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Lipid Metabolism and Insulin Signalling in Adipocytes

- enhanced autophagy in type 2 diabetes

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Cover: A transmission electron microscopy photograph showing a typical autophagosome in an adipocyte from a type 2 diabetic patient. Autophagosomes are formed in response to starvation and participate in digestion of intracellular components to release energy thus promoting cell survival during starvation in a process called autophagy. Autophagy is derived from Greek roots: *auto*, meaning “self”, and *phagy*, “to eat”.

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To my beloved family

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Abstract

Energy storage in the adipose tissue, to an extent leading to obesity, is associated with local as well as systemic insulin resistance. When insulin-producing beta-cells in the pancreas gradually fail to compensate, plasma levels of glucose rise and overt type 2 diabetes is diagnosed. Adipocytes are large cells, mostly consisting of one big central lipid droplet, with the surrounding plasma membrane full of small invaginations called caveolae. As caveolae contain the insulin receptor and several other insulin-signalling proteins, we have investigated several aspects of caveolae. We have also mapped mechanisms and defects in the insulin-signalling network in adipocytes from type 2 diabetic patients.

In paper I, we show that a subtype of caveolae has the capability to synthesize triglycerides from fatty acids and glycerol-3-phosphate. The triglyceride-synthesizing caveolae subtype also contains perilipin, suggesting the existence of a mechanism to protect newly made triglycerides from hydrolysis.

In paper II, we demonstrate that adipocytes from patients with type 2 diabetes have an attenuated insulin-stimulated phosphorylation of IRS-1 at Ser-307 (human sequence), which correlates with reduced insulin-stimulated phosphorylation of IRS-1 at tyrosine residues. Insulin-stimulated phosphorylation of IRS-1 at Ser-307 is dependent on the nutrient sensor TORC1. This finding indicates that adipocytes from type 2 diabetic patients have reduced TORC1 activity.

In paper III, we focus on the mechanisms for RBP4-induced insulin resistance. We also continue our mapping of insulin-resistance in adipocytes from type 2 diabetes. These cells exhibit, in addition to impaired insulin-stimulated glucose uptake and the defects presented in paper I, impaired insulin-stimulated phosphorylation of ERK. We do, however, not see any defects in PKB signalling. Neither do we see any enhanced insulin-stimulated phosphorylation of IRS-1 at Ser-312 (human sequence), a site that in mice is hyper-stimulated in response to high-fat feeding. Incubation with RBP4 recapitulates all defects we so far have seen in type 2 diabetes except reduced insulin-stimulated glucose uptake. These results are mirrored by blockade of endogenously produced RBP4 in the incubations with adipocytes from type 2 diabetic patients. In other words, RBP4-blocking antibodies restore all insulin-signalling defects we have found in adipocytes from type 2 diabetic patients, except insulin-stimulated glucose uptake.

In paper IV we show by several approaches that TORC1 activation is down-regulated in adipocytes from type 2 diabetic patients. The main finding is that there is enhanced autophagy in those adipocytes. Interestingly, autophagy may be a mechanism to enhance the breakdown of stored triglycerides in the adipocyte.

In conclusion, our data suggest that caveolae, in addition to being micro-domains for insulin-signalling are metabolic platforms. We describe defects in insulin-signalling in adipocytes from type 2 diabetic patients where the main finding is enhanced autophagy in these obese patients. The perceived starvation in adipose tissue might via secretion of adipokines, such as RBP4, have implications for local as well as systemic insulin-resistance.

Populärvetenskaplig sammanfattning

För första gången i världshistorien finns det fler överviktiga personer än undernärda. Enligt WHO är ungefär 1 miljard människor runt om i världen undernärda. Samtidigt finns nu ungefär 1,6 miljarder feta eller överviktiga personer. Till 2015 uppskattas denna siffra öka till 2,3 miljarder. Fetma ökar risken för både typ 2 diabetes och hjärt-kärlsjukdomar och som en följd av den snabbt ökande förekomsten av fetma ökar dessa sjukdomar på ett epidemiskt sätt.

Fetma är en direkt konsekvens av ett högt energiintag där det mesta av all extra energi lagras som olja i våra fettceller. Vid fetma har fettcellerna lagrat in så mycket olja att insulinresistens uppstår i cellerna, vilket ger insulinresistens både lokalt i fettvävnaden och i hela kroppen. Insulinresistens innebär att fettvävnad, muskler och lever inte reagerar lika bra på insulin som de vanligtvis gör. Muskler och fettväv behöver mer insulin för att ta upp socker ur blodet vid insulinresistens jämfört med ett normalt insulinkänsligt tillstånd. Insulinresistensen kompenseras i ett inledningsskede genom att betacellerna i bukspottkörteln producerar mer insulin. Insulinresistensen kan därför fortgå och utvecklas obemärkt under många år, och det är först när betacellerna slutar fungera som blodsockernivåerna stiger och typ 2 diabetes diagnostiseras. Insulinresistens och betacellernas bristande förmåga att under en lång tid kompensera för insulinresistens är därmed de bakomliggande orsakerna till typ 2 diabetes.

Min forskning syftar till att försöka förstå på vilket sätt fettceller från patienter med typ 2 diabetes är insulinresistenta och hur de har blivit det. Alla defekter som jag har hittat kan härledas till en minskad aktivitet hos proteinkomplexet TORC1. Detta komplex fungerar som cellens näringssensor och fungerar likadant hos såväl bakterier, bananflugor som människor. TORC1 aktiveras av en hög näringsnivå och inaktiveras av svält. När en cell svälter så bryter den ned sitt eget cellinnehåll för att försöka överleva.

Denna process kallas autofagi, ”självätande”, och bromsas av TORC1. I fettceller från diabetespatienter finner vi att TORC1 har en minskad aktivitet. Det leder till en mindre bromsande effekt på autofagi som därmed ökar. Vi finner alltså att fettceller från typ 2 diabetespatienter håller på att ”äta” sitt eget cellinnehåll.

Våra resultat visar att fettcellerna i fettvävnaden hos människor med typ 2 diabetes tror att de svälter fast de är sprängfyllda med olja och därmed har ett jättestort energilager. Detta kan ha betydelse för hela kroppens ämnesomsättning. Fettvävnaden rapporterar nämligen till resten av kroppen hur stort energilager som finns. Vid ett välfyllt lager signalerar fettväven till hjärnan att minska aptiten. Om fettväven signalerar svält så ökar aptiten.

Kan fetma uppfattas som svält av fettcellerna? Eller är det tvärtom, att det börjar med minskad TORC1 i fettcellerna. I en population är det en fördel att ha variationer i genomet för att öka chansen till överlevnad och det har föreslagits att det bör finnas individer med sparsamhetsgener (thrifty genes). En ärftlig minskad aktivitet av TORC1 i fettceller skulle möjligtvis passa i den beskrivningen. Om fettcellerna tror att de svälter kanske de signalerar till hjärnan att öka aptiten, och i vårt nutida samhälle med överflöd på mat leder det till fetma. Detta är emellertid frågor som det ännu inte finns något svar på men som framtida forskning säkert kommer att klargöra.

De här resultaten ställer saker på sin spets inom diabetesforskningen. En stor del av världens forskningsresurser används nu för att studera möss som har ett diabetesliknande tillstånd till följd av en långvarig fettrik diet. Insulinresistensen hos dessa möss beror på att näringssensor-komplexet TORC1 är överaktivt, alltså motsatt till det vi finner hos fettceller från typ 2 diabetiker. Diabetesforskning runt om i världen fokuserar därmed sin forskning på ett tillstånd som inte alls överensstämmer med typ 2 diabetes hos människor.

List of publications

This thesis is based on the following scientific papers;

- I. **Öst A.**, Örtegren U., Gustavsson J., Nyström F.H., and Strålfors P. (2005) *Triacylglycerol is synthesized in a specific subclass of caveolae in primary adipocytes*. J. Biol. Chem. 280:5-8.

- II. Danielsson A., **Öst A.**, Nyström F.H., and Strålfors P. (2005) *Attenuation of insulin-stimulated insulin receptor substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes*. J. Biol. Chem. 280, 34389-34392

- III. **Öst A.**, Danielsson A., Lidén M., Eriksson U., Nyström F.H., and Strålfors P. (2007) *Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes*. FASEB J. 21, 3696-704

- IV. **Öst A.**, Svensson K., Ruishalme I., Franck N., and Strålfors P. (2009) *Attenuated mTOR signaling and enhanced autophagy in adipocytes from obese patients with type 2 diabetes*. Submitted.

Publications not included in this thesis;

Karlsson M., Thorn H., Danielsson A., Stenkula K.G., **Öst A.**, Gustavsson J., Nyström F.H., and Strålfors P. (2004) *Co-localization of insulin receptor and insulin receptor substrate-1 to caveolae in primary human adipocytes. Cholesterol depletion blocks insulin signalling for metabolic and mitogenic control.* Eur J Biochem 271:2471-9.

Danielsson A., **Öst A.**, Lystedt E., Kjolhede P., Gustavsson J., Nyström F.H., and Strålfors P. (2005) *Insulin resistance in human adipocytes occurs downstream of IRS1 after surgical cell isolation but at the level of phosphorylation of IRS1 in type 2 diabetes.* FEBS J. 272:141-51.

Örtegren U., Yin L., **Öst A.**, Karlsson H., Nyström F.H., and Strålfors P. (2006) *Separation and characterization of caveolae subclasses in the plasma membrane of primary adipocytes, segregation of specific proteins and functions.* FEBS J. 273:3381-92.

Franck N., Stenkula K.G., **Öst A.**, Lindström T., Strålfors P., and Nyström F.H. (2007) *Insulin-induced GLUT4 translocation to the plasma membrane is blunted in large compared with small primary fat cells isolated from the same individual.* Diabetologia 50(8),1716-22

Örtegren U., Aboulaich N., **Öst A.**, and Strålfors P. (2007) *A new role for caveolae as metabolic platforms.* Trends Endocrinol Metab 18(9), 344-9

Al-Hilli SM., Willander M., **Öst A.**, and Strålfors P. *ZnO nanorods as an intracellular sensor for pH measurements* J Appl Phys 102

Danielsson A., Fagerholm S., **Öst A.**, Franck N., Nyström F.H., and Strålfors P. (2009). *Short-term over-eating induces insulin resistance in fat cells in lean human subjects.* Mol Med 15:228-34

Ahmad F., Lindh R., Tang Y., **Öst A.**, Ruishalme I., Strålfors P., Degerman E., and Manganiello V.C. *Differential regulation of adipocyte PDE3B in distinct membrane compartments in response to insulin and CL316243 (β 3-receptor agonist)*, Submitted

Abbreviations

ACC	Acetyl-CoA-carboxylase
BMI	Body mass index
ERK	Extracellular signal-regulated kinase
FASN	Fatty acid synthase
FOXO1	Forkhead box O1
GLUT4	Glucose transporter 4
HDL	High density lipoprotein
IRS	Insulin receptor substrate
LDL	Low density lipoprotein
PDK1	3'-phosphoinositide-dependent kinase-1
PGC1	Peroxisome proliferator-activated receptor gamma coactivator
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B, Akt
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PTB	Phosphotyrosine binding domains
RBP4	Retinol binding protein 4, plasma retinol binding protein
SH2	Src-homology 2
SREBP	Sterol Regulatory Element Binding Protein
TORC1	Rapamycin dependent mTOR-raptor complex
TORC2	Rapamycin independent mTOR-rictor complex

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Introduction

During the time I did the research for this thesis, WHO made the remarkable announcement that for the first time in history there are more overweight than underweight persons on this planet. About 1,6 billion people in the world are overweight or obese, compared to 1 billion that are undernourished. This is of course extremely unfair, and one can argue that spending time and money on research to understand an obesity-related disease is pure luxury. Everyone knows that obesity is treated with less food and more exercise. Nevertheless, it has been proven difficult to change eating habits and there might be inherited factors that make us more or less susceptible to high-energy food and a sedentary lifestyle. For a population in general, there is an advantage to have variations in the genome and the thrifty gene hypothesis proposes that there are individuals with gene variants set for sparser conditions. In addition, it's possible that metabolic conditions during earlier generations have made imprints in our genome (1). Clearly, the true complexity of metabolism is first now starting to unravel, with different tissues such as liver, muscle, brain and fat communicating and affecting each other, possibly even over generations.

Obesity and the metabolic syndrome

Regardless of the etiology of obesity, it has been shown that the risk for serious health consequences such as cardiovascular disease and type 2 diabetes increases progressively with obesity, measured as BMI (2, 3).

The World Health Organization (WHO) defines "overweight" as a body mass index (BMI) equal to or more than 25 kg/m², and "obesity" as a BMI equal to or more than 30 kg/m².

BMI is easily calculated by dividing body weight in kilograms with the squared body height in centimetres, $BMI = \text{weight} / \text{height}^2$.

Several reports show that it is mainly excessive visceral fat, also called abdominal or central obesity, that is associated with cardiovascular disease and diabetes (4, 5). In this respect, a problem with BMI is that it doesn't discriminate between fat that is located under the skin (subcutaneous fat) or fat embedded around internal organs (visceral fat). Visceral fat is better measured as the supine abdominal height, which is the height of abdomen measured midway between the top of the pelvis and the bottom of the ribs with the patients lying down, or as the sagittal abdominal diameter with the patients standing (6). Abdominal obesity often co-exist with risk factors for type 2 diabetes and cardiovascular disease in a condition called the metabolic syndrome.

The metabolic syndrome, also called syndrome X, is characterized by a group of metabolic derangements often occurring simultaneously. They include: abdominal obesity, atherogenic dyslipidemia (high triglycerides, low HDL cholesterol and high LDL cholesterol), elevated blood pressure and insulin resistance.

Insulin resistance and type 2 diabetes

After a meal, enhanced blood glucose is sensed by beta-cells in the pancreas that as a response secrete insulin and thereby stimulate muscle, fat and liver to take up and store fat, protein and sugar. Insulin moreover potently stimulates protein synthesis and cell growth, thereby being a major anabolic hormone. The onset of type 2 diabetes is always preceded by insulin-resistance in skeletal muscle and adipose tissue. In an insulin resistant tissue it takes more insulin to get the same response as in an insulin sensitive tissue. As long as insulin producing beta-cells in pancreas are functional, insulin resistance is compensated for by increased secretion of insulin. It is possible that long-term overproduction of insulin leads to beta-cell stress, which promotes formation of amyloid that leads to cell death (7, 8). It is first when beta-cells fail to compensate that blood glucose rises and the classical signs of diabetes with large volumes of urine, thirst and in a late phase, weight reduction is presented.

Overt diabetes mellitus is defined clinically by fasting or postprandial hyperglycemia or an abnormally increased glucose excursion in response to a defined glucose load. WHO's definition of diabetes is fasting blood glucose ≥ 7.0 mmol/l (126 mg/dl) or ≥ 11.1 mmol/l (200 mg/dl) 2 h after ingestion of a 75 g oral glucose load.

The definition of type 2 diabetes clearly divides subjects into diabetic or non-diabetic, but there is a gradually worsening of underlying problems, often over many years, with increasing insulin-resistance and in a later phase a decreased capacity of insulin-producing beta-cells to fully compensate. As insulin-resistance results in elevation of insulin concentrations, and in later phases glucose concentrations, the degree of insulin-resistance can be calculated by using fasting levels of plasma insulin and glucose. A high HOMA index correlates with high insulin-resistance and elevated risk of getting type 2 diabetes.

HOMA and QUICKI are measurements of insulin-resistance and insulin-sensitivity, respectively, that can be calculated using fasting insulin concentration (mU/l) and fasting glucose concentration (mmol/l)

$$\text{HOMA} = \frac{[\text{insulin}] \cdot [\text{glucose}]}{22,5} \quad \text{QUICKI} = 1 / (\text{LOG}([\text{insulin}] \cdot [\text{glucose}]))$$

What is the connection between obesity and systemic insulin resistance?

More than 80 % of all patients with type 2 diabetes are overweight (9) but, despite the strong correlation between obesity and insulin resistance, it has been difficult to find a mechanism to explain this. A large amount of adipocentric hypotheses have emerged to explain the apparent connection between obesity and systemic insulin-resistance, and I will discuss three of them; the ectopic fat syndrome, adipose tissue inflammation, and

adipose tissue as an endocrine organ. They are all possible explanations why enhanced obesity leads to local insulin-resistance in adipose tissue as well as systemic insulin-resistance. Finally, I will also discuss a more holistic view of insulin-resistance.

Ectopic fat syndrome

The number of fat cells is probably set in early adolescence and remain constant over lifetime even after marked weight reduction (10). In line with this, excessive food intake probably results in larger fat cells rather than increased number of cells, and several studies show that large adipocytes are more insulin resistant (11, 12). As adipocytes have a maximum size and therefore a maximum fat storage capacity, insulin resistance in adipocytes might be a protective mechanism to prevent fat overload.

In contrast to acquired obesity where both subcutaneous and visceral fat are overloaded, protease inhibitor treated patients with HIV have an expansion of the visceral fat while the subcutaneous fat is wasted (13), similar to patients with Cushing's disease. These conditions are both associated with insulin resistance. It has been shown that too little subcutaneous fat in experimental animals (14), as well as in humans (15), induces insulin resistance.

Systemic insulin resistance apparently can result from both too much or too little subcutaneous fat. In this background the overflow hypothesis has emerged; during times of excessive food intake, extra energy is stored as fat in adipose tissue and if absent, defective or insulin resistant conditions due to fat overload or other factors, energy must be stored elsewhere, in liver, muscle and as elevated levels of lipids in blood – a phenomenon described as ectopic fat deposition (16). Even though obesity predisposes to insulin resistance and type 2 diabetes, this scenario suggests that it is not obesity per se that is the problem, but the loss of capacity to store excess calories. In support for this view, treatment with PPAR γ activators such as rosiglitazone, which promote differentiation of adipocytes, alleviate systemic insulin resistance while enhancing adipose tissue mass.

The ectopic fat syndrome is tightly coupled to the lipotoxicity theory, which claims that for tissues other than adipose tissue, fat is toxic (17, 18). In support of the lipotoxicity theory there are compelling evidences showing that fatty acids induce insulin resistance in liver and muscle cells.

The ectopic fat syndrome suggests that the correlation between obesity and insulin resistance is due to adipocytes' inability to store lipids, which leads to elevated levels of plasma free fatty acids and concomitant uptake of free fatty acids in non-adipocyte cells, such as muscle and liver cells, where they induce insulin resistance. In other words, elevated levels of free fatty acids, a consequence of adipose tissue insulin resistance, cause systemic insulin resistance.

Adipose tissue as an endocrine organ

Another main theory for the connection between obesity and systemic insulin resistance is based on the relatively recent acceptance of fat tissue as a nutrient sensor and endocrine organ. Adipokines, hormone-like substances such as leptin, adiponectin, RBP4, interleukin-6, resistin, visfatin, and TNF- α (19), are secreted from fat tissue in response to different stimuli and regulate and fine-tune whole body metabolism. One of the most studied adipokine is leptin, which is secreted in proportion to fat tissue mass and signals to the brain to down-regulate appetite when stores of energy are plenty. In almost all cases of obesity the levels of leptin are normally elevated, and administration of extra leptin does not promote any weight loss (20). But in the few cases of obesity with leptin deficiency, administration of leptin dampens appetite and patients rapidly lose weight.

Retinol binding protein 4 (RBP4) binds and transports vitamin A in serum and is therefore extensively studied in the context of retinol metabolism. RBP4 is mainly secreted from the liver, which holds the largest vitamin A reserve, but it is also secreted from adipocytes but to a lesser extent (21). Adipocyte-secreted RBP4 was discovered to induce systemic insulin resistance by Kahn and colleagues (22), when they were looking for mechanism for insulin-resistance in muscle and liver tissue in adipocyte-specific GLUT-4 knockout mice. In mice, over expression or injection of recombinant

RBP4 causes systemic insulin resistance, while genetic deletion of RBP4 results in enhanced insulin sensitivity, suggesting a causal relationship between RBP4 and insulin resistance (22).

In humans the concentration of RBP4 is positively correlated to BMI and plasma insulin concentration, and is in some cases lowered by exercise (23-25). There are, however, several reports that don't find any correlation between RBP4 and insulin resistance and the usefulness of RBP4 as a diagnostic marker for insulin resistance is strongly questioned (26-29). The discrepancy might be explained by the higher RBP4-to-retinol ratio in serum from type 2 diabetic patients (30). Apparently, some RBP4 doesn't carry retinol and this subpopulation is bigger in type 2 diabetes, as well as in patients suffering from chronic renal failure (31). It is possible that different antibodies used to measure RBP4 concentration have a higher affinity for one of the subpopulations of RBP4 thus creating conflicting results (32).

An obvious link between obesity and systemic insulin resistance is adipocyte-secreted adipokines, which have the potential to have diagnostic value as well as constitute drug targets to reduce insulin resistance.

Adipose tissue inflammation

Type 2 diabetes patients have slightly elevated plasma levels of acute phase proteins, such as serum amyloid A, C-reactive protein, cortisol, and interleukin-6, indicating a low-grade inflammation process (33). Moreover, many of the problems clustered with type 2 diabetes, such as insulin resistance and atherogenic dyslipidaemia, are also normal features of acute-phase reactions. It has therefore been postulated that the metabolic syndrome in fact is a direct consequence of immune system activation. Adipocytes secrete both pro- and anti-inflammatory cytokines, and large adipocytes secrete more pro-inflammatory adipokines than small cells (34, 35), possibly linking obesity with low-grade inflammation. In line with this, adipose tissue from obese mice, as well as subcutaneous human fat from obese subjects, are infiltrated by macrophages (36, 37) and, at least in mice, macrophage activation definitely plays a major role in obesity-induced insulin-resistance (38). Active macrophages secrete a cocktail of pro-

inflammatory cytokines, which will affect adipocytes to secrete pro-inflammatory adipokines. It is also true that, the other way around, pro-inflammatory adipokines will attract and activate macrophages creating a self-sustaining inflammatory milieu.

So how are triglyceride over-load and large fat cells coupled to inflammation? This is not at all clear but it has been speculated that it might be through the endoplasmic reticulum (ER) stress pathways, which are overlapping and intertwined with inflammation pathways and activated in adipose tissue and liver of obese mice (39). It is possible that ER senses obesity-induced metabolic-stress and transfers it to both insulin resistance and inflammatory responses through JNK signalling. On the other hand, it is possible that ER-stress is not the prime event but a consequence of hypoxia, inflammation or nutrient deprivation, all well-known inducers of ER-stress.

Regardless of the etiology, obesity is clearly associated with low-grade inflammation in adipose tissue, with concomitant elevation of acute phase proteins and pro-inflammatory cytokines in plasma that could induce systemic insulin resistance.

A holistic view of insulin resistance

Obesity doesn't always lead to insulin resistance, many (20 - 60 %) obese subjects are insulin sensitive (40-43). So obesity might be a major factor in the development of insulin resistance, but it is not a determinant. In addition to obesity, there must be other factors involved in the development of insulin resistance.

Adipose tissue is not unique in its ability to affect other organs. This is clearly demonstrated by the fact that many tissue-specific genetic knockout models in mice give systemic effects. Mice with muscle-specific knockout of the insulin receptor have normal levels of glucose and insulin but enhanced free fatty acid levels (44). Interestingly, the insulin resistance in muscle seems to redistribute substrates to adipocytes resulting in increased fat deposition in adipose tissue, which unexpectedly is highly insulin sensitive. Muscle-specific insulin receptor knock-out mice have many

properties associated with type 2 diabetes; enhanced visceral adipose tissue, elevated fatty acids in the blood and muscle insulin resistance, but they are not diabetic. The anti-diabetic effect might be ascribed to the insulin sensitive adipose tissue that consists of small well differentiated adipocytes (45).

Liver-specific knockout of the insulin receptor promotes systemic insulin resistance and glucose intolerance, which is indicative of the central function of the liver to control whole body metabolism (46). Moreover, Shoelson and colleagues have shown that liver steatosis and inflammation can be the primary source of systemic factors that lead to the development of systemic insulin resistance (47). Their transgenic mouse with liver-specific over-activation of NF-kappaB has normal weight, but is systemically insulin resistant. So even if inflammation is a local and highly regulated event, it can at some point be transferred to systemic insulin resistance.

Many paths can lead to systemic insulin resistance and there are considerable inter-tissue communications to coordinate whole-body metabolism in diverse situations such as eating, fasting, exercise and trauma. As an illustrative example; skeletal muscle secretes IL-6 and the plasma level rises up to 100-fold during exercise (48), possibly promoting the beneficial effects of exercise, such as improved systemic insulin sensitivity (49). Clearly, analogously to adipocytes, muscle cells secrete substances that have effect on whole body metabolism.

Caveolae as signalling and metabolic platforms

Adipose tissue consists mainly of adipocytes but there are also other cells types, such as endothelial cells, nerves cells, and macrophages. In addition, adipocytes secrete collagen that, like brick-and-mortar, attaches cells to each other. Adipocytes are large cells, in obese patients sometimes as large as 200 μm in diameter, but normally somewhere around 90 μm . More than 95% of the total cell volume consists of a large oil droplet that pushes the nucleus against the plasma membrane (Fig. 1). The distance between the plasma membrane and the lipid droplet ranges from less than 50 nm, where it is as thinnest, to 1000 nm where the nucleus is protruding, which is the place with the thickest cytosol. In this tiny space organelles, such as mitochondria, are squeezed in.

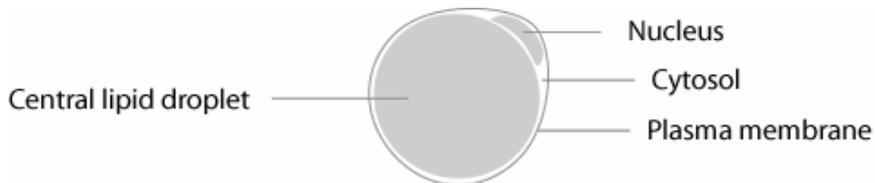


Figure 1 Adipocytes are large cells with a big central lipid droplet and a thin rim of cytosol beneath the plasma membrane. Where the nucleus is protruding the cytosol is a little thicker, but still not more than 1000 nm.

Adipocytes have an abundance of cholesterol-rich invaginations, called caveolae, in the plasma membrane. From the outside caveolae look just like small holes no larger than 10-20 nm, but on the inside they bulge in like small light bulbs, ranging from 25-150 nm in diameter (50). Considering that the cytosol in some areas is only "a caveola" thick, it is no surprise that caveolae sometimes make physical contact with the central

lipid droplet (Fig 2). These caveolae micro-domains have multiple functions. In adipocytes there are at least three different kinds of caveolae with different lipid and protein compositions, reflecting their diverse functions in insulin signalling and metabolism (51). Since so many proteins involved in signalling localize in caveolae, it has been proposed that caveolae are signalling platforms, micro-domains where signalling proteins gather and interact (52). In addition, it has been proposed that caveolae might be metabolic platforms for both lipid and glucose metabolism (53).

Caveolae as insulin signalling platforms

In rat adipocytes, the insulin receptor is situated in caveolae (54), and more specifically in caveolae with high density and very-high density, but not in caveolae with low density (51). Caveolin or caveolae most probably have a stabilizing role for the insulin receptor since caveolin-1 knockout mice have a 90% decrease of insulin receptor protein in adipocytes without any changes in mRNA levels (55). Upon insulin stimulation caveolin-1 is rapidly phosphorylated on tyrosine residues (56), which coincides with the appearance of insulin receptor containing caveolae and/or caveosomes in the endosomal fraction (57). Since endocytosed insulin receptors are phosphorylated, it is possible that down-stream signalling might occur in the vesicles in the endosomal fraction as well as in the plasma membrane. In line with this, it has been shown that tyrosine phosphorylated IRS-1 as well as activated PI3K is internalized simultaneously with insulin receptors (58, 59) in a high molecular weight (>3000 kDa) complex (59). In addition to the insulin receptor, IRS-1 (60, 61) and PDE3B (62) have been reported to localize to caveolae.

After insulin-stimulation, GLUT4 first rapidly translocates to the plasma membrane and after 15 min, probably through a lateral movement in the plasma membrane, to caveolae. At the same time the cell increases the uptake of glucose, indicating that GLUT4 is functional first when located in caveolae (63). Immunogold electron microscopy and cell fractionation confirms that GLUT4 is mainly located in caveolae (64). There are, however, some conflicting data about localization of GLUT4 in caveolae (reviewed in (65)).

Caveolae as metabolic platforms (Paper I)

High-density caveolae harbour several fatty acid binding proteins besides caveolin and disruption of caveolae inhibits fatty acid uptake. Furthermore, this specific subclass of caveolae have the capacity to synthesize triglycerides from fatty acids (Paper I), a task historically ascribed to the endoplasmic reticulum (ER). It is difficult to estimate how much fatty acids that are esterified in caveolae compared to ER *in vivo*, but *in vitro* data show that caveolae have the same capacity as ER (Paper I). Esterified fatty acids, triglycerides in lipid droplets, are protected from hydrolysis by perilipin. Interestingly, triglyceride-synthesizing caveolae, but not the other two sub-types of caveolae, contain perilipin. Moreover, insulin increases and isoproterenol decreases the amount of perilipin B in the plasma membrane (66), suggesting that the metabolism of triglycerides in caveolae is hormonally regulated.

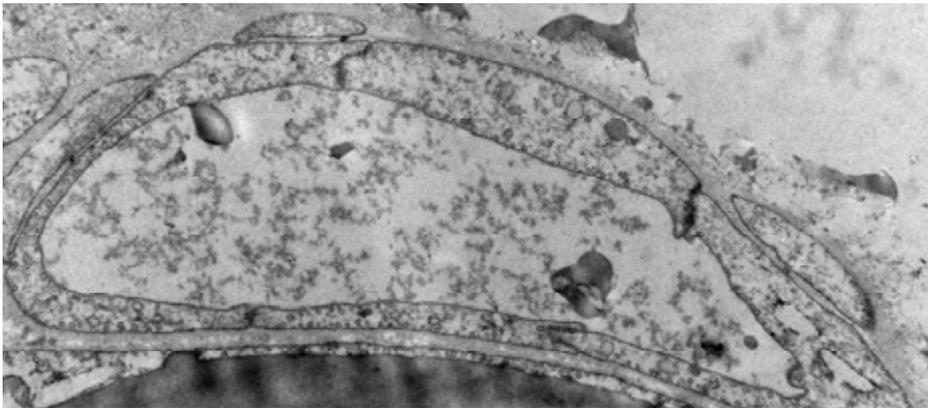


Figure 2a This transmission electron microscopy picture shows a capillary and in the lower part of the picture an adipocytes. The dark part of the adipocyte is the central lipid droplet that is covered by a thin rim of cytoplasm.

How triglycerides formed in caveolae are transported to the central lipid droplet isn't known, but for large parts of the cell it is possible that no transport is needed because the cytosol is so thin that caveolae actually make contact with the central lipid droplet (Paper I) (Figure 2).

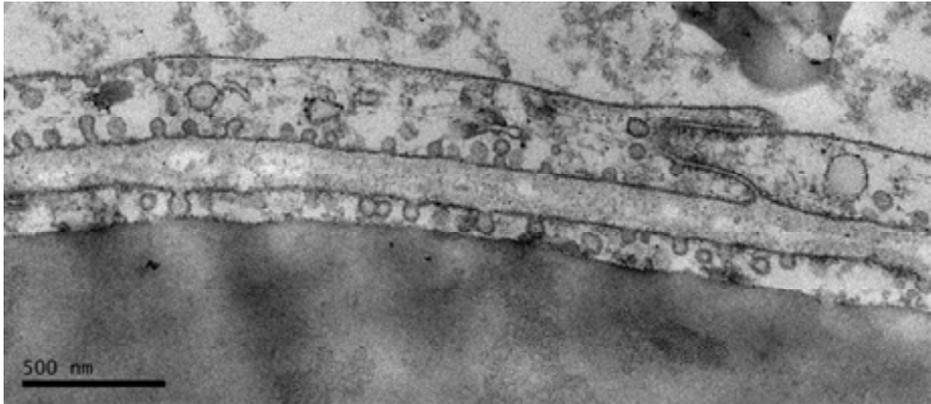


Figure 2b A close up of 2a. The upper part is part of an endothelial cell and the lower part shows a part of an adipocyte. Both cells have invaginations in their plasma membranes that are called caveolae, which means little caves.

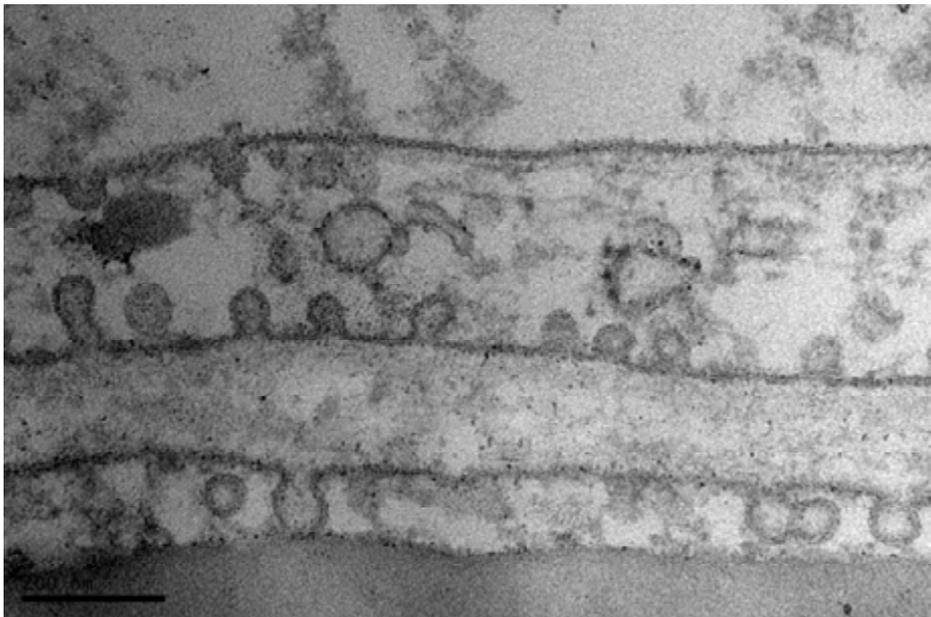


Figure 2c A close up of 2b. In this picture it is evident that the cytosol in some areas of the adipocyte is only a caveolae thick. In this case caveolae-synthesized triglycerides could be transferred to the central lipid droplet by direct physical contact.

Insulin signalling in adipocytes

Insulin signals nutrient availability and when insulin binds its receptor on the adipocytes this information is via a highly complex network of signals transferred to anabolic reactions, such as protein synthesis and most importantly, uptake and esterification of fatty acids. When dissecting the intracellular signalling pathways involved in this transfer of information there has been a clear tendency of thinking in terms of linear cascades. However, with accumulating knowledge, it is now possible to get a hint of a larger picture with feedback signals and interacting signalling pathways, which make up a complex web of signalling proteins. With this knowledge, it is appropriate to see signalling proteins as hubs that receive information from a variety of pathways and the sum of this information is then passed on to yet another set of signalling proteins. One problem with this view is that it is extremely difficult to get the whole picture, so in this limited summary of insulin signalling there is first a description of the major hubs, and then the control of glucose uptake and lipid and protein synthesis by insulin is described as pathways. To get the detailed picture of each hub, data from a variety of cell types are included, but in the pathways, if not clearly stated otherwise, only findings in adipocytes are discussed. Since not much is known about autophagy in adipocytes, this part consists of data from a variety of cell types.

The IRS-1 hub

The insulin receptor (IR) belongs to a family of receptors with intrinsic tyrosine kinase activity. Upon insulin binding, the receptor dimerizes and the two β -subunits reciprocally phosphorylate each other on tyrosine residues to create specific binding

sites for proteins containing Src-homology 2 (SH2) or phosphotyrosine binding (PTB) domains. IR also phosphorylates a variety of proteins, such as insulin receptor substrate (IRS), shc and cbl, which are attracted to the phosphotyrosine residues in IR. IRS is a typical docking protein that, when activated by phosphorylation at tyrosine residues, provides binding for a number of signalling proteins with SH2 domains, such as PI3K Class 1A, Grb2, and SHP2 (67).

There are four subtypes of IRS involved in insulin signalling IRS1-4. IRS3 is not expressed in humans (68) and IRS4 appears to play a limited role in the metabolic effect of insulin. The specific roles of IRS-1 and IRS-2 have been dissected using knock-out mice and seem to be tissue specific (69). IRS-1 knock-out mice have retarded growth and elevated levels of insulin characteristic of peripheral insulin resistance, but these mice are not diabetic most probably due to compensatory β -cell hyperplasia. Muscle and adipose tissue from IRS-1 knock-outs display reduced insulin-stimulated PI3K activity and glucose uptake, whereas liver PI3K activity is normal due to compensatory action of IRS-2 (70). Apparently, IRS-2 doesn't have any compensatory role in adipose or muscle tissue regarding insulin-stimulated PI3K activity and glucose uptake (69), but it has been reported that IRS-2 specifically controls lipid metabolism via the PKB isoform 2 in muscle (71). IRS-2 knock-out mice show minimal growth retardation and no changes in insulin-stimulated glucose uptake in muscle and adipose tissue (69).

All together, IRS-1 is probably the main isoform involved in insulin-stimulated glucose uptake in muscle and fat, whereas IRS-1 and IRS2 have overlapping roles in insulin-signalling in liver.

Tyrosine phosphorylation of IRS-1

IRS-1 is a fairly large protein with a calculated molecular mass of 132 kDa, but due to extensive phosphorylation it migrates as 185 kDa during sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the N-terminal part of IRS-1 there is a PH domain that targets IRS-1 to PIP₃, generated by PI3K in the plasma membrane in response to insulin (Fig. 3). Close to the PH domain is a PTB domain that is attracted to phosphotyrosine on the insulin receptor. Together these two domains ensure correct

targeting of IRS-1 in response to insulin. When in correct position close to each other, the intrinsic tyrosine kinase activity of the insulin receptor phosphorylates IRS-1 on multiple tyrosine residues (72). Interestingly, human IRS-1, but not rat IRS-1, co-localizes with the insulin receptor in caveolae even in the absence of insulin (60, 61). Insulin-stimulation thus induces a two-fold increase of plasma membrane associated IRS-1 in human adipocytes but more than a ten-fold increase in rat adipocytes (61).

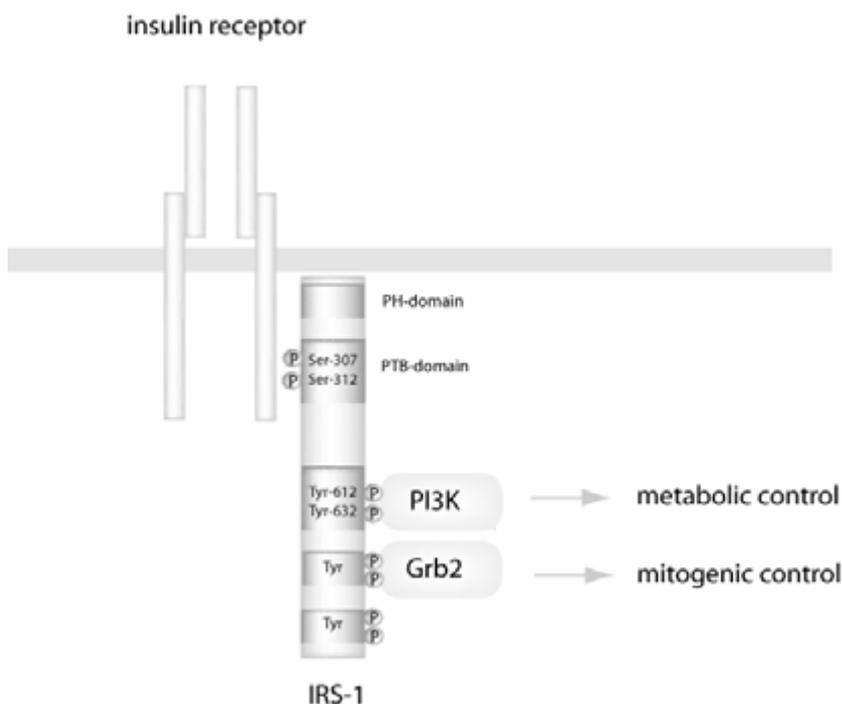


Figure 3 A schematic illustration of human IRS-1. Upon insulin stimulation the insulin receptor phosphorylates IRS-1 at tyrosine residues. Specific phospho-tyrosines provide binding sites for proteins with a SH2 domain. PI3K and Grb2 both have SH2 domains and are attracted to phospho-tyrosines of IRS-1. By binding to IRS-1, PI3K and Grb2 start the signalling cascade of insulin metabolic and mitogenic control, respectively. Insulin-stimulated feedback phosphorylation of IRS-1 at serine residues regulates insulin receptor tyrosine phosphorylation of IRS-1. Adapted from (73).

IRS-1 have ~20 potential tyrosine phosphorylation sites. Many of the tyrosine residues are found in common Tyr-phosphorylated consensus motifs (YMXM or YXXM) that bind SH2 domains of their effector proteins. IRS-1 and IRS-2 contain about nine YMXM-motifs that, when tyrosine phosphorylated, provide potential sites binding for SH2 domains in p85, the regulatory part of PI3K. Binding of p85 to IRS-1 brings PI3K close to the plasma membrane near its substrate thereby propagating the insulin signal. Since creation of these binding sites is crucial for metabolic insulin signalling, several investigators have tried to pin-point which motifs that have highest affinity for p85. It has consistently been shown in mice that phosphorylation on Tyr-608 and Tyr-628 (corresponding to Tyr-612 and Tyr-632 in human IRS-1) bind p85. In addition, some reports also suggest phosphorylation on Tyr-939 and Tyr-658 (72, 74-76). The SH2 domain of Grb2 binds preferentially Tyr-895 in IRS-1 (murine sequence), thereby transmitting insulin's mitogenic signalling via activation of SOS-ras-ERK, (72, 77). Interestingly, in adipocytes insulin appear to signal mitogenic control largely via IRS-1 (78). Hence, by offering binding sites for PI3K and Grb2, tyrosine phosphorylations of IRS-1 are most important for further insulin-signalling (Fig. 3). Protein phosphatases, for example protein tyrosine-phosphatase 1B (PTP-1B) and Src Homology Phosphatase 2 (SHP2), antagonize insulin action by catalyzing dephosphorylation of tyrosine residues on the insulin receptor, IRS-1, and/or other key proteins in the insulin signalling pathway (79, 80).

Serine phosphorylation of IRS-1

In vitro experiments with purified insulin receptor and recombinant IRS-1 show that the intrinsic tyrosine kinase activity of the activated insulin receptor phosphorylates IRS-1 on tyrosine residues only (81). On the other hand, in vivo experiments show that insulin stimulation also results in multiple serine phosphorylations, suggesting that IRS-1 by feedback mechanisms is a substrate for insulin-activated serine kinases (81)(Fig. 3). In addition, several non-insulin dependent protein kinases, such as JNK, phosphorylate IRS-1 on serine residues (82). Most serine phosphorylations are considered to be inhibitory for insulin-signalling, but there are several examples where serine phosphorylation correlates positively with tyrosine phosphorylation (83-86).

The PI3K Class 1A hub

PI-3 kinase plays an important role in insulin control of many regulated metabolic processes, including glucose uptake, general and growth-specific protein synthesis and glycogen synthesis. There are three main classes of PI3K (Table 1) (87), of which class 1A for long has been known to play a pivotal role in insulin signalling. The third class of PI3K is involved in amino acid sensing and control of TORC1 mediated regulation of autophagy, which is integrated with the insulin signalling pathway. In this text PI3K refers to the class 1A type.

PI3 Kinase	Main substrate	Second messenger	Domain attracted to second messenger
Class I	PIP2	PIP3	Pleckstrin homology (PH)
Class II	PI, PIP	PIP, PIP2	
Class III	PI	PIP	FYVE domain

Table 1 There are three main classes of PI3K. Class IA is most important in insulin signalling.

PI3K Class 1A is composed of a 110 kDa catalytic subunit (p110) and a 85 kDa regulatory subunit (p85), which upon binding to phosphorylated tyrosine residues on IRS-1 get in close proximity to its lipid substrate in the inner leaflet of the plasma membrane. Binding of the SH2 domain in p85 to phosphorylated YMXM-motifs in IRS-1 activates the associated catalytic domain. This activation is maximal when both of the SH2 domains are occupied (88). PI3K phosphorylates the membrane lipid phosphatidyl-inositol-3,4-bisphosphate (PIP2) to phosphatidyl-inositol-3,4,5-trisphosphate (PIP3), offering binding sites for proteins with PH domains, including PKB (89) and PDK1 (90) (Fig. 4). Even though PI3K can bind to phosphotyrosine on IR directly, IRS-1 provides extra SH2 domains thereby increasing possible binding sites for PI3K. Moreover, PIP3 in the plasma membrane generated by PI3K provides binding sites for IRS-1, enhancing its attraction to the area. In this way there is a mutual

attraction of IRS-1 and PI3K class 1A to areas in the plasma membrane where they are mandatory for proper propagation of insulin-signalling (91, 92).

The action of PI3K is reversed by lipid phosphatases. Two phosphatases targeting PI(3,4,5)P₃ have been described in adipocytes. One is SHIP2 that removes the 5' phosphate and the other is PTEN that removes the 3' phosphate to give PI(3,4)P₂ and PI(4,5)P₂, respectively (93-95).

The PKB hub

PDK and PKB are cytosolic PH-domain-containing kinases that are attracted to PIP₃ generated in micro-domains in the plasma membrane (96). PKB is first phosphorylated at Thr-308 by PDK1 and after being phosphorylated at Ser-473 by the rapamycin-insensitive mTOR-rictor complex (TORC2) it is completely active (97) (Fig. 4). Once activated PKB can detach from the plasma membrane and translocate to different compartments to control its downstream substrates (98, 99). PKB targets are GSK, rapamycin-sensitive raptor-TORC1 and AS160, promoting glycogen synthesis, protein synthesis and GLUT4 translocation, respectively. In addition, PKB phosphorylates PDE3b and FOXO. Since PKB is in control over so many metabolic pathways it is an important hub in insulin signalling (73).

PKB is dephosphorylated and inactivated by protein phosphatase-2A (100). Protein phosphatase-2A is in that way a negative regulator of insulin-stimulated phosphorylation of PKB and subsequent metabolic effects (101).

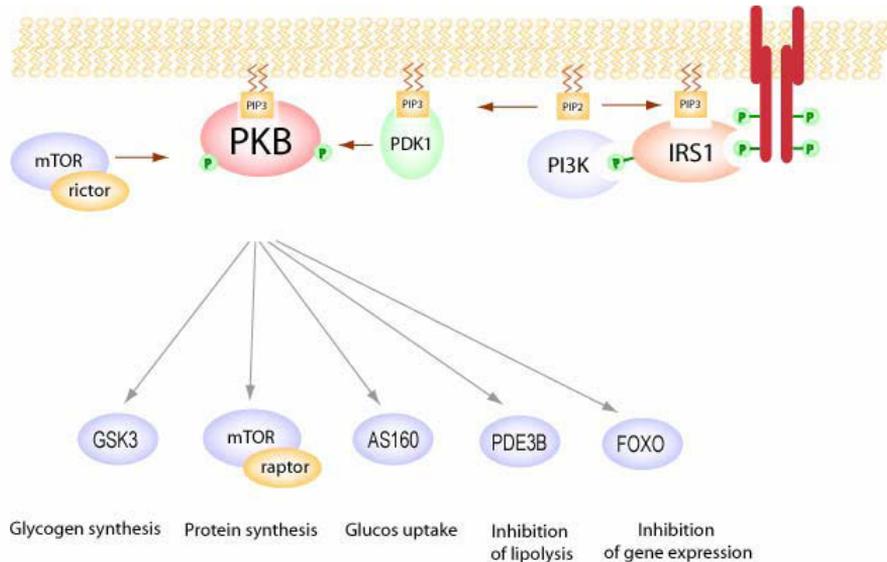


Figure 4 Upon insulin-stimulation the insulin receptor is auto-phosphorylated on tyrosine residues thereby providing binding sites for IRS-1. As a docking protein, IRS-1 provides binding sites for additional proteins such as PI3K that by binding to IRS-1 comes close to its substrates in the plasma membrane. PI3K phosphorylates the plasma membrane lipid PIP2 to PIP3 that attracts PKB and PDK1. In close proximity, PDK1 phosphorylates PKB at Thr-308. PKB is then further phosphorylated by rapamycin-insensitive mTOR-rictor complex (TORC2) at Ser-473. When fully activated PKB phosphorylates several substrates that control cellular metabolism.

The ras-ERK hub

Insulin is well known to have a mitogenic effect that is transmitted by the Grb2-SOS-Ras-ERK pathway. This insulin-signalling pathway, similarly to that of metabolic control, is dependent on IRS-1 (102-104). Adipocytes are terminally differentiated cells that don't divide, so in adipocytes this pathway is probably more focused on the regulation of transcription and translation. Transcription is stimulated via the transcription factors ELK1 (73) and AP1 (105), whereas translation is mediated via p90 ribosomal protein S6 kinase and activation of TORC1 signalling (106).

ERK is involved in adipogenesis where it is needed during an early proliferation step, but it also needs to be turned off to allow terminal differentiation (107).

The TORC1 hub

The rapamycin-sensitive raptor-mTOR complex (TORC1) is a primitive nutrient sensor that under poor growth conditions is turned off resulting in enhanced autophagy. During favourable growth conditions TORC1 is turned on, thus promoting protein and lipid synthesis. TORC1 is under control by the TSC1-TSC2 complex that acts as a GTPase-activating protein (GAP) for Rheb. As an intact complex, TSC1-TSC2 thus stimulates the conversion of Rheb-GTP into the inactive Rheb-GDP. Dissociation of the complex leads to accumulation of Rheb-GTP that binds to and activates TORC1 leading to enhanced protein synthesis and support of cell growth (108)(Fig.5).

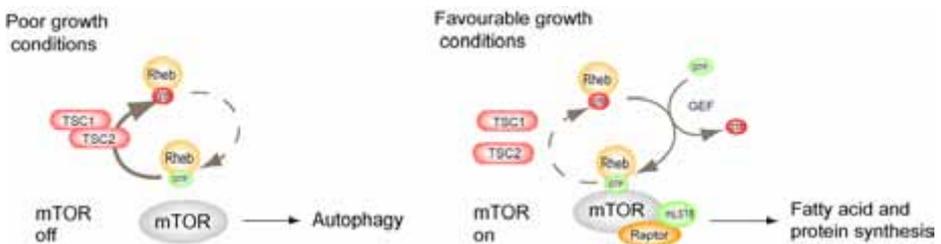


Figure 5 During poor growth conditions TSC1 and TSC2 form a complex that promotes formation of Rheb-GDP thereby preventing activation of the rapamycin-sensitive mTOR-raptor complex (TORC1). During favourable growth conditions the TSC1-TSC2 complex is dissociated, which results in accumulation of Rheb-GTP that binds to and activates TORC1. Adapted from (109)

Insulin inhibits the formation of the TSC1-TSC2 complex leading to TORC1 activation and cell growth, through both PKB/akt and ERK, while REDD1 and AMKP, sensing hypoxia and energy shortage, respectively, activates the TSC1-TSC2 complex leading to inhibition of TORC1 and cell growth (109). In addition to Rheb-GTP, TORC1 is activated by amino acids and ATP via hVps34, a Class 3 PI3K (110, 111). The mechanisms are still unclear but seem to require increased production of PtdIns(3)P. Once activated, TORC1 activates PPARgamma, SREBP, S6K and PGC1 while inhibiting autophagy (Fig. 7 and fig. 9).

Insulin stimulation of glucose uptake

Glucose is a fundamental source of energy and during resting conditions the brain accounts for 80 % of whole-body consumption. Glucose is stored in the liver and muscle, but only the liver has the capability through glycogenolysis and gluconeogenesis to release glucose to the blood to maintain constant levels of glucose during fasting. After a meal, excess glucose is taken up mainly by liver, muscle and fat cells through specific glucose transporters. Liver cells have mainly glucose transporters 2 (GLUT2) that are constitutively situated at the plasma membrane while muscle and fat cells mainly have intracellular stores of glucose transporters 4 (GLUT4), which translocate to the plasma membrane only when cells are stimulated with insulin. For this to happen several parallel events need to be executed, orchestrated by insulin-signalling proteins. Despite massive work, for the last 30 years, there are still a lot of gaps in what we know about insulin-signalling and GLUT4 translocation and fusion with the plasma membrane. For review see (112).

Insulin signalling to control GLUT4

There are two major signalling pathways for insulin to stimulate glucose uptake that have been investigated in greater detail. One is the IRS1-PI3K pathway described earlier as separate hubs. Two important targets of PI3K, PKB and an atypical protein kinase C λ/ζ (PKC λ/ζ), have been shown to be involved in insulin stimulation of glucose uptake. Over expression of a dominant-interfering PKB mutant inhibits insulin-stimulation of GLUT4 translocation, while over expression of a membrane-bound form of PKB results in increased insulin-stimulation of GLUT4 translocation and glucose uptake, demonstrating the pivotal role of PKB in insulin stimulated glucose uptake (113, 114). Of all putative PKB substrates involved in GLUT4 translocation, only the Rab GTPase-activating protein AS160 has been examined in greater detail (115). In the basal state AS160 is associated with GLUT4 vesicles, where it has an inhibitory role, but dissociates from GLUT4 vesicles in response to insulin (116). It is possible that the release of AS160 from GLUT4 vesicles relieves the inhibition of Rab proteins, including Rab10, thereby stimulating GLUT4 vesicle fusion with the plasma membrane

(116, 117). Over expression of mutant AS160 that cannot be phosphorylated by PKB inhibits GLUT4 translocation (118). PKC λ/ζ activation is also PI3K and PDK1 dependent (119) and needed for insulin stimulation of glucose uptake (120).

The second pathway in insulin stimulation of glucose uptake is the activation of caveolae/raft associated TC10. Insulin receptor tyrosine phosphorylation offers binding sites for the CAP-Cbl complex that enables tyrosine phosphorylation of Cbl to recruit a complex of CrkII and the guanine-nucleotide-exchange factor CG3. Upon its translocation to lipid rafts, CG3 catalyzes the activation of the small G protein TC10 (112, 121). Over- expression of dominant-interfering TC10 mutants inhibits insulin-stimulated GLUT4 translocation and glucose uptake (122, 123).

GLUT4 translocation to the plasma membrane

Determined by microscopy, GLUT4 is under basal conditions mainly located in perinuclear tubulo-vesicular structures and in puncta throughout the cytosol (124, 125). The perinuclear structures co-localize with elements from the endosomal recycling compartment and the trans-Golgi network and are considered to be a pool of GLUT4 that is not acutely responsive to insulin (126). The insulin-responsive pool doesn't co-localize with any of the markers above and it has proven difficult to exactly define the biochemical nature of these structures. They are named GLUT4 specialized compartment (SC) or GLUT4 storage vesicles (GSV). Both compartments are most likely not static but consist of continuously cycling components. It has been proposed that in the basal state, GLUT4 cycles mainly between the perinuclear structures and the cytosolic puncta, and when stimulated with insulin the flow is rerouted to cycle between the intra-cellular and plasma membrane compartments. In human adipocytes it is possible that this translocation step plays a less significant role considering that the cytosol is only 100-200 nm thick. The GSV vesicles are already in close proximity to the plasma membrane and insulin simply stimulates docking and fusion of GLUT4 vesicles to the plasma membrane.

So how is the insulin signalling coupled to the vesicle trafficking machinery? Several studies has shown that PI3 kinase is mandatory for insulin-stimulation of GLUT4 translocation (127), but this might not be very surprising considering that PI3K regulates many down-stream proteins. Whereas PKB is active at the plasma membrane, another downstream target of PI3K, PKC λ/ζ , is, in HeLa cells, selectively activate on endosomes (128). PKC λ/ζ is mandatory for GLUT4-vesicle binding to microtubule (129) and most likely have a role in shifting the flow from the GSV-endosomal cycling to the plasma membrane (Fig 6).

At 19 °C, when insulin stimulates PI3 kinase activity but not PKB activity, GLUT4 vesicles accumulate close to the plasma membrane without fusing with it, indicating that PKB most probably has a role in promoting docking/fusion of GLUT4 vesicles with the plasma membrane (130). It is possible that PKB, or more specifically PKB isoform 2, regulates GLUT4-vesicle associated AS160 phosphorylation that relieves the inhibition of Rab proteins and thereby enables association between GLUT4 vesicles and the plasma membrane (131) (Fig 6).

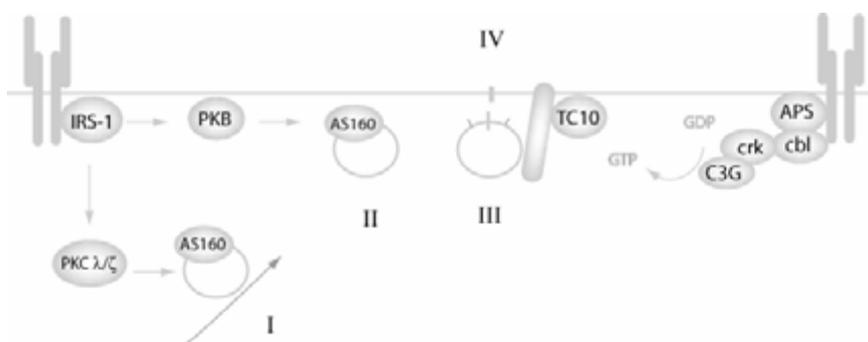


Figure 6. Insulin-stimulated GLUT4-vesicle translocation. (I) PKC λ/ζ , which is activated by IRS-1, promotes binding of the GLUT4 vesicle to microtubule and the translocation to the plasma membrane. (II) Plasma membrane activated PKB phosphorylates AS160 resulting in the release of AS160 from the GLUT4 vesicle uncovering rab proteins that are involved in fusion events. (III) The GLUT4 vesicle is attracted to the activated TC10-complex in caveolae thereby coming in close proximity to the plasma membrane. (IV) Fusion is mediated with SNARE and rab proteins.

Insulin stimulation of lipid synthesis

Insulin promotes storage of excess energy as triglycerides in adipocytes. Dietary triglycerides are transported from the intestine in chylomicrons and hydrolysed by lipoprotein lipase (LPL) in the capillaries. Fatty acids are then by active transport and/or passive diffusion transferred over the endothelial cells to the adipocytes. Excess glucose is stored partly as glycogen and, when plenty, mainly as triglycerides. In this case, glucose is converted to pyruvate by glycolysis and via the first step in the tricarboxylic acid cycle in mitochondria converted to citrate. ATP-citrate lyase (ASLY) converts cytosolic citrate into acetyl-CoA that by fatty acid synthase (FASN) are condensed to long-chain fatty acids. Fatty acids are then subsequently esterified with glycerol-3-phosphate to triglycerides. It has been debated if human adipocytes have the capability to synthesis fatty acids from glucose but it must now be acknowledged that it probably is on par with the livers' fatty acid synthesis capacity (132). Several *in vivo* studies support that high calorie diet or insulin infusion up-regulates the fatty acid synthesis capacity in human subcutaneous adipocytes (133-135), probably reflecting the role of adipocytes in converting excess glucose to fatty acids. When synthesized in liver, fatty acids are delivered to adipocytes as triglycerides in very low density lipoproteins via the same mechanism as chylomicrons.

In adipose tissue, the expression of sterol regulatory element binding protein 1c (SREBP-1c), which controls the expression of ASLY and FASN, is low during fasting conditions but dramatically elevated upon feeding or insulin administration (135-137). This master regulator of lipid metabolism was recently shown to be TORC1 dependent, in *Drosophila* and human retinal pigment epithelial cells, putting insulin-stimulated lipid synthesis under control by the IRS-1-PI3K-mTOR pathway (Figure 6) (138). An extra twist on insulin-stimulated lipid synthesis is that TORC1 via YY1-PGC1 α controls mitochondrial biogenesis in skeletal muscle (139), thereby enhancing the cells capacity to convert pyruvate to citrate providing substrates for fatty acid synthesis. In this scenario it should be noted that adipocytes' potential for lipogenesis is closely correlated to the amount of mitochondria (140). SREBP-1c also increases the transcription of GLUT4 thereby enhancing the potential for insulin-stimulated glucose

uptake (141). Considering that glucose via glycolysis and mitochondrial conversion from pyruvate to citrate is substrate for fatty acid synthesis, it makes sense that GLUT4, mitochondrial biogenesis and fatty acid synthesis are co-ordinately up-regulated in response to insulin and nutrients. In addition to being substrate for fatty acids synthesis, glucose is also converted to glycerol-3-phosphate that is needed for fatty acid esterification to triglyceride. Inhibition of TORC1 inhibits the expression of FASN and insulin-stimulated glucose uptake concomitantly (142) (Fig. 7).

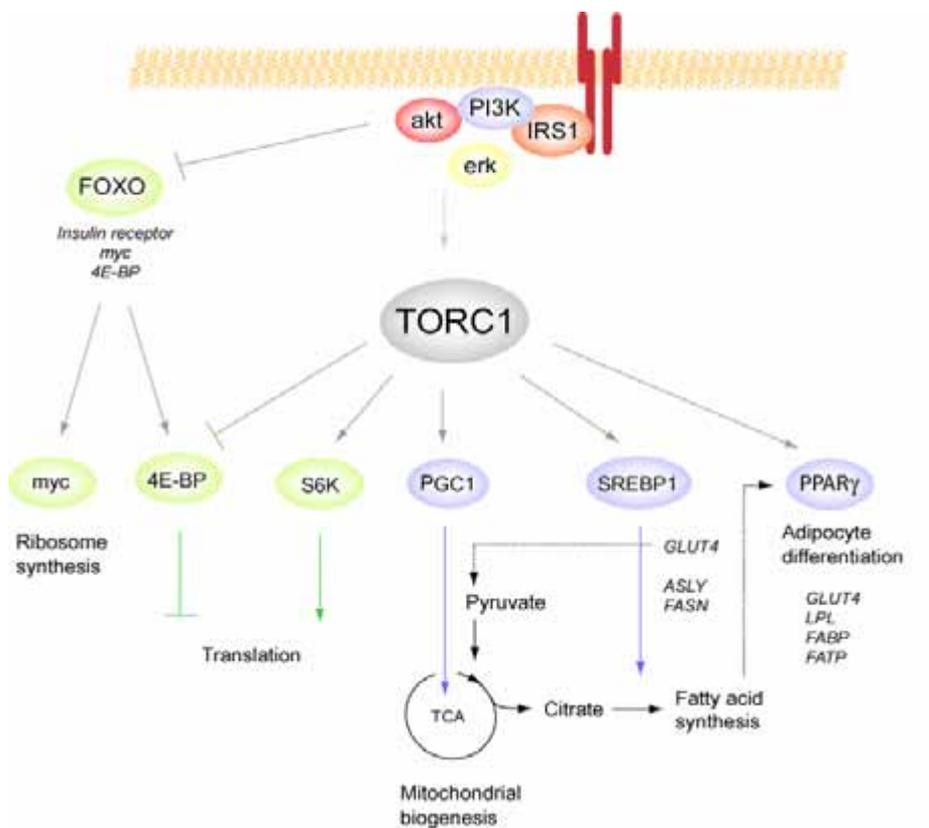


Figure 7 TORC1 controls signalling proteins involved in protein and lipid synthesis. Adapted from (138)

Furthermore, SREBP-1c controls the expression of PPARgamma, which is another master regulator of lipid synthesis and adipocyte differentiation. LPL, FABP and FATP, are targets of PPARgamma and are involved in hydrolysis of lipoprotein triglycerides and fatty acid uptake. As fatty acids are activating ligands for PPARgamma, newly produced fatty acids, stimulated by SREBP-1c, may further activate PPARgamma. In addition to fatty acids, PPARgamma is activated through phosphorylation by TORC1. Consequently, insulin activates PPARgamma in at least three different ways; SREBP1 controls the expression, TORC1 controls the activity by phosphorylation, and newly synthesised fatty acids may act as activating ligands, which emphasize the cooperative and additive behaviour in this pathway. In support for the pivotal role of TORC1 in adipocytes' differentiation and lipid metabolism, it has been shown that rapamycin inhibits adipogenesis (142, 143) while TORC1 activation by TSC1-TSC2 deficiency promotes adipogenesis, even in the absence of insulin (144) (Fig. 7).

Insulin stimulation of protein synthesis

As adipocytes are professional fat handling cells, insulin-stimulated triglyceride synthesis has been dissected in adipocytes by several research groups while there are almost no studies done on insulin-stimulated protein synthesis in adipocytes.

Generally, insulin stimulates protein synthesis by; (1) enhancing the biogenesis of ribosomes, (2) down-regulation of 4E-BP that inhibits the initiation of translation, and by (3) phosphorylation and activation of S6K that recruits eIF4B to promote translation (145). These events emerge mainly from the IRS1-PI3K pathway that via PKB and PKB/ERK activates FOXO and TORC1, respectively. In a basal state FOXO is located in the nucleus where it transcribes 4E-BP to create rising levels of 4E-BP, which inhibit initiation of translation in the absence of insulin. Insulin phosphorylates FOXO, thereby promoting its translocation out of the nucleus, turning transcription of 4E-BP off. Whereas FOXO controls the amount of 4E-BP mRNA, TORC1 control 4E-BP activity. TORC1 phosphorylate 4E-BP leading to its dissociation from the cap complex, thus promoting assembly of the eIF4F complex and recruitment of ribosomes. In addition TORC1 activates S6K, which participates in the recruitment of eIF4B to the eIF4F complex.

There are, however, indications of an adipocyte specific up-regulation of ribosome biogenesis during starvation or calorie-restriction, likely reflecting the need for adipocytes to be metabolically active in order to mobilize stored lipids during nutrient deprivation (146, 147). In *Drosophila*, feeding enhances myc expression in muscle tissue, while myc expression in the fat body is inhibited by a FOXO-dependent mechanism (146). Since myc is known to regulate ribosomal RNA synthesis (148, 149) it is possible that such a FOXO-dependent adipocyte-specific mechanism ensures that adipocytes remain metabolically active during starvation.

Insulin inhibition of autophagy

In general, autophagy is a primitive response to starvation in which cellular material is broken down to secure an uninterrupted supply of oxidizable substrates, thus safeguarding cell survival. Autophagy has not been examined in adipocytes and it is therefore not at all known what role autophagy plays in adipocytes. Since adipocytes largely consist of a big oil droplet one would think that it should take long before this cell type starves. It is possible that autophagy in adipocytes, rather than being a way for the cell to survive, is important in the breakdown of triglycerides for release of fatty acids during whole body starvation, thus promoting whole body survival. In *drosophila* larva, starvation induces a rapid induction of autophagy in the fat body thereby promoting degradation of lipid droplets and release of stored energy (150). Autophagy is vital for survival during starvation and inhibition of autophagy leads to profound nutrient sensitivity (150). In addition, it has very recently been found that in mouse hepatocytes proteins involved in autophagy are associated with lipid droplets and that autophagy regulates lipid stores (151). It is therefore possible that, in addition to triglyceride hydrolysis by the hormone sensitive lipase and adipose tissue triglyceride lipase, autophagy induced lipid droplet degradation in lysosomes contributes to the release of free fatty acids during times of whole-body fasting or starvation.

Inhibition of autophagy is under control by extra-cellular nutrients and, more importantly in multicellular organisms, growth factors such as insulin (152). Insulin signals nutrient availability and inhibits autophagy via PI3K-TORC1. In autophagosome biogenesis, proteins from the autophagy related genes (atg proteins) form a vesicle of unknown origin to a cup-shaped structure with double membranes, a phagophore (153) (Fig. 8). During an elongation phase atg8/LC3 is recruited to and conjugated with phosphatidylethanolamine in the phagophore membrane. The growing phagophore engulfs cytosolic components, including organelles, eventually enclosing them to form a vesicular autophagosome. When the autophagosome fuses with a lysosome, lysosomal acid hydrolases degrade the content of the autolysosome and macromolecules are released into the cytosol through permeases in the membrane. In the short run autophagy enhances starvation tolerance, but in extreme it will eventually lead to cell death.



Fig 8 In autophagosome biogenesis a cup-shaped phagophore, with a double bi-layer membrane, grows to form an autophagosome. During this elongation phase intracellular organelles are trapped inside the autophagosome and when fusing with a lysosome the content inside the autophagosome is digested and released through permeases in the membrane.

The details of TORC1 inhibition of autophagy is mostly studied in yeast where it has been shown that active TORC1 phosphorylate atg13, which inhibits the binding to atg1, thereby inhibiting the formation of atg13-atg1 complex needed for autophagy (154).

Insulin resistance in human adipocytes from type 2 diabetic patients

Attenuation of a positive feedback mechanism to IRS-1 (Paper II-III)

We find that in adipocytes from type 2 diabetic patients the EC50 for insulin-stimulated tyrosine phosphorylation of the insulin receptor is normal (155), despite reduced levels of insulin receptor protein (156), but the EC50 for tyrosine phosphorylation of IRS-1 is substantially higher than normal (Paper II). This means that adipocytes from type 2 diabetic patients need higher insulin concentrations to induce the same degree of phosphorylation/activation of IRS-1. The normal EC50 value of insulin receptor autophosphorylation/activation indicates that, in type 2 diabetes, IRS-1 must be regulated by other mechanisms than reduced activity of the receptor (Paper II)(155). The reduced insulin-stimulated tyrosine phosphorylation of IRS-1 cannot be explained by reduced amounts of IRS-1 protein (Paper II) (156). There is however a report that claims that there are reduced amounts of IRS-1 protein in adipocytes from type 2 diabetic patients (157). Down-stream of IRS-1, we find that adipocytes from type 2 diabetic patients have reduced insulin-stimulated activation of ERK (Paper III), but maintained activation on PKB (156), and reduced insulin-stimulated glucose uptake (155).

There are several kinases that by phosphorylation of IRS-1 at serine residues regulate the insulin receptor's capability to phosphorylate IRS-1 at tyrosine residues. Most serine phosphorylations of IRS-1 are considered to be inhibitory to IRS-1 function, but there are several examples where serine phosphorylation correlates positively with tyrosine phosphorylation (83-86). Interestingly, insulin-stimulated phosphorylation at Ser-307, which correlates positively with insulin-stimulated tyrosine phosphorylation of IRS-1, is

attenuated in adipocytes from type 2 diabetic patients (Paper II). It is not known which serine kinase that executes the Ser-307 phosphorylation, but since it is rapamycin-dependent and probably not mTOR (158), a good candidate is S6K that is located downstream of TORC1 in the insulin-signalling cascade. Indeed, isolated S6K has been shown to phosphorylate IRS-1 at Ser-307 in vitro (159).

Interventions that attenuate insulin-induced Ser-307 phosphorylation, such as starvation, incubation with RBP4, or rapamycin, correlate well with reduced insulin-stimulated tyrosine phosphorylation of IRS-1 (Paper II,III)(158), mimicking the situation in adipocytes from type 2 diabetic patients (Paper II). There are, however, conflicting reports, suggesting that phosphorylation at Ser-307 has a negative effect on insulin-stimulated tyrosine phosphorylation of IRS-1 (160, 161). Since phosphorylation of IRS-1 at Ser-307 is inhibited by rapamycin (a specific inhibitor to TORC1) and by starvation, which via several mechanisms inhibits TORC1, it has been suggested that this positive feedback fulfils a role in coordinating insulin signalling with nutrient availability (158).

It appears that phosphorylation of IRS-1 at Ser-307 is associated with signalling for mitogenic control via ERK, rather than metabolic control via PKB (Fig. 9). In support of this idea: (1) replacement of Ser-307 with alanine reduced insulin-stimulated phosphorylation of S6K and DNA synthesis (158); (2) incubation of adipocytes with RBP4, which attenuates Ser-307 phosphorylation of IRS-1, reduces insulin-stimulated ERK phosphorylation without affecting PKB activity or glucose uptake (Paper III); (3) adipocytes from type 2 diabetic patients display reduced insulin-stimulated phosphorylation of IRS-1 at tyrosine and Ser-307 concurrently with reduced insulin-stimulated ERK phosphorylation (Paper II,III); and (4) adipocytes from type 2 diabetic patients incubated with RBP4-blocking antibodies, which inhibit autocrine RBP4 signalling, show enhanced insulin-stimulated Ser-307 phosphorylation of IRS-1 and enhanced phosphorylation of ERK, without any effects on glucose uptake (Paper III).

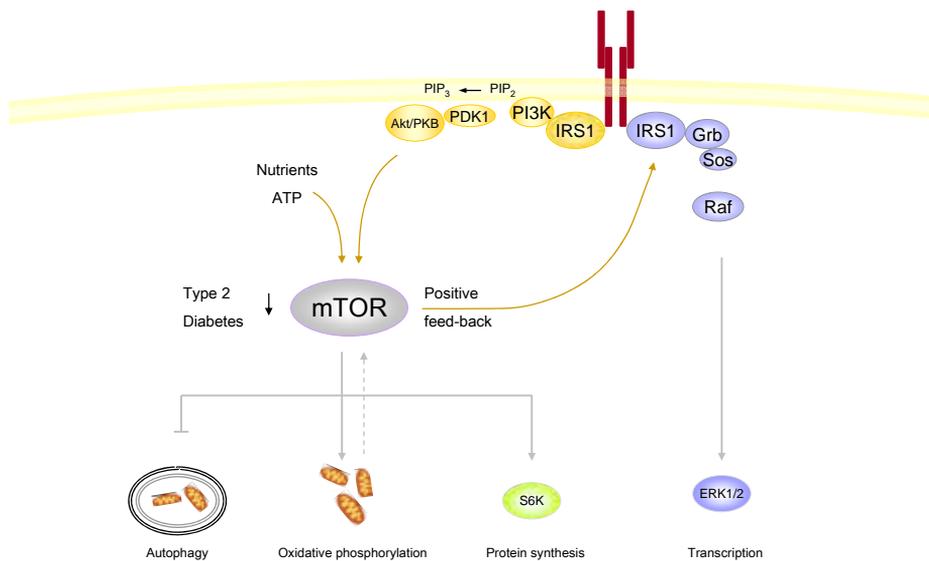


Figure 9. In adipocytes from type 2 diabetic patients there is a reduced insulin-sensitivity for tyrosine phosphorylation of IRS-1 and phosphorylation of ERK, but not of PKB/akt. The reduced insulin-sensitivity of tyrosine phosphorylation of IRS-1 appears to be the result of reduced positive feed-back signal from TORC1. Reduced TORC1 activity is demonstrated as enhanced autophagy, reduced amount of mitochondria, and attenuated insulin-stimulated phosphorylation of S6K at Thr-389.

Surprisingly, RBP4-induced reduction of insulin-stimulated tyrosine phosphorylation of IRS-1 is not transmitted to reduced PKB phosphorylation (Paper III). This might be explained by two pools of IRS-1 (Fig. 9) or that only phosphorylation of IRS-1 at tyrosine residues specific for Grb2 is affected. Regardless of the mechanism, there are a number of examples with reduced insulin-stimulated tyrosine phosphorylation of IRS-1, with maintained insulin-stimulated PKB phosphorylation, in the literature. Truncated IRS-1, consisting of residues 1-309 only, fails to promote PI3K activity but still activates PKB (162). In muscle from type 2 diabetic patients there is reduced insulin-stimulated tyrosine phosphorylation of IRS-1, with maintained insulin-stimulated PKB phosphorylation (163). Moreover, overexpression of PTP1B resulted in a 50-60% decrease in insulin-stimulated tyrosine phosphorylation of insulin receptor and IRS-1,

which was transferred to a reduced phosphorylation of ERK, whereas PKB and glucose uptake were not affected (79).

In conclusion, in adipocytes from type 2 diabetic patients we see an attenuated insulin-stimulated phosphorylation of IRS-1 at Ser-307 and subsequently a reduced insulin-stimulated tyrosine phosphorylation of IRS-1. Simultaneously we see reduced insulin-stimulated phosphorylation of ERK, normal insulin-stimulated phosphorylation of PKB, and reduced insulin-stimulated glucose uptake. These effects can be explained by reduced TORC1 activity, implying that adipocytes from type 2 diabetic patients, despite the correlation to obesity, are in a starvation mode (Fig 9). Consistent with a hypo-active TORC1, we do not find elevated phosphorylation of IRS-1 at Ser-312 in human adipocytes from type 2 diabetic patients (Paper III).

Most studies of phosphorylation of IRS-1 at serine residues are made in mice or cell lines derived from mouse. When comparing data from human and mouse studies it is important to be aware of the different nomenclature. Ser-307 in human IRS-1 corresponds to Ser-302 in mouse IRS-1 and Ser-312 in human IRS1 corresponds to Ser-307 in mouse IRS-1 (Fig. 10)

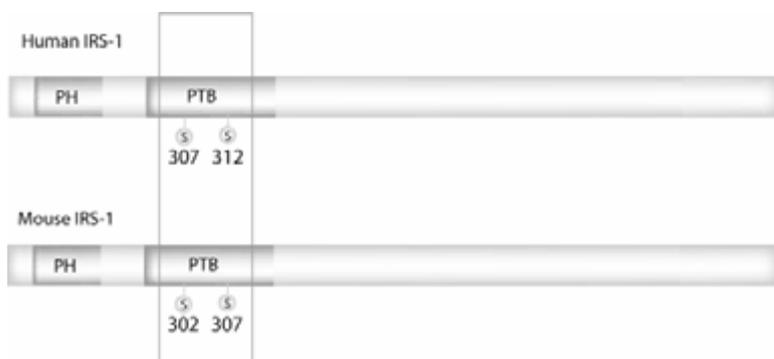


Figure 10 Schematic illustrations of human and mouse IRS-1. Ser-307 in human IRS-1 corresponds to Ser-302 in mouse IRS-1 and Ser-312 in human IRS1 corresponds to Ser-307 in mouse IRS-1.

In contrast to our data that don't show a correlation between attenuated insulin-stimulated phosphorylation of IRS-1 at Ser-307, PKB phosphorylation and glucose uptake, a study in mouse C2C12 muscle cells shows that phosphorylation of IRS-1 at Ser-302 (corresponding to human Ser-307) is coupled to enhanced PKB phosphorylation and enhanced glucose uptake (86). In the same study, it is also shown that phospho-Ser-302 is a binding site for PKC λ/ζ . Interestingly, PKC λ/ζ is involved in insulin stimulation of GLUT4 translocation and glucose uptake. PKC λ/ζ is mainly active in the endosomal fraction (128), where it mediates functional coupling between rab4 on the GLUT4 vesicle and the motor protein kinesin, thus promoting transport of GLUT4 via microtubules to the plasma membrane (129). Activation of PKC λ/ζ by insulin is dependent on PI3K and PDK1 located in the plasma membrane (119). Considering that PKC λ/ζ doesn't have a SH2 domain, it is possible that the binding to phospho-Ser-307 in IRS-1 is crucial for correct targeting close to PDK1. In support of this, we see an attenuation of insulin-stimulated phosphorylation of PKC λ/ζ in adipocytes from type 2 diabetic patients (unpublished data, A. Danielsson)

It has been proposed that nutrient overload, which negatively affects insulin signalling through enhanced TORC1-S6K mediated phosphorylation of IRS1 at serine residues, is the cause of insulin-resistance in type 2 diabetes (164). This is not what we see in adipocytes from type 2 diabetic patients. We see an attenuated TORC1-dependent positive feedback to IRS-1, indicative of reduced TORC1 activity. Our results in human adipocytes do, however, not exclude the existence of a negative TORC1-dependent feedback signal to IRS-1. Danielsson et. al. have shown that in human adipocytes phosphorylation at Ser-307 and Ser-312 of IRS-1 is separated in time. Insulin-stimulated phosphorylation at ser-307 reaches its maximum in only a few minutes, while insulin-stimulated phosphorylation at Ser-312 has its maximum first after 30-60 minutes (165). Phosphorylation of Ser-312, moreover, requires much higher concentration of insulin compared with phosphorylation of Ser-307 (165).

Reduced TORC1 activity and increased autophagy (Paper IV)

In adipocytes from type 2 diabetes patients the activation of TORC1 in response to insulin is reduced as determined by the phosphorylation state of mTOR at Ser-2448 and the TORC1 substrate S6K at Thr-389 (Paper IV). Consistent with the finding of reduced TORC1 activity, adipocytes from type 2 diabetic patients also exhibit increased autophagic activity, determined as punctate LC3 appearance, and increased amount of autophagosomes, determined by their typical morphological appearance in the electron microscope (Paper IV). In addition, lipofuscin-particles are almost absent in adipocytes from type 2 diabetic patients, but increase with age in non-diabetic patients. Activation of autophagy with rapamycin over-night strongly reduces the number of lipofuscin-particles, suggesting that the reduction seen in the diabetic state is due to enhanced autophagy (Paper IV).

TORC1 controls mitochondrial oxygen consumption and oxidative capacity (166), at least partly through a YY1-PGC1alpha transcriptional complex (139). Indicative of reduced TORC1 activity in adipocytes from type 2 diabetic patients, we found reduced levels of PGC1alpha mRNA and reduced amounts of the mitochondrial electron transport protein UQCRC2, simultaneously with reduced mitochondrial volume determined by electron microscopy (Paper IV).

In conclusion, reduced insulin-stimulated phosphorylation of S6K at Thr-389, reduced amount of PGC1alpha mRNA, with concurrently reduced mitochondrial function and volume, and enhanced autophagy, clearly indicate that there is reduced TORC1 activity in adipocytes from type 2 diabetic patients.

Multiple studies have conclude that excess nutrients, hyperinsulinemia, or other manipulations that result in over-activation of the TORC1-S6K pathway induce insulin resistance characterized by low levels of IRS-1 protein (160, 167-171). This is not what we see in adipocytes from type 2 diabetic patients. In human diabetic adipocytes, we find maintained levels of IRS-1 (155) and a hypo-active TORC1-S6K pathway, which is consistent with the discovery of an attenuated TORC1-dependent positive feedback to

IRS-1 (Paper II). In this scenario, active TORC1 is needed to propagate insulin signalling by enhancing tyrosine phosphorylation of IRS-1, and inhibition of TORC1 by rapamycin leads to insulin-resistance (Fig. 9).

Supportive of our findings, long-term treatment with rapamycin in humans to prevent rejection of kidney transplants causes worsening of insulin resistance and in some cases newly onset of diabetes (172), with hypo-activation of insulin-induced tyrosine phosphorylation of IRS-1 in the presence of maintained protein levels of IRS-1 (15), which mimics the situation we see in adipocytes from type 2 diabetic patients. It should also be noted that rapamycin extends life-span in mice, which usually is a characteristic of reduced insulin signalling (173). Moreover, long-term treatment with rapamycin protects against high-fat diet related obesity in rat, consistent with the pivotal role of TORC1 in adipocyte differentiation. Interestingly, long-term rapamycin treatment simultaneously induces glucose intolerance characterized by reduced phosphorylation of TORC1 and S6K (174).

Dysregulation of TORC1 activity

Short-term over-eating results in enhanced TORC1 activity

Over-eating and obesity clearly increases the risk for the development of insulin resistance and it is easy to assume that insulin resistance is a pathological condition since it increases the risk for type 2 diabetes, but it is quite possible that it is a normal physiological response. In support of this notion it has been shown that young lean healthy humans on a high calorie diet for only four weeks became mildly systemically insulin resistant and showed signs of liver steatosis (175). Subcutaneous fat biopsies were taken before and after the intervention and mRNA levels were compared with micro-array technique. These data were combined with data from a study with obese patients that were on a very-low-calorie-diet for 16 weeks followed by 2 week re-feeding period (176). Genes were selected that were up- or down-regulated after the very-low-calorie-diet and had the opposite behaviour after re-feeding and after high calorie diet (147). The majority of mRNA that fulfilled these criteria could be grouped into either protein or fatty acid synthesis pathways. Clearly, high calorie diet up-regulates and low calorie diet down-regulates fatty acid synthesis capacity in subcutaneous human adipocytes.

When it comes to mRNA coding for proteins involved in protein synthesis the picture is more complicated. High-calorie diet down-regulates and low-calorie diet up-regulates genes involved in ribosome biogenesis and protein synthesis (4E-BP) (147). As 4E-BP is an inhibitor of translation and availability of ribosomes must be conducive for translation, it is difficult to interpret what the total outcome will be. In other words, during starvation the capacity for protein synthesis is higher but also more strongly inhibited.

So what nutrient sensing pathways can be involved in response to high and low calorie diet respectively? Many of the genes coding for proteins involved in fatty acid synthesis are under control of SREBP-1c, a lipid synthesis master-regulator that in turn has been shown to be under control of the nutrient and stress sensor TORC1 (138, 177).

Consequently, both TORC1 and SREBP-1c are activated by insulin and feeding, while inhibited by starvation. In addition to genes of lipid synthesis, it has been shown that SREBP-1c controls GLUT4 expression in adipocytes (141). GLUT4 expression in adipocytes is, similarly to genes in lipid synthesis, down-regulated during fasting (178, 179). It makes sense that the nutrient sensor TORC1 in adipocytes enhances the capacity for glucose uptake and fatty acid synthesis during high calorie eating. During starvation, on the other hand, inhibition of TORC1 is important to induce autophagy, which in adipocytes might be involved in lipid droplet break-down and release of stored energy (Paper IV)(151).

Ribosomal biogenesis is controlled by TORC1 and another nutrient sensor, FOXO. It has been shown that they probably converge on Myc for coordination (146). In *Drosophila*, there is FOXO-dependent up regulation of mRNA involved in ribosome biogenesis selectively in the fat body during starvation, while in muscle tissue the corresponding mRNA is as expected down regulated (146). This tissue-specific difference may reflect that fat tissue is highly active during starvation to maintain whole body homeostasis, while muscle protein synthesis is down-regulated to save energy. It is possible that a similar mechanism exists in human adipocytes. If so, FOXO could explain the fluctuation of proteins involved in ribosomal biogenesis during feeding and fasting. Nuclear FOXO also transcribes 4E-BP, which is up-regulated by low-calorie diet and down-regulated by re-feeding and by high-calorie diet.

Even though other pathways can't be excluded; high calorie diet up-regulation and low calorie diet down-regulation of fatty acid synthesis capacity in subcutaneous human adipocytes correlate intimately with and can be control by TORC1 activity. In parallel, high calorie diet down-regulation and low calorie diet up-regulation of mRNA coding for proteins involved in ribosomal biogenesis and for 4E-BP can be explained by FOXO activity (Fig. 11).

Interestingly, both TORC1 and FOXO have been shown to be involved in the development of insulin resistance. High-fat feeding in mice markedly enhances TORC1 dependent phosphorylation of S6K, resulting in an S6K dependent phosphorylation at

Ser-307 (180), which has been shown to decrease insulin-stimulated phosphorylation of IRS-1 by various mechanisms (181). When it comes to FOXO, it has been shown that in drosophila and mouse cells insulin represses FOXO1 dependent transcription of the insulin receptor, thus creating a direct negative feed-back in insulin signalling (182)(Fig. 11). In other words, insulin stimulation results in less insulin receptors that can induce insulin resistance in an uncomplicated way, at least in drosophila and mice.

The feeding-induced down-regulation of insulin receptors is also apparent in rat and human adipocytes. Rats that are food deprived over-night rapidly down-regulate the level of insulin receptor mRNA when fed (183). One hour after feeding, the insulin receptor mRNA is reduced by more than 20%, and after 8-16 hours it is reduced to half. At the same time the insulin binding capacity drops to similar levels. In parallel, mRNA for the lipogenic enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) are robustly increased. This elevation is slower than the down-regulation of insulin receptor mRNA, starting at 4 hours and reaching its maximum after 16-24 hours. Adipocytes from humans on a high carbohydrate diet for two or four weeks, present reduced insulin binding (184) and reduced insulin receptor protein levels (156), respectively.

Apparently, insulin resistance in the adipose tissue as a response to feeding can be a physiological event explained by known nutrient sensing pathways. But what is the function of adipose insulin resistance in feeding? Several explanations can be envisioned; 1) it is a way to terminate the signal, 2) elevation of insulin receptors during fasting allows a quick response when eating after a period of fasting, and/or 3) adipocyte insulin resistance results in higher plasma levels of nutrients and insulin, promoting a higher anabolic state. Supportive of this view is that short-term overeating in healthy humans results in insulin resistance concurrent with elevated muscle mass in the absence of exercise (175).

In adipocytes, fasting or a low calorie-diet promotes insulin sensitivity via elevation of insulin receptor mRNA, but there is also a parallel reduction of the amount of GLUT4 mRNA, creating a period of time for increased insulin sensitivity. Prolonged fasting or

starvation should eventually promote insulin resistance due to GLUT4 reduction (178) and attenuated insulin-stimulated phosphorylation of IRS-1 at Ser-307 (human sequence)(Paper II).

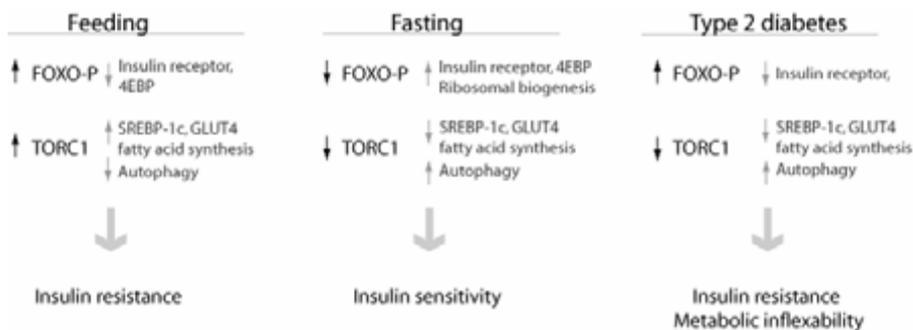


Figure 11 Feeding and fasting induce insulin resistance and insulin sensitivity, respectively. Insulin resistance in adipocytes from type 2 diabetic patients presents reduced levels of SREBP-1c mRNA, inconsistent with obesity related over-eating. Prolonged fasting or starvation, though, should eventually result in insulin resistance due to down regulation of GLUT4 and attenuation of insulin-stimulated phosphorylation of IRS-1 at Ser-307.

Adipocytes from type 2 diabetic patients have attenuated TORC1 activity

There are numerous studies showing reduced levels of insulin receptor protein in adipose tissue from type 2 diabetic patients, possibly reflecting activated FOXO due to obesity related over-eating (see table 2) (Fig. 11). TORC1-SREBP-1c dependent genes, on the other hand, are inappropriately down-regulated considering the obesity related over-eating and high insulin levels (see table 3)(Fig. 11). Moreover, consistent with reduced TORC1 activity, adipocytes from diabetic patients do not respond with the expected rise in SREBP-1c in response to insulin, which results in metabolic inflexibility. Analogously to SREBP-1c, GLUT4 is normally down-regulated during fasting and up-regulated by feeding and in response to insulin, but in adipocytes from type 2 diabetic patients there are reduced amount of GLUT4 mRNA, inconsistent with obesity related over-eating and high insulin levels (Fig. 11)(185, 186). It has been

shown that GLUT4 actually is under control of SREBP-1c, thereby explaining the coordinated regulation of GLUT4 and SREBP-1c during fasting and eating (141).

Short Description	Year	Ref
Reduced insulin binding in T2D	1977	(187)
Reduced insulin binding in T2D	1981	(188)
Reduced insulin binding and tyrosine activity in T2D	1987	(189)
Reduced insulin receptor tyrosine activity in T2D	1987	(190)
Reduced amount of insulin receptor protein in T2D	2009	(156)
Reduced amount of insulin receptor protein in overweight	2009	(191)

Table 2 Adipocytes from type 2 diabetic patients have reduced amounts of insulin receptors, which is indicative of phosphorylated FOXO and consistent with obesity-related over-eating.

Gene	Short description	Ref
FAS, ACC, SREBP-1c	Reduced mRNA in obese subjects	(133)
SREBP-1c	Reduced mRNA and blunted up-regulation in response to insulin in muscle and adipose tissue from T2D patients	(135)
SREBP-1c	Blunted up-regulation in response to insulin in adipose tissue from T2D patients	(134)
SREBP-1c	Reduced mRNA in obese and diabetic patients	(192)
SREBP-1c	Reduced mRNA in diabetic patients	(193)
FAS	Elevated mRNA but reduced protein in obese patients	(194)
ACLY, ACACA, FASN, PPARG, DGAT1, DGAT2	Lipogenic genes correlates negatively to BMI and cell size	(195)
FAS, SREBP-1c	Reduced mRNA in obese subjects	(196)
FAS	Reduced mRNA and blunted up-regulation in response to two weeks of high carbohydrate eating in obese subjects	(197)
	Strong negative correlation between fat cell lipogenesis and HOMA-IR	(198)
FAS, DGAT1	mRNA correlates positively with insulin sensitivity	(199)

Table 3 Adipocytes from type 2 diabetic patients have reduced amounts of SREBP-1c mRNA indicative of reduced TORC1 activity, which is inconsistent with obesity related over-eating.

As a consequence of reduced TORC1-SREBP-1c activity, there is enhanced autophagy in adipocytes from type 2 diabetic patients (Paper IV). Moreover, other targets of TORC1, such as PGC1alpha mRNA levels and insulin-stimulated phosphorylation of S6K at Thr-389, are down-regulated in adipocytes from type 2 diabetic patients (Paper IV). It has been shown that TORC1 via YY1-PGC1alpha controls mitochondrial biogenesis (139) and there are reports of reduced amount of mitochondria in adipose tissue from type 2 diabetic patients (Paper IV)(200-206). Altogether, loads of evidence point to reduced TORC1 activity in adipocytes from type 2 diabetic patients.

The etiology of reduced TORC1 activity

The etiology of insulin resistance and type 2 diabetes is probably multifaceted with both inherited and acquired components. Relatives of type 2 diabetic patients have a higher risk to develop diabetes, but life-style clearly plays a central role (207). This has been known for long and it has been proposed that there are inherited factors that make individuals more sensitive to high calorie eating. One can also think about it as parallel events that add up.

Middle-aged, non-obese and non-diabetic relatives of type 2 diabetic patients are systemically insulin resistant (208) with reduced expression of both PPARgamma and SREBP-1c in their adipose tissues (209). Down-regulated TORC1-SREBP-1c activity can therefore be one of the inherited components in insulin resistance. Consistent with an inherited down-regulated TORC1-PGC1alpha activity, young lean insulin-resistant offspring of parents with type 2 diabetes have impaired mitochondrial activity in muscle (205). Moreover, a twin study with monozygotic and dizygotic twins confirms that there is an inherited part in PGC1alpha expression in muscle that at least partly is influenced by a PGC-1 α Gly482Ser polymorphism (210).

In a Finnish twin study with 2 453 young healthy monozygotic twin-pairs only 14 pairs exhibited large weight difference between the siblings, confirming that there are genetic factors in the development of obesity (206). The obviously acquired obesity in one of the twins correlated with systemic insulin resistance, larger adipocytes, reduction of

mitochondrial DNA, and disturbed mitochondrial metabolism in the adipose tissue. Simultaneously, several pathways involved in inflammation were up-regulated in the adipose tissue of the obese twin. The result from this twin study complements earlier studies that has shown that large adipocytes correlate with enhanced expression of pro-inflammatory adipokines (34, 35). Moreover, large adipocytes are also associated with reduced capacity for fatty acid synthesis (195) and there is a strong correlation between mitochondrial DNA and the adipocytes' ability to synthesize fatty acids (140). Altogether, long-term overeating-associated obesity results in larger adipocytes that are insulin resistant with reduced amount of mitochondrial DNA and reduced capability to synthesize fatty acids. As TORC1 regulates mitochondrial biogenesis via PGC1alpha and fatty acid synthesis capacity via SREBP-1c, it is possible that acquired obesity and concurrent insulin resistance in adipocytes is associated with reduced TORC1 activity. These data suggest that, in contrast to short-term overfeeding (Fig. 11), obesity associated long-term overfeeding results in down-regulation of TORC1. One speculation is that TORC1 activation is down-regulated as a consequence of long-term down-regulation of insulin receptor mRNA.

It can be envisioned that acquired insulin resistance, consisting of both a physiological insulin resistance associated with short-time over-eating and a pathological insulin resistance associated with long-time over-eating, adds up with the inherited insulin resistance to create a pathological situation with high insulin resistance, which eventually challenges the insulin producing beta-cells.

Nutrient sensing and secretion of RBP4

The amount of RBP4 in plasma correlates with systemic insulin resistance (23) and RBP4 induces insulin-resistance in adipocytes (Paper III). Considering the close correlation between obesity and insulin resistance in humans, and that adipocyte-secreted RBP4 induces insulin-resistance in mice (22), it is possible that adipocyte-secreted RBP4 is causative in insulin resistance. But what controls RBP4 secretion from adipocytes? And is there a connection to reduced TORC1 activity?

In mice, adipocyte specific knockout of GLUT4 enhances the transcription of RBP4 (22), and in humans there is an inverse correlation between GLUT4 and RBP4 mRNA in both subcutaneous (23) and visceral adipocytes (211). GLUT4 has been shown to be down-regulation in adipocytes from obese mouse models of diabetes (212) as well as from patients with type 2 diabetes (185, 186). These data made Kahn and colleagues suggest that there is a “mechanistic link between reduced adipocyte GLUT4, elevated RBP4 and insulin resistance.” (23). Interestingly, in addition to obesity and insulin resistant states, fasting induces a rapid down regulation of GLUT4 in rat adipocytes (178, 179). If there is a GLUT4-RBP4-insulin resistance axis, it might have evolved as an energy saving mechanism to restrict glucose uptake by peripheral tissue during sparse conditions. Since obesity is highly correlated to insulin resistance and GLUT4 levels in adipocytes, it has been proposed that obesity might be mistaken for starvation (213).

In *Drosophila*, it has been shown that the homolog to RBP4, NLaz, is critical for metabolic adaptation during stress (214). NLaz loss-of-function mutants rapidly lose their stores of glycogen and lipids during starvation and are relatively short-lived, while over expression of NLaz promotes longevity, insulin resistance, smaller flies and stress tolerance. Considering that NLaz is expressed in the fat body, is up-regulated in response to starvation and promotes systemic insulin resistance, it fulfills all the criteria of being the nutrient sensing and systemic energy-saving mechanism suggested to exist in mammals (Fig 12). Intriguingly, *Drosophila* NLaz is under control by starvation and JNK, which both control TORC1 activity. It remains to see, though, if TORC1 directly regulates RBP4 expression and secretion.

In vitro experiment shows that RBP4 induces insulin resistance in human adipocytes from non-diabetic subjects. In adipocytes from type 2 diabetic patients blocking antibodies against RBP4 restore insulin signalling (Paper III). These data suggest that RBP4 acts in an autocrine manner to locally induce insulin resistance (Fig 12). As RBP4 attenuates TORC1 dependent phosphorylation at Ser-307 of IRS-1 in human adipocytes, it is possible that there is an autocrine feedback loop that sustains the insulin resistant milieu (Paper III)(Figure 12).

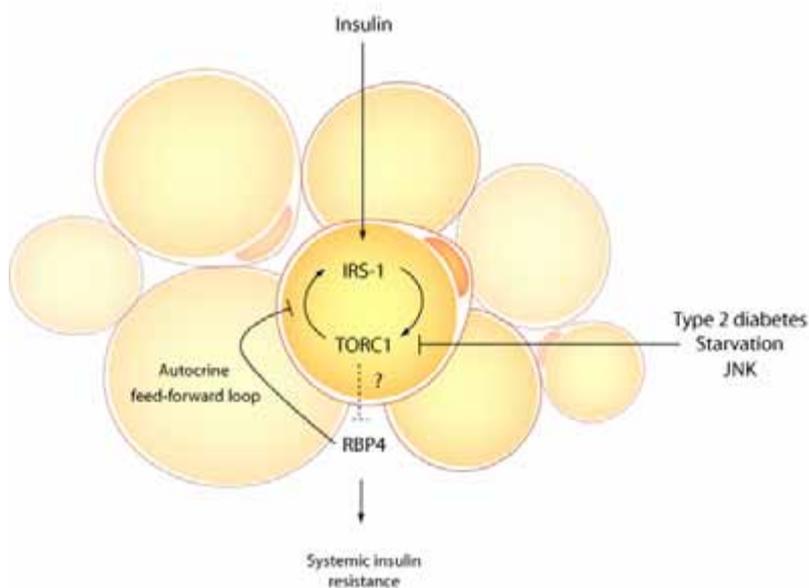


Figure 12 Role of adipocyte secreted RBP4 in local and systemic insulin resistance. NLaz, the drosophila analogue to RBP4, is secreted from the fat body in response to starvation and promotes systemic insulin resistance and starvation tolerance (214). In adipocytes from diabetic patients, there is reduced TORC1 activity (Paper IV) and RBP4 induces insulin resistance in an autocrine fashion by attenuation of the TORC1-dependent insulin-stimulated phosphorylation at Ser-307 of IRS-1 (Paper II, III).

In *Drosophila*, starvation simultaneously induces autophagic-mediated release of nutrients from the fat body (150) and a fat body-mediated humoral energy-saving inhibition of whole body insulin sensitivity (215). Analogously, it is possible that the perceived starvation in adipocytes from type 2 diabetic patients is transferred to enhanced secretion of RBP4, which induces systemic insulin resistance, a mechanism that probably has as a function to spare energy during starvation.

Concluding remarks and future perspective

We present evidence that adipocytes from type 2 diabetic patients exhibit hypoactive TORC1 and attenuation of insulin-stimulated phosphorylation of IRS-1 at Ser-307, which results in reduced tyrosine phosphorylation and activation of IRS-1. As a result of reduced TORC1 activity, adipocytes from type 2 diabetic patients exhibit enhanced autophagy, a primitive response to starvation where the cells digest intracellular components to provide substrates for energy production. In other words, adipocytes from type 2 diabetic patients, which due to obesity are over-loaded with fat, show signs of starvation. Since adipocytes modulate whole body metabolism by secretion of adipokines, the perceived starvation in adipocytes might have implication for whole body metabolism.

Is obesity mistaken for starvation? Or is it the other way around - reduced TORC1 activity leads to obesity. The thrifty gene hypothesis suggests that in a population there is an advantage to have individuals with genes set for scarce conditions. Reduced TORC1 activity in adipocytes could fulfil such a function. If so, adipose tissue would signal low energy reserves to the brain, to increase appetite, which in our environment with abundance of food leads to obesity. These are, however, unanswered questions that require further research.

Much of the experimental diabetes research around the world uses mouse models to study insulin resistance. In mice, high fat diet-induced insulin-resistance in adipose tissue is characterized by hyperactive TORC1. This is the opposite to what we find in adipocytes from type 2 diabetic patients. The diabetes research society thereby uses resources to study a model that is not in agreement with insulin resistance in type 2 diabetes.

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During the time I did the research for this thesis, my mother got the diagnosis type 2 diabetes bringing it to a more personal level for me. Hopefully, the combined efforts from scientist around the world will lead to a greater understanding of type 2 diabetes opening up for better treatments for my mother and the other 150 million persons that suffer from this disease. I'm glad to be a part of it.

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