentrations of EGF is endocytosed through CME while at higher concentrations the receptor employs both CME and a clathrin-independent pathway sensitive to filipin [67]. Another example is the receptor for TGF-β that internalize through CME, in order to regulate signal transduction, or through caveolae, for receptor degradation [66].

After dissociation from insulin, the IR is recycled to the plasma membrane while insulin is being degraded [85,86]. Internalization of the IR is actually necessary to explain the observed insulin signaling dynamics for phosphorylation of IR and IRS1 in primary human adipocytes [87] indicative of the essential role endocytosis plays in insulin signaling.

The insulin receptor substrate 1

Insulin receptor substrate proteins (IRSs) constitute a family of proteins that all contain an N-terminal PH domain followed by a PTB domain and a C-terminal part that varies in length [31]. IRS1 and IRS2 are expressed in a number of tissues including adipose, muscle and liver tissue. IRS3 has only been found in rodents, while IRS4 is mainly expressed in thymus and hypothalamus [31]. IRS1 and IRS2 probably have redundant roles in insulin signaling but while IRS1 may predominate in adipocytes and skeletal muscle, IRS2 may be more important in liver [88,89].

Both the PH and the PTB domains are essential for IRS1 interaction with the IR, and IRS1 binds via the PTB domain to the juxtamembrane part of IR [90] (Fig. 5). C-terminal to the PTB domain in the amino acid sequence is the so called SAIN-domain (Shc and IRS1 NPXY binding domain)[91]. Raptor in mTORC1 binds the SAIN domain and may position IRS1 so that mTOR can phosphorylate IRS1 at Ser636/639 [91].

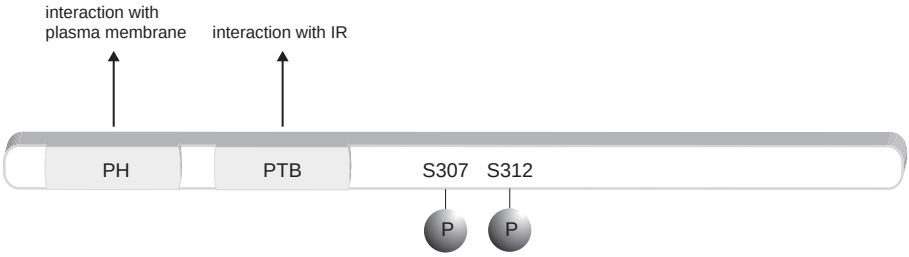

Figure 5. Structure of human IRS1.

Human IRS contain a N-terminal PH domain followed by a PTB domain. The C-terminal varies in length between different IRS-isoforms and contain multiple potential tyrosine, serine and threonine phosphorylation sites. Depicted in the figure is the phosphorylation of two serine sites, Ser307 and Ser312, studied in paper (II, III, IV).

IR phosphorylates IRS1 on multiple tyrosine residues and already within a few minutes after insulin stimulation of a human adipocyte, tyrosine phosphorylation of IRS1 peaks, and is followed by a lower quasi-steady-state level of phosphorylation at tyrosine residues (paper III)[87].

Tyrosine phosphorylation of IRS1 propagates the insulin signal from activated IR to downstream targets, while serine and threonine phosphorylation of IRS1 may function mainly as regulators of IRS1 activity in response to feedbacks and cross-talk with other signaling pathways. There are more than 200 serine and threonine residues on IRS1, where approximately 70 are found in canonical kinase phosphorylation motifs but only a fraction of these residues have been thoroughly characterized [31]. Due to the extensive phosphorylation IRS1 migrates as a protein of ~185kDa during SDS-polyacrylamide gel electrophoresis although unmodified it has a molecular weight of only 131 kDa.

For several of the serine phosphorylation sites on IRS1 there is a lack of consensus as to whether they function as positive or negative regulators of insulin signaling.

A number of papers have indicated a positive impact of serine phosphorylation on IRS1 activity. Phosphorylation of IRS1 at Ser307 and Ser312 has been described to play a positive regulatory role in insulin signaling, as discussed below. Other examples include insulin induced phosphorylation of IRS1 at Ser629 and at Ser1223 that both have been correlated with enhanced tyrosine phosphorylation [92,93].

A number of serine sites have been suggested to have a negative effect on tyrosine phosphorylation of IRS1. These include Ser307 [94], Ser312 [38,94-97], Ser408 [98], Ser789 [99] and Ser1101 [100]. Serine phosphorylation has been suggested to inhibit the interaction between IRS1 and the juxtamembrane region of IR [95], to inhibit binding of IRS1 to PI3K [100] and to lead to degradation of IRS1 [101].

A number of kinases have been suggested to phosphorylate IRS1 at serine sites, including c-Jun N-terminal kinase (JNK), IκB kinase (IKK), S6K (discussed below), mTOR, PKC isoforms α, θ, δ and ζ , glycogen synthase kinase 3 and MAP kinases as summarized in ref. [97].

Contradictory findings of inhibitory or enhancing effects on insulin signaling for serine/threonine phosphorylation of a specific site may result from temporal effects, protein localization differences or different model systems. After studying the temporal phosphorylation pattern of IRS1 at Ser318 it was proposed that this site could actually both enhance and attenuate tyrosine phosphorylation of IRS1 in a time-dependent manner, which adds another level of complexity to the insulin signaling system [102]. In a follow-up paper [97], the same group showed that insulin

phosphorylation of IRS1 at Ser307 recruits PKCζ to IRS1, and PKCζ in turn phosphorylates Ser318 at early time points. However, the continued phosphorylation of Ser318 was ascribed to a mTOR-dependent kinase. Phosphorylation of IRS1 at Ser312, on the other hand, was independent of the other two phosphorylation sites.

Phosphorylation of IRS1 at Ser312 was long considered to attenuate insulin signaling, as shown in different model systems [103-105]. However, with the use of knock-in mice it was shown that *in vivo* Ser312-phosphorylation actually played a positive role in the regulation of insulin signaling [106].

Phosphorylation of IRS1 at Ser307

Phosphorylation of IRS1 at Ser307 is of particular interest due to its seemingly important role in the pathogenesis of insulin resistance and type 2 diabetes (discussed in further detail below).

In human primary adipocytes we have found that insulin induces a rapid phosphorylation of IRS1 at Ser307, which is followed by an elevated quasi steady-state level of phosphorylation (paper III, IV). In paper III we performed a systems wide analysis of the entire insulin signaling network to control of glucose uptake and protein synthesis. By collecting dynamic and steady-state data in a consistent fashion for the insulin signaling network and analyzing these data in a systems wide fashion using mathematical modeling we established the critical importance of an mTOR-mediated positive feedback to phosphorylation of IRS1 at Ser307 for maintaining insulin sensitivity and signal strength throughout the insulin signaling pathway.

Other groups have also suggested a positive correlation between Ser307 phosphorylation and tyrosine phosphorylation of IRS1. For example, work using different cell lines including 3T3-L1, CHO-T cells and murine muscle cells indicates a positive correlation between phosphorylation of IRS1 at Ser307 and tyrosine phosphorylation of IRS1 [97,107,108]. Also, upon overexpression of IRS1 S307A in 32D cells, insulin induced tyrosine phosphorylation of IRS1 was attenuated and was paralleled by a poor binding of the p85α-subunit of PI3K to IRS1 as well as diminished phosphorylation of S6 and 4E-BP [107].

Insulin and IGF-1 stimulated phosphorylation of IRS1 at Ser307 is inhibited after treatment with rapamycin, shown in human adipocytes and in four different cell lines including 3T3-L1 [107,109]. Also, in CHOIR/IRS1 cells, amino acid and glucose starvation reduced basal and insulin stimulated phosphorylation of IRS1 at Ser307, while reintroducing amino acids or glucose to the medium normalized the insulin response without effecting PKB phosphorylation [107]. These experiments indicate that a downstream kinase of mTORC1, or possibly mTOR (in mTORC1) itself, mediates the insulin stimulated phosphorylation of IRS1 at Ser307.

Ser307 lies in a consensus sequence **R**X**R**XX(**S**/**T**) that is recognized by several protein kinases belonging to the AGC family of protein kinases, including p70-S6K1 and PKB [108,110,111]. Not surprisingly, S6K1 has been considered to be the protein kinase catalyzing the phosphorylation of IRS1 at Ser307 in response to insulin. In support of this hypothesis, S6K1 phosphorylation of IRS1 at Ser307 has been shown *in vitro* [110,112]. Also, insulin-stimulated phosphorylation of IRS1 at Ser307 was reduced in knockdown experiments of S6K1 and S6K2 [110,112].

While S6K1 can phosphorylate IRS1 at Ser307 under certain conditions this does not seem to be the case in response to insulin stimulation in the human primary adipocyte. Through a number of approaches we show in paper IV that another mTORC1-dependant protein kinase than S6K1 catalyzes the insulin induced phosphorylation of IRS1 at Ser307. This conclusion is based on (i) time resolved data which show large timeframe differences in the phosphorylation of S6 and IRS1 at Ser307 in response to insulin. Also, (ii) through overexpression of a dominant-negative form of S6K1 we found a reduced insulin-stimulated phosphorylation of S6 while phosphorylation IRS1 at Ser307 was unaffected. In addition, (iii) inhibition of S6K activity by PF-4708671 decreased phosphorylation of S6, while phosphorylation of IRS1 at Ser307 was unaffected. Taken together, these findings support that S6K does not catalyze the insulin-induced phosphorylation of IRS1 at Ser307 in primary mature human adipocytes.

In paper IV, we also performed *in vitro* experiments with an mTOR-immuno-precipitate to determine if mTOR itself could be the catalyzing protein kinase. Thus, we immunoprecipitated mTOR from lysates of insulin stimulated human adipocytes and incubated with a Ser307-containing IRS1 peptide. The Ser307-peptide comprises the IRS1 amino acid residues 288-314. With this setting, we could detect an insulin-induced phosphorylation of the peptide at Ser307 only with LC-MS/MS.

As this was the only site phosphorylated we concluded that the mTOR-immunoprecipitate contains a Ser307-specific protein kinase. But with the use of three different inhibitors (Torin1, PF-4708671 and Akt1/2) we concluded that neither mTOR, S6K1 nor PKB was the major protein kinase in the mTOR-immunoprecipitate that phosphorylates IRS1 at Ser307 *in vitro*.

Previously, additional serine/threonine protein kinases apart from the kinases investigated in paper IV have been suggested to phosphorylate IRS1 at Ser307. JNK was suggested as a potential protein kinase for Ser307 phosphorylation [94], but was shown not to be involved in insulin or IGF-1 induced phosphorylation of IRS1 at Ser307 in mouse embryonic fibroblasts [107]. Several isoforms of PKC have been suggested as protein kinases for serine phosphorylation of IRS1, for example PKC δ that was shown to phosphorylate Ser307 *in vitro* alongside a number of other serine sites in IRS1 [113]. It remains to identify the protein kinase that phosphorylates IRS1 at Ser307 after insulin stimulation of primary human adipocytes, and this protein kinase may be found in the mTOR-immunoprecipitate isolated in paper IV.

mTORC1 and mTORC2

The two mTOR signaling complexes, mTORC1 and mTORC2, are key nodes for regulation by insulin of a number of fundamental cellular processes. Both complexes contain the serine/threonine protein kinase mTOR, mLST8, the mTOR inhibitor deptor and the scaffolding proteins tti1/tel2 [114]. mTORC1 also contains the protein raptor and the proline-rich Akt substrate (PRAS40). Raptor mediates the binding of mTOR to signaling motifs (TOS motifs) on S6K1 and 4E-BP [91]. PRAS40 has an inhibitory function on mTORC1 activity that is relieved after insulin stimulation through release of PRAS40 from the complex [115,116]. mTORC2 also contains the scaffolding proteins rictor and mSin1, as well as protor 1/2 [114].

Both mTOR complexes respond to growth factor signals and mTORC1 is also sensitive to amino acid availability, energy status, stress and oxygen availability of the cell. mTORC1 coordinates inputs and regulates cell growth in terms of lipid and protein synthesis, energy metabolism, control of lysosome biogenesis and the regulation of autophagy. mTORC2 in turn regulates metabolic responses, cell survival and reorganization of the cytoskeleton through e.g. engaging AGC kinases such as PKB, PKC and SGK1 [114].

Rapamycin is a commonly used inhibitor of mTORC1 and is highly specific for mTORC1 during short incubations [114,117]. Rapamycin forms a complex with the 12kDa FK506-binding protein (FKBP12) that binds and inhibits mTOR activity [118]. Short-term rapamycin treatment of primary human adipocytes isolated from subcutaneous adipose tissue reduced insulin sensitivity for phosphorylation of IRS1 at tyrosine and Ser307 [109] and for glucose uptake [119].

Long-term treatment with rapamycin seems to affect mTORC2 activity through reduced mTORC2 assembly, as shown in mice [120] and cell lines [121]. Long-term rapamycin treatment decreased phosphorylation of PKB at Ser473 in adipose tissue of mice [121], and in 3T3-L1 adipocytes where rapamycin treatment for ≥48h affected rictor-mTOR assembly [122]. There was no change in phosphorylation of PKB at Ser473 after short-term rapamycin treatment (40min) of human subcutaneous adipocytes supporting the time-specific inhibition of mTORC1 by rapamycin (paper IV), although this is in contrast to findings by Pereira et al. [119]. Rapamycin-FKBP12 is unable to bind to pre-formed mTORC2 but can bind newly synthesized free mTOR, providing a possible explanation for the observed long-term mediated decrease in formation of mTORC2 [121] and decreased phosphorylation of PKB at Ser473.

Other mTOR inhibitors are in use, for example Torin1. Torin1 inhibits the mTOR active site through competing for ATP, thus inhibits both mTOR-complexes [117].

S6K1 and S6K2

S6K1 and S6K2 are two highly homologues proteins belonging to the family of AGC serine/threonine protein kinases. Both S6 kinases are found in two isoforms, the gene for S6K1 encodes p70-S6K1 and p85-S6K1 [123], while the gene for S6K2 encodes p54-S6K2 and p56-S6K2 [124]. p70-S6K1 and S6K2 phosphorylate the 40S ribosomal subunit protein S6 after insulin stimulation [124].

S6K isoforms differ in their intracellular localization. p70-S6K1 is predominately found in the cytosol whereas p85-S6K1 contains a nuclear localization signal motif that directs it to the nucleus [125]. Also the S6K2 isoforms are mostly confined to the nucleus [126]. After EGF-stimulation of HEK293 cells, p70-S6K1 was found to translocate from the cytosol to the plasma membrane while p54-S6K2 remained in

the nucleus [126]. Although the catalytic domain displays high homology between p70-S6K1 and p54-S6K2 the amino- and C-terminal differ, which possibly reflects different function and regulation of the proteins [124].

Full activity of S6 kinases follows after phosphorylation of a number of amino acid residues, including phosphorylation of Thr229 by PDK1 in the activation loop [127] and Thr389 in the hydrophobic motif by mTORC1 [128] (p70-S6K residue numbering).

After a lag-time of a couple of minutes insulin induced a slowly increasing phosphorylation of p70-S6K1 at Thr389 in primary human adipocytes, which was paralleled by a slow increase in phosphorylation of its substrate S6 at Ser235/236 (paper III, IV).

In HEK293-cells EGF-stimulation fully activated p70-S6K after 30 min while p54-S6K2 required 2h for maximal activation [126]. Insulin and nutrient stimulation of HeLa-cells also resulted in a slowly increasing phosphorylation of p70-S6K at Thr389, displaying a lag-phase for the first minutes followed by a steady increase in phosphorylation until 120 min [117].

Insulin signaling in insulin resistant states

Introduction to insulin resistance

The insulin resistant state is characterized by an impaired cellular response to insulin as compared to in the normal, insulin sensitive state. In an insulin resistant state both or either the sensitivity or the responsiveness for insulin is altered. For example, a decrease in insulin sensitivity can be expressed as a right-shift in an insulin dose-response curve leading to a higher half-maximal value (EC_{50}) (e.g. in paper III, Fig. 2, *a2*, type 2 diabetes insulin dose-response curve for IRS1-Yp). While the decrease in insulin responsiveness can be expressed as a reduced insulin stimulated steady-state phosphorylation/activity of the signal mediating proteins (e.g. paper III, Fig. 2, *d2*, type 2 diabetes insulin time-course curve for IRS1-S307p).

There are a number of different methods to measure insulin sensitivity and resistance in humans [129]. These include the golden standard *in vivo*, the hyperinsulinemic euglycemic glucose clamp and the indirect, in clinical use Oral Glucose Tolerance Test (OGTT). Insulin resistance can also be measured with simple indices such as the homeostasis model assessment (HOMA) index and the quantitative insulin sensitivity check index (QUICKI). The HOMA-index correlates insulin resistance with fasting concentrations of insulin and glucose in the blood, where a higher value indicates insulin resistance [130]. As a measure of insulin sensitivity the QUICKI-index can be used [131]. HOMA is calculated according to Equation 1, and QUICKI according to Equation 2, where insulin is the fasting insulin concentration (mU/l) and glucose is the fasting glucose concentration (mmol/l).

$$HOMA = ([insulin] * [glucose])/22.5 \qquad (eq. 1)$$

$$QUICKI = 1/(log[insulin] + log[glucose]) \qquad (eq. 2)$$

Insulin resistance develops gradually in adipocytes and is paralleled by an increased excretion of insulin from β-cells in the pancreatic islets of Langerhans to meet the demands of the adipose tissue, resulting in hyperinsulinemia. Insulin resistance in adipocytes precedes the development of insulin resistance in other insulin responsive tissues such as skeletal muscle and liver. Peripheral insulin resistance increases the demand on the pancreatic β-cells to secrete even more insulin, and at some point the exhausted β-cells fails to secrete the adequate amounts of insulin. Failure of the β-cells may result from β-cell loss in combination with altered function, possibly mediated by the deposition of islet amyloid, reviewed in ref. [132]. The lack of insulin needed to maintain glucose homeostasis result in hyperglycaemia, and overt type 2 diabetes can be diagnosed.

Type 2 diabetes and the metabolic syndrome

WHO estimates that across the globe 364 million people have diabetes, whereas the numbers of type 2 diabetes constitutes the great majority. It has been called a worldwide "tsunami of diabetes" [2]. The number of people with type 2 diabetes will continue to rise, and the disease is estimated to affect around half a billion adults worldwide in 2030 [1]. In Sweden 386 000 adults were estimated to have type 2 diabetes in 2011 [1]. This figure is expected to rise the coming years and reach around 6% of the Swedish population in 2030 [1,133]. Global numbers are expected to increase mostly in low and middle-income countries in the age group of 40-60 years. High-income countries will in comparison experience a lower increase in new cases of type 2 diabetes [1].

According to WHO, type 2 diabetes can be diagnosed after two fasting samples of blood glucose of ≥7.00 mmol/l, or if blood glucose ≥11 mmol/l after a OGTT (plasma glucose samples 2-hours after a glucose load of 75 g, in adults), or if a value of HbA_{1C} ≥6.5 %.

Insulin resistance is one important cornerstone in a cluster of metabolic abnormalities or risk factors referred to as the metabolic syndrome [134]. Other risk factors included in the metabolic syndrome are obesity, dyslipidemia and hypertension [134]. An individual with the metabolic syndrome has an increased risk of developing type 2 diabetes and cardiovascular disease [134].

Genetics and epigenetics

Type 2 diabetes may be a polygenic disorder comprising defects at the level of insulin sensitivity and insulin secretion that combined with the environment influence the development of the disease. At present, the disease in around 90 % of subjects with type 2 diabetes cannot be explained by genetic variants, although approximately 40 genes have been found that are associated with type 2 diabetes [135]. So far, most of the genetic variants found have been linked to β-cell function and a few to insulin sensitivity including the *IRS1* gene encoding IRS1 and *PPARG* encoding peroxisome proliferator-activated receptor γ (PPARγ) [135].

Epigenetics involve regulation of gene expression through DNA methylation, histone modifications and nuclear RNAi, reviewed in ref. [136]. These changes are reversible in their nature and can be inherited, but do not involve changes in the nucleotide sequence. A number of studies have shown that the intrauterine environment affects insulin secretion and insulin resistance. For example, both the development of overt type 2 diabetes and age of onset have been shown to be affected by the environment in the uterus, where both undernutrition as well as overnutrition of the foetus is associated with an increased risk of developing type 2 diabetes, reviewed in ref. [137].

The mechanisms of epigenetic involvement in the transmission of a propensity for obesity, insulin resistance and type 2 diabetes, as well as their importance constitute an important area for present research.

Insulin signaling in subjects with type 2 diabetes

The insulin resistant state found in type 2 diabetes is evident as an altered activity of most signaling intermediates in the insulin signaling network in adipocytes.

In paper III we analyzed insulin signaling in isolated adipocytes from subjects with type 2 diabetes and compared with control subjects. Most intermediates in the signaling pathway displayed a reduced insulin sensitivity of protein phosphorylation in the diabetic state. We could not detect any difference in response time to insulin in the time-course dynamics of insulin induced protein phosphorylation. However, there was a consistent and explicit decrease in steady-state levels of protein phosphorylation with only one exception, the phosphorylation of PKB at Ser473.

In paper III we found that the insulin sensitivity of phosphorylation of IR at tyrosine is unaffected in type 2 diabetes (also in [138]) while the amount of IR protein is decreased to 55% of controls (paper II). Reduced levels of IR were also found in adipose tissue from obese subjects and in mice models [139], although another report found unaltered levels of IR in adipocytes from subjects with type 2 diabetes [88].

Steady-state levels of tyrosine phosphorylation of both IR and IRS1 in response to insulin were reduced in the diabetic state, and insulin sensitivity for tyrosine phosphorylation of IRS1 was reduced (paper III)[109,138].

Previously we have found the amount of IRS1 to be unaltered in adipocytes from subjects with type 2 diabetes (paper II) [109]. However, this finding is in contrast to findings by others e.g. [88,140] where a decrease in IRS1 amount by on average 70% was reported. Also, in subgroups of massively obese and healthy individuals with a genetic predisposition to type 2 diabetes a reduction of both protein and gene expression of IRS1 was found [141,142].

The qualitative behavior of insulin stimulated phosphorylation of IR and IRS1 was examined in paper III using a minimal model of the IR-IRS1 subsystem [87]. With this minimal modelling approach, a reduction in the amount of IR protein could explain the reduced steady-state level of IRS1 phosphorylation, but not the shift in insulin sensitivity for tyrosine phosphorylation of IRS1. Further, a reduced amount of IRS1 (to 50%) could not explain the attenuated insulin sensitivity for tyrosine phosphorylation of IRS1 or the reduced insulin stimulated steady-state

phosphorylation of IR in type 2 diabetes. However, introducing a positive or negative feedback to IRS1 could mimic the reduced insulin sensitivity for phosphorylation of IRS1 in the diabetic state.

Further, in paper III, a detailed mathematical model of the insulin signaling pathway to control of glucose uptake and protein synthesis was formulated for the non-diabetic state that could describe the phosphorylation behavior of proteins in the signaling pathway. To mimic the type 2 diabetic state three diabetes parameters were introduced into the model. These parameters were (i) decreased levels of IR, (ii) decreased levels of GLUT4 and (iii) an mTORC1-mediated feedback to IRS1.

Decreased levels of GLUT4 were introduced in the model as levels of GLUT4 have been found to be reduced in adipocytes in insulin resistant states and type 2 diabetes [140,141,143,144]. Through introduction of the three diabetes parameters, the model could relatively faithfully reproduce the experimental data from the diabetic state. The modeling approach allowed evaluation of the effects on the signaling pathway of the individual diabetes parameters. Neither reduced amount of IR nor of GLUT4 could reproduce insulin signaling in the diabetic state. However, a reduced mTORC1-dependant positive feedback to IRS1 had a major impact on the insulin signaling pathway and affected every level of the signaling pathway. These findings stress the crucial role of a reduced mTORC1 activity for insulin resistance in the diabetic state.

A number of findings indicate a reduced mTORC1 activity in human subjects with type 2 diabetes. These include the decreased insulin stimulated phosphorylation of p70-S6K (paper III)[145], S6 (paper III) and IRS1 at Ser307 (paper III)[109,145], and an up-regulated autophagy and impaired mitochondrial function in type 2 diabetes [146]. Interestingly, decreased mTORC1 activity could also lead to reduced GLUT4 transcription via reduced activation of the SREBP1 [43,44,147] and reduced levels of IR via increased autophagy [139,146].

Obesity and insulin resistance

A person is defined by WHO as being obese if the Body Mass Index (BMI) is higher or equal to 30 (kg/m^2) (Table 1).

Table 1. Classification of adults according to BMI.

BMI (kg/m^2)	**<18.50**	**18.50-24.99**	**25-29.99**	**≥30**
Classification	Underweight	Normal range	Overweight	Obese

The adipose tissue can expand with weight gain either by increasing the size of the adipocytes (*hypertrophic* obesity) and/or through increased number of adipocytes (*hyperplastic* obesity) (Fig. 6).

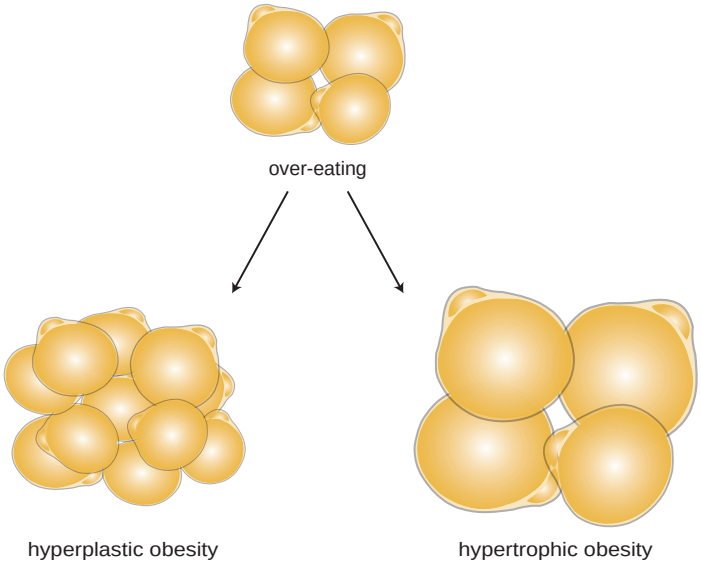

Figure 6. Hyperplasia vs hypertrophy.

Hyperplastic obesity is characterized by an increased numbers of adipocytes. Hypertrophic obesity is characterized as an increased size of the adipocytes, and is associated with insulin resistance and a risk for the development of type 2 diabetes.

Hypertrophic obesity

Several studies have shown a correlation between the size of adipose cells and insulin resistance. For example, size of the adipocytes have been found to be inversely correlated to systemic insulin sensitivity, GLUT4 gene expression, secretion of adiponectin and serum RBP4 [148]. Increased size of large adipocytes was associated with insulin resistance in moderately obese/overweight individuals and in first-degree relatives of subjects with type 2 diabetes [149,150] and large adipocytes have been shown to be an independent risk factor for type 2 diabetes [151]. However, Mundi et al. [152] showed that the size of the subcutaneous adipocytes is not as useful as the total amount of subcutaneous and visceral adipose tissue in the prediction of fasting levels of insulin, glucose and triacylglycerol in a cohort of healthy and slightly overweight females and males.

A few studies have analyzed small and large subcutaneous adipocytes from the same human subject. Large adipocytes display an elevated lipolytic capacity compared to smaller [153]. Larger adipocytes also secrete higher amounts of pro-inflammatory (e.g. leptin, IL-6, TNF-α and MCP-1) than anti-inflammatory adipokines (IL-10) when compared to smaller adipocytes [4,154]. Neither protein expression nor insulin-stimulated activation of IR, IRS1 and Akt1 were found to differ between smaller and larger human adipocytes, while GLUT4 translocation to the plasma membrane was unresponsive to insulin in the larger adipocytes [3].

Hyperplastic obesity

It has been shown that the number of adipocytes is increasing during childhood and adolescence to be held at a relatively constant number during adulthood [155]. Obese individuals have more adipocytes than lean individuals [155,156]. In adults the adipocytes that have undergone necrosis or apoptosis are suggested to be replaced by the differentiation of preadipocytes to mature adipocytes [157].

However, the turnover of adipocytes in subjects classified as having a hypertrophic adipose mass was shown to be slower than in subject with hyperplastic adipose mass [158]. Another report showed that increased BMI and mean adipose cell size correlated to a reduced capability of preadipocytes to differentiate towards mature adipocytes [159]. Also, in moderately overweight/obese subjects, insulin resistant individuals had

a larger pool of small cells than insulin sensitive individuals [149]. The larger pool found in the insulin resistant state was suggested to reflect the decreased capability of the small cells to differentiate into mature adipocytes [149]. Moreover, adipose-derived stem cells isolated from subcutaneous adipose tissue from morbidly obese subjects displayed a reduced differentiation potential compared to stem cells from normal weight subjects [160].

Benefits of an unlimited hyperplastic expanded adipose mass were shown in a mice model where adiponectin was overexpressed in the leptin deficient mouse model of ob/ob mice [161]. These mice developed an extreme hyperplastic obesity of the subcutaneous adipose tissue. The hyperplastic obesity was accompanied by significantly enhanced insulin sensitivity alongside decreased lipid content of the liver, normalized pancreatic islet morphology and decreased macrophage infiltration of the adipose tissue [161].

Interestingly, the drug pioglitazone (a glitazone used in treatment of type 2 diabetes) increases insulin sensitivity, at least in part, through differentiation of preadipocytes. Pioglitazone activates PPARγ and promotes adipogenesis, as the number of small adipocytes increase after treatment with pioglitazone of rat and human adipocytes [162,163].

Obesity and systemic insulin resistance

There is a link between obesity and systemic insulin resistance although not all obese individuals are insulin resistant [5,164]. In fact, sub-groups of obese individuals display the same insulin sensitivity as normal-weight individuals [164]. However, around 70-90% of subjects with type 2 diabetes are obese or overweight [165] and BMI can predict the development of type 2 diabetes [166].

There are different adipocentric hypotheses trying to explain the relationship between obesity and systemic insulin resistance and type 2 diabetes. These include ectopic fat deposition due to limitation in adipose mass expandability, the low-grade inflammatory state and the adipokine hypotheses, reviewed in ref. [167].

Adipose mass expansion and ectopic fat deposition

It has been hypothesized that with obesity and increased size of the adipocytes the adipose mass will eventually become unable to expand much further through the storage of excess lipids, which will result in accumulation of lipids ectopically and as a consequence give rise to insulin resistance [167-169].

Adipocytes that under normal conditions have a mean size of about 100 μm in diameter can as the individual gain weight increase significantly in size [5]. Potentially, mechanisms that limit cell size can be essential to avoid adipocytes from bursting and causing necrosis that in turn may lead to massive inflammation [170]. Indeed, in adipose tissue from obese individuals a marked increase in necrotic adipocytes were found and these were surrounded by pro-inflammatory macrophages [171]. In paper III we propose that mTORC1-signaling senses an enlarged cell size and inhibits further growth of the adipocytes. Limitations in size of the mature adipocytes combined with low differentiation of preadipocytes imply that excess fatty acids need to be disposed elsewhere than in the adipose tissue. Thus causing increased fatty acid deposition ectopically, in muscle, liver and pancreas, which may induce systemic insulin resistance.

In support of this hypothesis, a recent paper [172] showed that adipose tissue in obese subjects display lowered fatty acid uptake from chylomicrons after a meal compared with lean subjects and also a decreased triacylglycerol storage capacity. However, the release of non-esterified fatty acids from the subcutaneous adipose tissue was in the same range in obese and lean subjects [172]. In the same subjects, there were indications of enhanced triacylglycerol storage in the liver [172].

Inflammation and insulin resistance

Insulin resistant adipose tissue is associated with a chronic low-grade inflammation. In adipose tissue from obese humans and mice, macrophage infiltration is elevated and also the secretion of inflammatory mediators such as TNF-α, IL-6 and IL-1β [170,171]. TNF-α has been suggested to effect insulin signaling through stress mediators such as JNK and IKK, where JNK may phosphorylate IRS1 on inhibitory serine sites thus inhibiting phosphorylation of IRS1 at tyrosine and insulin signaling [38,104].

The role inflammation plays in the development and worsening of insulin resistance is still under intensive investigation. Interestingly, recent papers of overfeeding in

mice and humans support the concept that inflammation is secondary to development of insulin resistance [173-175]. For example, overfeeding of human subjects for one month resulted in significant weight gain and peripheral insulin resistance, but the adipose tissue did not show any increase in macrophage or immune cell infiltration [174]. Further, in another study of overfeeding of human subjects [175], subjects developed insulin resistance while the subcutaneous adipose tissue lacked macrophage infiltration and inflammatory gene expression was unaffected.

Overfeeding and insulin resistance

In paper II we addressed the effects of short-term overfeeding on insulin signaling. Lean volunteers were examined before and at the end of a 4-week period of consuming a high-calorie diet. Subjects remained lean according to BMI, but gained significantly in weight and body fat and developed a moderate systemic insulin resistance at the end of the study. Subcutaneous adipocytes were isolated from the study subjects before and at the end of study and insulin signaling was analyzed.

A number of similarities between insulin signaling in adipocytes from subjects with type 2 diabetes and from the study subjects on high-calorie diet were found. In both groups the amount of IR was decreased, in the diet-group to 57% of baseline and in subjects with type 2 diabetes to 55% compared to controls. The reduced amount of IR in the insulin resistant states confirms previous findings in adipocytes from obese insulin resistant subjects and obese subjects with type 2 diabetes that display reduced binding of insulin compared to controls, indicative of a reduced IR number [176,177]. A reduced amount of IR was also found in visceral adipose tissue from obese subjects [139]. FOXO1 binds to the IR promotor during fasting conditions resulting in increased amounts of IR [50]. Thus the feeding conditions with elevated fasting levels of insulin compared to baseline, experienced by the high-calorie diet-group could result in decreased amount of IR due to reduced activation of IR transcription by FOXO1. Reduced FOXO1 mediated transcription of IR could also serve as an explanation for the reduction in amount of IR found in type 2 diabetes. In the diabetic mouse model *db/db* mice the nuclear amount of FOXO1 in white adipose tissue is 50% lower compared to control mice [178].

Insulin sensitivity for phosphorylation of IRS1 at tyrosine was impaired in both groups and also the maximal effect of insulin in the diet-group at the end of the study (shown in paper III also for type 2 diabetes, Fig.2, *a2* and *b2*). Phosphorylation of IRS1 at Ser307 was impaired at the end of the study in the diet group, which corresponds to findings in type 2 diabetes subjects (paper III)[109]. The amount of IRS1 was not affected by the diet or in type 2 diabetes compared with controls. Insulin-stimulated phosphorylation of IRS1 at Ser312 was unaffected by the diet, which has also been found previously in subjects with type 2 diabetes [145].

No change was observed in the insulin sensitivity for phosphorylation of PKB at Thr308 neither at the end of diet nor in type 2 diabetes. Surprisingly, diet increased insulin sensitivity and responsiveness for phosphorylation of MAP-kinases ERK1/2. Increased activity of ERK1/2 might be explained by the expanding adipose mass due to the ongoing weight-gain of the subjects. On the other hand, in adipocytes from subjects with type 2 diabetes basal levels of ERK1/2 have been found to be elevated two-fold compared to controls, while insulin sensitivity for phosphorylation of ERK1/2 was reduced [140,145].

Due to leanness of the study subjects we could obtain enough adipose tissue from only 6 subjects out of 18 subjects originally participating in the overfeeding study. But the whole cohort of 18 subjects was studied with respect to effects on the liver [179]. After diet the subjects had increased amounts of hepatic triglycerides as well as elevated and pathological levels of serum alanine aminotransferase (ALT) [179]. Levels of ALT are coupled to non-alcoholic fatty liver disease and insulin resistance [179].

Gene expression in subcutaneous adipocytes from the overfeeding subjects was compared to gene expression in subcutaneous adipose tissue from subjects that were re-feeded after a period of caloric restriction [180]. In both groups, caloric intake up-regulated 52 genes, including genes for enzymes involved in *de novo* fatty acid synthesis, while 50 genes were down-regulated, including genes encoding 4E-BP1/2 and ribosomal proteins [180].

Others have also analyzed gene expression profiles in adipocytes from lean individuals subjected to overfeeding [175,181]. Alligier et al. [175] found that moderate overfeeding resulted in up-regulation of genes involved in lipid metabolism and

storage, as well as increased vascularization of the adipose tissue and remodeling of the extracellular matrix.

In a number of studies, the effects of overfeeding human subjects have been analyzed with respect to systemic insulin sensitivity, changes in adipose cell size and number, and secretion of adipokines [174,175,182-190].

Moderate weight gain after overfeeding a normal or high-fat diet for one month [174] or two months [175] did not affect mean adipocyte cell size. However, overfeeding for two months [189], as well as massive overfeeding during several months, was reported to increase the mean subcutaneous adipocyte cell size [183]. Numbers of subcutaneous adipocytes was found to be unaltered by moderate and massive overfeeding [175,183]. Tchoukalova [189] also reported unaltered numbers of subcutaneous adipocytes after overfeeding, while the number of lower-body (femoral) adipocytes was actually increased.

Overfeeding on a high-fat diet for only 5 days induced hepatic insulin resistance and hyperinsulinemia while glucose disposal was unaffected [187]. Tam et al. [174] showed that moderate weight gain after a month on a high-fat diet also resulted in systemic insulin resistance. In a study by Erdmann et al. [186] overfeeding for four and a half months also left glucose disposal unaffected while hyperinsulinemia and systemic insulin resistance developed. These studies show that overfeeding, even for short periods, induce insulin resistance and compensatory mechanisms to maintain glucose homeostasis.

Levels of leptin have been found to be elevated following short-term overfeeding [184,187,190], while reports vary concerning the levels of adiponectin [187,190].

Wnt-signaling was found to be inhibited after moderate overfeeding for two months [175]. Wnt-signaling controls commitment of mesenchymal stem cells to the adipose lineage and also inhibits differentiation of preadipocytes to mature adipocytes [191]. Likely, the ongoing weight gain of the overfeeding subjects results in enhanced maturation of preadipocytes to mature adipocytes. However, already acquired hypertrophic obesity correlates with the opposite - increased Wnt-signaling [191].

In summary, overfeeding can induce both systemic insulin resistance and affect insulin signaling, mimicking the situation in isolated adipocytes from subjects with type 2 diabetes (paper II). Relatively short-term overfeeding periods was in some studies found both to increase adipocyte mean size [183,189] and adipocyte number in a depot specific way [189]. Also, shorter periods of overfeeding can be sufficient to alter secretion of adipokines [184,187] and to affect Wnt-signaling [175].

Comparatively few studies are available on over-eating by human subjects and the experimental protocols vary. Variation are found in the length of the overfeeding periods, the amount of excessive calorie intake and the composition of the diet in combination with unchanged or restricted exercise protocols. This makes it complicated to interpret the experimental data, and to what degree a specific variable contributes to an observed effect is difficult to discern. Nonetheless, even very short periods of overfeeding can trigger adaptive mechanisms of insulin resistance and insulin secretion [187,188]. For obvious reasons, fewer studies have related overfeeding to insulin signaling and insulin resistance compared to the large number of studies that have analyzed weight reduction. However, a wealth of information can be obtained from overfeeding studies, including the mechanisms involved and the role inflammation plays in the development of weight gain, obesity and insulin resistance and possibly the link to genetic and epigenetic predisposition.

Mathematical modeling of biological systems

Introduction to systems biology

In order to take advantage and make full use of the increased amount of detailed and dynamic experimental data on signaling networks there is a need for systematic approaches to analyze the data. One approach to decipher biological data on dynamic systems is to use mathematical models and computational methods, i.e. "systems biology". Systems biology is a rather new and rapidly growing field [192]. With the use of mathematical modeling, a whole system can be taken into account instead of analyzing merely individual events, and thus non-trivial conclusions can be drawn about complex biological processes. Exemplified by the binding of insulin to the IR that have been studied using mathematical modeling [30], insulin signaling normally and in diabetes (paper III) and in prediction of drug targets discussed in [193].

Formulation of a mathematical model

Most of the mathematical models published in biology are based on ordinary differential equations (ODEs) [192]. ODEs are useful to describe the dynamics of biological systems as well as other systems. To be able to describe the dynamics of a system, we need quantitative experimental data that contain detailed kinetic information, such as time-resolved data. In addition to ODEs there are a number of other modeling methods, including Boolean and Bayesian modeling [193].

In the process of creating a mechanistic mathematical model that describes a biological system we use experimental data and prior knowledge of the system and combine this knowledge into formulation of hypotheses (i.e. model structures) to test as possible explanations to the experimental data.

In paper III, a mechanistic mathematical model was used to describe insulin signaling from the IR to control of glucose uptake and protein synthesis. ODEs were used to describe the protein interactions in the insulin signaling network as the experimental data consisted of detailed dynamic, as well as steady-state, experimental data.

The mathematical formulation of a model using ODEs can be illustrated with the simple reaction of insulin binding to its receptor (Fig. 7).

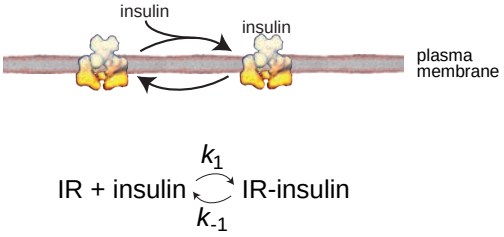

Figure 7. Formulation of a mathematical model from a mechanistic hypothesis.
Illustration of a hypothesis, insulin binds to the insulin receptor with the rate constant k_1 and forms the complex IR-insulin. The concentration of IR-insulin is reduced by the reverse reaction with the rate constant k_{-1}.

In Equation 3 the hypothesis outlined in Figure 7, is described with an ODE.

$$\frac{d([IR \cdot insulin])}{dt} = k_1[IR][insulin] - k_{-1}[IR \cdot insulin] \qquad (eq. 3)$$

Equation 3 describes the time-dependent change of the concentration of the insulin bound state of the receptor (IR-insulin) assuming mass-action kinetics, where the parameters (kinetic rate constants) are k_1 and k_{-1}. In paper III, mass action-kinetics was assumed to describe the kinetics of a reaction, unless the explanation of data required Michaelis-Menten or Hill expressions in which case these were used.

When a model has been constructed with a set of differential equations that describe every reaction in the system, initial conditions of the different states need to be given as well as parameter values. Parameter values are usually unknown, or if they are known, have an intrinsic uncertainty due to the experimental conditions and methods used to obtain them [194]. In paper III parameter values were treated as

unknown but limited within a range of widely but realistically set boundaries. To find the best parameter values, optimization was made in MATLAB [195] through extensive fitting to experimental data.

Optimization of the model also involves modification of the model structure, e.g. adding more states or changing the kinetic expressions, for simulations of the model to follow the behavior of the experimental data.

Detailed mechanistic models, of the kind presented in paper III for the insulin signaling network, are descriptive and therefore non-conclusive [196]. A conclusive model on the other hand contains a minimal number of states, and thus is referred to as a minimal model. One example of a minimal model is the IR-IRS1 subsystem analyzed in paper III, which was adapted from [87]. A minimal model can be rejected through the use of core predictions - and in this way strong conclusions can be drawn about the model system [194]. A core prediction is a feature found for all acceptable parameter combinations in a given model [87]. For example, in a study of IR and IRS1 dynamics in rat adipocytes different mechanistic model structures were tested [197]. For one of the model structures the core prediction required that less than 10% of IR should remain in the plasma membrane after 10 min of insulin stimulation. However, experimental data showed that less than 10 % was internalized upon insulin stimulation and the model could thus be rejected based on the core prediction.

Material and methods

Detailed experimental protocols can be found in each paper, and in the following section only selected aspects of the material and methods will be discussed.

Model system – rat adipocytes (paper I)
Epididymal adipocytes from Harlan Sprague-Dawley rats were isolated and analyzed. The Harlan Sprague-Dawley rat is one of the most commonly used outbred strains in animal research. Epididymal adipose tissue is a depot of white adipose tissue that is easily accessible in relatively large quantity from male rats.

Model system – human adipocytes (paper II-IV)
In paper II subcutaneous adipose tissue was obtained from male and female study subjects through needle aspiration in the umbilical region. The leanness of the study subjects restricted the amount of adipose tissue that could be aspirated. In paper II-IV subcutaneous adipose tissue slices was obtained from male and female subjects that underwent elective surgery at the University Hospital in Linköping-Norrköping. Subjects were classified into a control group or into a group of patients with type 2 diabetes according to a diagnosis of type 2 diabetes. In paper II, subjects with type 2 diabetes were included if they also fulfilled criteria for the metabolic syndrome. In paper III, subjects with type 2 diabetes were included if they had a BMI > 27 kg/m^2 i.e. were overweight or obese. In paper IV, no patients with T2 diabetes were included and the BMI of subjects ranged from 18-28 kg/m^2.

Isolation and treatment of rat and human adipocytes (paper I-IV)
Adipocytes were isolated after collagenase digestion of tissue pieces. Rat adipocytes were analyzed directly following isolation (paper I). Prolonged incubation of the rat adipocytes was not used as murine adipocytes have been shown to experience increased fatty acid release, decreased glucose uptake and down regulation of a number of important genes in the insulin signaling pathway with increasing time in

culture [138,198]. In human adipocytes on the other hand, our group has previously shown that the surgical procedures used to obtain human adipose tissue induce insulin resistance in the adipocytes, which can be reversed by overnight incubation of the cells [138]. However, the insulin resistant state found in cells from type 2 diabetic subjects is maintained also after overnight incubation [138]. Thus, isolated human adipocytes were incubated overnight before analysis (paper II, III, IV). In paper IV adipocytes were transduced with adenoviral vectors and incubated for 48 h to overexpress the protein before analysis of the adipocytes.

SDS-PAGE and immunoblotting (paper I-IV)

With the use of SDS-polyacrylamide gel electrophoresis proteins were separated, transferred to a PVDF membrane and identified using specific antibodies. Immunoblotting is a semi-quantitative technique that can provide site-specific phosphorylation data relatively easily. For quantification of the immunoblotting data the concentrations of antibodies used were titrated to provide a linear response. In paper I, equal amount of protein from each sample were loaded in each lane on the gel. In paper II-IV, samples were loaded according to constant total cell volume and the readout from each lane was normalized to the respective lane´s content of β-tubulin or actin in order to be able to compensate for variation in sample loading.

Isolation of subcellular fractions (paper I)

Isolation of subcellular fractions using sucrose density gradient centrifugation is a commonly used technique and can provide relatively pure subcellular fractions. In paper I the plasma membrane and endosomal fractions were isolated. Adipocytes were subject to very mild homogenization to ensure the integrity of the endosomal membranes. Through repeated centrifugation steps lipids, cell debris and nuclei could be removed and a plasma membrane containing fraction as well as an intracellular endosomal membrane containing fraction were isolated. The plasma membrane containing fraction was loaded on a 1.12M sucrose cushion and subjected to ultracentrifugation, after which the plasma membrane fraction constituted a milky-white layer on top of the cushion and could be collected. The intracellular endosomal membrane containing fraction was loaded on top of a 0.67M and 1.05M discontinuous sucrose gradient. After ultracentrifugation the interphase between the two sucrose cushions was collected and referred to the as the endosomal fraction. The endosomal fraction was either prepared for immunocapture, transmission electron microscopy or for SDS-PAGE and immunoblotting. The plasma membrane fraction was pelleted and subjected to SDS-PAGE and immunoblotting.

Immunocapture using magnetic beads (paper I)

With the use of antibodies immobilized on magnetic beads it is possible to specifically isolate antigen-containing targets such as membrane vesicles. In paper I we isolated vesicles from the endosomal fraction with the use of magnetic Dynabeads M-450 (Dynal, Oslo, Norway). First, the endosomal fraction was incubated with primary antibodies directed either against the IR (rabbit polyclonal) or Tyr14-phosphorylated caveolin-1 (mouse monoclonal). Second, the secondary anti-rabbit or anti-mouse, respectively, antibodies were added. The immunocaptured samples were then subjected to SDS-PAGE and immunoblotting.

Transmission electron microscopy (paper I)

To study cellular structures with very high resolution we used transmission electron microscopy. In paper I the endosomal fraction was adsorbed to carbon formvar covered nickel grids, prefixed and immuno-labelled. Primary antibodies were detected with gold-labeled secondary antibodies. Following immuno-labelling the specimen were fixed in glutaraldehyde, and fixed and contrast-stained using osmium tetraoxide. Grids were washed, freeze-dried and coated with tungsten and thereafter the endosomal fraction was analyzed with transmission electron microscopy.

Glucose uptake (paper III)

Insulin stimulated glucose uptake was measured in isolated adipocytes. Cells were pre-incubated with insulin after which 2-deoxy-D-[1-^{3}H] glucose was added. Glucose uptake was measured as the accumulated amount of intracellularly 2-deoxy-D-[1-^{3}H] glucose, as the radioactive-labeled glucose derivative is phosphorylated but not further metabolized.

Overexpression of proteins (paper IV)

With the use of adenovirus exogenous DNA can be delivered to non-dividing cell types such as the mature adipocyte. Delivered viral DNA will not integrate in the host genome, thus the expression of recombinant protein will be transient. In paper IV we used adenovirus to express dominant negative S6K1 (T389A) in human primary adipocytes. As controls we used expression of EGFP and untransfected cells to ascertain that overexpression of a protein in general or viral infection *per se* did not cause the observed effects.

Immunoprecipitation, IRS1-Ser307-kinase assay and mass spectrometry (paper IV)
Immunoprecipitation can be used to isolate and concentrate a protein from a complex solution using antibodies directed toward the protein of interest. In paper IV we used immunoprecipitation to isolate and concentrate mTOR and mTOR associated proteins from human primary adipocyte lysates. Lysates were incubated with antibodies toward mTOR followed by binding to Protein G PLUS-Agarose beads. After washing, the immunoprecipitates were prepared either for SDS-PAGE or for IRS1 Ser307-kinase assay. In paper IV, an IRS1 Ser307-peptide (IRS1 amino acids 288-314) was incubated with mTOR-immunoprecipitates. Thereafter, peptides were cleaved by trypsin and prepared for LC-MS/MS. Separation of the peptide fragments was done in a HPLC system followed by tandem MS analyses. With this method, phosphorylation of the IRS1 Ser307-peptide could be identified and quantified.

Summary of the papers

The section below gives a short summary of each paper included in this thesis.

Paper I

The aim of this study was to examine the internalization dynamics of the insulin activated IR and to determine by which route the receptor internalizes in primary rat adipocytes.

Within minutes of insulin stimulation of primary rat adipocytes IR in the plasma membrane were auto-phosphorylated, and an increased number of phosphorylated receptors appeared in an endosomal fraction. A maximum of 10% of the total number of IR in the plasma membrane was found in the internalized compartment. Within the same time frame, insulin stimulation mediated tyrosine phosphorylation of caveolin-1 at Tyr14 in the plasma membrane, and an increased amount of phosphorylated caveolin-1 was detected in the endosomal fraction. After immunocapture of endosomal vesicles with antibodies against phosphorylated caveolin-1 at Tyr14 we found the IR, and after immunocapture with antibodies against the IR we found caveolin-1 in the same vesicles. Also, using transmission electron microscopy and immunogold labeling of the endosomal fraction the IR and caveolin-1 were found to be co-localized to the same endosomal vesicles. Incubation of the adipocytes with an inhibitor of clathrin coated pit endocytosis inhibited the internalization of the transferrin receptor while leaving the insulin induced internalization of the IR unaffected both at high and low concentrations of insulin.

In conclusion, after insulin stimulation of primary adipocytes the IR rapidly internalize primarily by way of caveolae.

Paper II

The aim of this study was to investigate insulin signaling in subcutaneous mature adipocytes from lean subjects on a high-calorie diet and compare with findings from subcutaneous mature adipocytes from patients with type 2 diabetes.

Lean human volunteers were put on a high-calorie diet for 4 weeks. Subjects nearly tripled their total energy intake per day. Physical activity was restricted to 5000 steps a day. On average the subjects remained lean according to their BMI but substantially increased their body fat and developed a mild systemic insulin resistance. Insulin signaling was studied in isolated subcutaneous adipocytes from the study participants before and after 4 weeks on the diet. Subcutaneous adipocytes were also obtained from subjects with type 2 diabetes and the metabolic syndrome, which were compared with adipocytes from a control group of subjects without type 2 diabetes. The amount of IR protein was found to be significantly reduced both in study subjects and subjects with type 2 diabetes, while IRS1 amount was unaffected. A decrease in insulin sensitivity of adipocytes from the group on diet was demonstrated at the level of IRS1 as insulin sensitivity for phosphorylation of IRS1 at tyrosine was decreased and the maximal insulin-induced phosphorylation both at tyrosine and at Ser307 was lowered. Insulin sensitivity for phosphorylation of IRS1 at Ser312 and of PKB at Thr308 was not affected in adipocytes from either the group on diet or from subjects with type 2 diabetes. Both insulin sensitivity and insulin responsiveness for phosphorylation of the MAP-kinases ERK1/2 were enhanced in adipocytes from the group on diet.

In conclusion, lean healthy subjects that dramatically over-eat for a short period of time develop a small systemic insulin resistance. Moreover, insulin signaling in adipocytes from subjects on diet displayed insulin resistance with similarities to insulin signaling in adipocytes from subjects with type 2 diabetes.

Paper III

The aim of this study was to deepen the understanding of insulin signaling in type 2 diabetes through the construction of a first comprehensive and dynamic mathematical model of insulin signaling to control of glucose uptake and protein synthesis, with experimental data gathered in a consistent manner from human adipocytes.

Insulin signaling was analyzed in human mature adipocytes from patients with or without type 2 diabetes. The IR/IRS1 activated signaling pathway in control of glucose uptake via PKB/AS160 was analyzed as well as the control of protein synthesis via mTORC1/S6K/S6. Insulin sensitivity as well as time-resolved data for insulin stimulated phosphorylation of key regulatory sites in the respective signaling proteins was obtained and glucose uptake was measured. At all analyzed levels of the pathways the proteins displayed altered signaling in type 2 diabetes compared to non-diabetic controls, either as reduced insulin sensitivity and/or as decreased steady-state phosphorylation. A systems biology approach was used to analyze the experimental data. First, analysis of a minimal model of the IR-IRS1 subsystem showed that introducing a downstream feedback to IRS1 was necessary to be able to describe the reduced insulin sensitivity of phosphorylation of IRS1 at tyrosine residues that was found in the diabetic state. Second, a comprehensive mathematical model was formulated using ODEs that could well describe the dynamic and steady-state behavior in the experimental data of the insulin signaling network. We found that decreasing the amount of IR and GLUT4 protein to 55% and 50% respectively as well as decreasing a positive feedback from mTOR to IRS1 to 15%, resulted in a model that could reproduce insulin signaling in the diabetic state. Importantly, the positive feedback from mTORC1 to phosphorylation of IRS1 at Ser307 was shown to be crucial for normal insulin signaling. These findings suggest that mTOR senses and limits adipocyte expansion through the regulation of IRS1 activity, thus introducing an insulin resistant state in the adipocyte as a protective mechanism to avoid uncontrolled adipocyte growth.

In conclusion, a mathematical model was developed using a large experimental data set of insulin signaling in mature adipocytes obtained from patients with or without type 2 diabetes. Analysis of the model revealed that the insulin resistant state found in adipocytes from subjects with type 2 diabetes could largely be reproduced through decreased mTOR activity towards IRS1.

Paper IV

The aim of this work was to investigate the protein kinase that catalyzes the insulin-stimulated and mTORC1-mediated phosphorylation of IRS1 at Ser307.

S6K1 has previously been found to phosphorylate IRS1 at Ser307 *in vitro*. In this work we analyzed phosphorylation of IRS1 at Ser307 in response to insulin in primary human adipocytes. Time course dynamics for phosphorylation of IRS1 at Ser307 displayed very different characteristics compared to the phosphorylation of S6K1 and its *bona fide* substrate S6. Overexpression of a dominant negative form of S6K1 as well as inhibition of S6K1 with PF-4708671 inhibited S6 phosphorylation while leaving phosphorylation of IRS1 at Ser307 unaffected. An mTOR immunoprecipitate was isolated from whole cell lysate and found to phosphorylate S307 uniquely in a peptide corresponding to amino acid residues 288-314 of human IRS1. Phosphorylation of the peptide by the mTOR immunoprecipitate was not primarily due to the activity of mTOR, S6K1 or PKB as shown with selective inhibitors.

In conclusion, S6K1 is not the protein kinase that phosphorylates IRS1 at Ser307 in primary human adipocytes in response to insulin and the identity of the physiological kinase remains to establish.

Future perspectives

To gain a deeper understanding of insulin signaling in adipocytes a number of aspects addressed by the papers in this thesis call for future investigation.

With regard to endocytosis of the IR details of the regulation of internalization of the IR is lacking. Characterization of caveolin-1 containing endosomes both structurally and functionally is needed. Also, to establish the importance of localization and temporal regulation of IR and downstream proteins in the insulin signaling pathway remains. That internalization of receptors is an important mechanism and part of signaling regulation is a concept that is generally accepted but, at least for the IR, much remains to be examined in order to understand its role in detail.

In the context of the relation between weight gain, insulin resistance and type 2 diabetes it would be interesting and potentially very informative to study short- and long-term effects of overfeeding on insulin responsive tissues from a larger group of human subjects.

Details of how mTOR senses and limits adipocyte size remains to be investigated in detail. As discussed in paper III the aim to restore insulin sensitivity in the already enlarged adipocytes may actually lead to necrosis and prevent the intended alleviation of insulin resistance. As suggested in paper III a productive way to alleviate insulin resistance may be to recruit new, “empty” insulin sensitive adipocytes in the adipose tissue. Thus understanding the molecular mechanisms behind the differentiation of preadipocytes and commitment of mesenchymal stem cells to the adipose lineage in general and specifically in insulin resistant states [159,191] is of great importance.

It remains to establish the physiological kinase responsible for the insulin induced phosphorylation of IRS1 at Ser307. In the light of the vital role this serine-site seems to play in fine-tuning the tyrosine phosphorylation of IRS1 and thus downstream insulin signaling this remains an important task. It will be of interest to identify the protein kinase in the mTOR-immunoprecipitate obtained in paper IV. Also, not much is known about the interplay and sequential activation and deactivation of the large number of potential phosphorylation sites on IRS1.

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