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Glucose and lipid metabolism in insulin resistance

– an experimental study in fat cells

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CONTENTS

ABSTRACT

Type 2 diabetes is usually caused by a combination of pancreatic β-cell failure and insulin resistance in target tissues like liver, muscle and fat. Insulin resistance is characterised by an impaired effect of insulin to reduce hepatic glucose production and to promote glucose uptake in peripheral tissues. The focus of this study was to further elucidate cellular mechanisms for insulin resistance that may be of relevance for type 2 diabetes in humans. We used rat and human adipocytes as an established model of insulin's target cells.

Glucocorticoids, e.g. cortisol, can induce insulin resistance in vivo. In the present study, pretreatment of rat adipocytes in vitro for 24 h with the cortisol analogue dexamethasone produced a downregulation of glucose uptake capacity as well as a marked depletion of cellular insulin receptor substrate 1 (IRS-1) and protein kinase B (PKB), two proteins suggested to play a critical role in the intracellular signal transduction pathway of insulin. The amount of phosphorylated PKB in response to acute insulin treatment was decreased in parallel to total PKB content. The basal rate of lipolysis was enhanced, but insulin's antilipolytic effect was not consistently altered following dexamethasone pretreatment.

Alterations in blood glucose as well as insulin levels may be of great importance for cellular as well as whole-body insulin resistance. High glucose (≥15 mM) for 24 h induced a decrease in glucose uptake capacity in rat adipocytes and IRS-1 content was reduced whereas IRS-2 was increased. Long-term pretreatment with a high insulin concentration downregulated insulin binding capacity and when combined with high glucose, it produced a pronounced reduction of cellular IRS-1 and 2 content together with insensitivity to insulin's effect to activate PKB and a decrease in glucose uptake capacity. A common denominator for a decrease in glucose uptake capacity in our rat adipocyte studies seems to be a decrease in IRS-1 content.

Adipocytes from type 2 diabetes patients are insulin-resistant, but in our work the insulin resistance could be reversed by incubation of the cells at a physiological glucose level for 24 h. Insulin resistance in fresh adipocytes from type 2 diabetes patients was associated with in vivo insulin resistance and glycemic level and with adipocyte cell size and waist-hip ratio (WHR).

As a potential mechanism for postprandial dyslipidemia in type 2 diabetes, we examined the nutritional regulation of subcutaneous adipose tissue lipoprotein lipase (LPL) activity. It was upregulated by ~40-50 % after a standardised lipid-enriched meal and this was very similar in type 2 diabetes patients and control subjects, suggesting that the postprandial hypertriglyceridemia found in type 2 diabetes is not explained by an altered nutritional regulation of LPL in subcutaneous fat.

In conclusion, the present work provides evidence for novel interactions between glucocorticoids and insulin in the regulation of glucose metabolism that may potentially contribute to the development of insulin resistance. High levels of glucose and insulin produce perturbations in the insulin signalling pathway that may be of relevance for human type 2 diabetes. Cellular insulin resistance may be secondary to the diabetic state in vivo, e.g. via glucotoxicity. This is supported by our finding that insulin resistance in adipocytes from type 2 diabetes patients can be reversed after incubation at a physiological glucose level.

Key words: adipocyte, insulin resistance, type 2 diabetes, insulin signalling, glucose uptake, insulin, glucose, dexamethasone, insulin receptor substrate, protein kinase B, GLUT4, lipoprotein lipase.

LIST OF PAPERS

I) **Burén J, Liu H-X, Jensen J, Eriksson JW** 2002 Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes. Eur J Endocrinol 146: 419-429

II) **Burén J, Liu H-X, Lauritz J, Eriksson JW** 2002 High glucose and insulin in combination cause insulin receptor substrate-1 and 2 depletion and protein kinase B desensitisation in primary cultured rat adipocytes. Possible implications for insulin resistance in type 2 diabetes. Eur J Endocrinol, in press

III) **Burén J, Lindmark S, Renström F, Eriksson JW** 2002 In vitro reversal of hyperglycemia normalizes insulin action in fat cells from type 2 diabetes patients. Is cellular insulin resistance caused by glucotoxicity in vivo? Metabolism, in press

IV) **Eriksson JW, Burén J, Svensson M, Olivecrona T, Olivecrona G** 2002 Postprandial regulation of blood lipids and adipose tissue lipoprotein lipase in type 2 diabetes patients and healthy control subjects. Atherosclerosis, in press

ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ADA	adenosine deaminase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BMI	body mass index
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
DMEM	Dulbecco's modified Eagle's medium
EC_{50}	concentration producing half-maximal effect
FFA	free fatty acid
GFAT	glutamine fructose-6-phosphate amidotransferase
GLUT	glucose transporter
HbA_{1c}	Glycosylated hemoglobin A
HOMA-IR	homeostasis model assessment insulin resistance index
HL	hepatic lipase
IR	insulin receptor
IRS	insulin receptor substrate
LBM	lean body mass
LPL	lipoprotein lipase
mRNA	messenger ribonucleic acid
p70S6K	p70 ribosomal S6 kinase
PDK	phosphatidylinositol dependent protein kinase
PIA	N^6-(R-phenylisopropyl) adenosine
PI3-K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel
SEM	standard error of the mean
VLDL	very low density lipoprotein
WHR	waist to hip ratio

INTRODUCTION

Insulin resistance was first described in the 1930s when Himsworth reported diabetes patients who did not respond to insulin treatment (Himsworth 1936). Since then, our knowledge has increased considerably but still the underlying mechanisms are far from being fully understood. Insulin resistance has a strong predictive value with respect to development of type 2 diabetes and together with decreased insulin production from the β-cells of the pancreas it provides the pathophysiological background for the disease (Reaven 1988). Initially, β-cells compensate for insulin resistance by increasing insulin secretion and hyperinsulinemia develops. However, as time goes by, the β-cell function becomes altered and fails to compensate for increasing insulin resistance and, thus, blood sugar levels start to rise (Purrello 2000) and eventually clinical diabetes is established (Fig. 1).

This study aims at shedding some light on the cellular mechanisms behind insulin resistance that may be of relevance for type 2 diabetes in humans. For this purpose we have used adipocytes from rats and humans, since fat is a well-established model of insulin's target tissues that is suitable for experimental studies on carbohydrate and lipid metabolism.

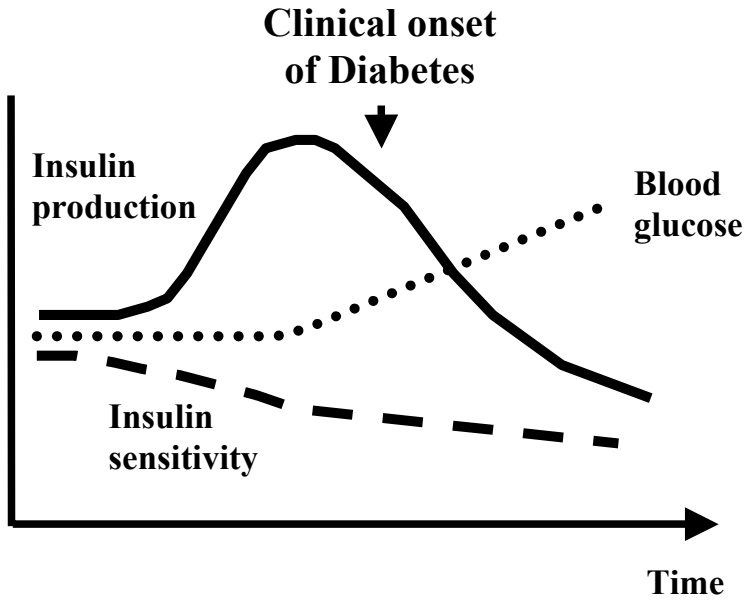

Figure 1.
Schematic illustration of the development and progression of type 2 diabetes.

Insulin resistance

Insulin resistance is a common denominator of many diseases in Western societies (Reaven 1988; DeFronzo 1991) and it is a central component in the so-called metabolic syndrome. Other proposed names are “syndrome X” and “the insulin resistance syndrome”. The definition of this syndrome has been extensively discussed. In 1998 the World Health Organization (WHO) recommended a definition of the metabolic syndrome that includes insulin resistance, impaired glucose tolerance (IGT) or type 2 diabetes as a necessary component combined with at least two among obesity, dyslipidemia, hypertension and microalbuminuria (Alberti 1998).

Insulin resistance can be defined as an impaired effect of a certain amount of insulin in target tissues, i.e. mainly muscle, fat and liver. Insulin resistance can manifest itself as either unresponsiveness or insensitivity to insulin. Unresponsiveness implies that there is an impaired maximal effect of insulin. Insensitivity, on the other hand, means that a higher insulin concentration than normal is necessary to produce a certain effect, i.e. the dose-response curve for insulin is shifted to the right (Kahn 1978). In most conditions of insulin resistance, there is a combination of unresponsiveness and insensitivity.

The role of insulin in glucose and lipid turnover

Glucose turnover

Glucose is the carbohydrate that is most commonly utilized for energy production in mammals. The brain needs glucose continuously and too low concentrations of glucose in the blood can result in seizures, unconsciousness and death. On the other hand, severely elevated blood glucose can lead to a medical emergency, and a chronically elevated glucose level in the blood may cause long-term organ damage, e.g. coronary, cerebrovascular and peripheral vascular disease, nephropathy, neuropathy and retinopathy. Consequently, it is important for the body to keep blood glucose levels within narrow limits. This is accomplished by a finely tuned hormonal system regulating glucose uptake by peripheral tissues and glucose production by the liver. During fasting, liver glycogen is broken down to glucose by glycogenolysis and released into the blood. Glycogen is also stored in muscle, but glucose derived from muscle glycogen cannot be transported into the circulation. Following prolonged fasting, glycogen is depleted and there is an increased synthesis of glucose, i.e. gluconeogenesis, from amino acids and glycerol in the liver. Even during prolonged starvation, gluconeogenesis prevents the occurrence of severe hypoglycemia.

After a meal, blood glucose levels increase and insulin plays a major role in keeping blood glucose levels within a narrow range, usually ~4-7 mM. The β-cells of the pancreas respond to increasing glucose levels by releasing insulin into the blood. Insulin affects glucose turnover in many tissues. In liver, insulin inhibits glycogenolysis and gluconeogenesis (DeFronzo 1987). In skeletal muscle, that accounts for approximately 75 % of insulin-mediated glucose disposal after a glucose challenge (DeFronzo 1981; DeFronzo 1985), insulin promotes glucose uptake into cells. When glucose has crossed the cell membrane it is immediately phosphorylated by hexokinase II. Glucose-6-phosphate is then used for glycogen synthesis or glycolysis. Insulin is involved in the regulation of glycogen synthase as well as key enzymes in glycolysis. After glycolysis, some glucose is converted to lactate that is released into the blood and then utilized for gluconeogenesis in the liver.

Insulin-stimulated glucose disposal in adipose tissue is quantitatively of minor importance compared to muscle. However, insulin has a potent effect to inhibit adipose tissue lipolysis and the release of glycerol and free fatty acids (FFAs) into the blood and this has major implications for glucose homeostasis. Increased levels and oxidation of FFAs are thought to contribute to development of muscle insulin resistance (Randle 1994; Boden 1997). In liver, FFAs blunt insulin's effects on hepatic glucose metabolism, and FFAs increase endogenous glucose production both by stimulating key enzymes and by providing energy for gluconeogenesis (Foley 1992). Moreover, glycerol released during triglyceride hydrolysis serves as a gluconeogenic substrate (Nurjhan 1992). Consequently, resistance to the antilipolytic action of insulin in adipose tissue results in excessive release of FFAs and glycerol that may have deleterious effects on glucose turnover and homeostasis.

Lipoprotein turnover

The majority of lipids in blood do not circulate in their free form. FFAs are bound to albumin, whereas triglycerides, phospholipids and cholesterol are transported in the form of lipoprotein complexes. The lipoproteins differ in size and lipid content, and the lipoprotein families are classified in terms of their density, as determined by centrifugation (reviewed by Mathews 1990) (Table 1). The lipoproteins consist of a hydrophobic core of triglycerides and cholestorol esters surrounded by phospholipids and proteins, so-called apoproteins.

Exogenous fat from a meal is digested and absorbed by the intestine and released into the blood stream as triglyceride-rich chylomicrons. Chylomicrons are formed in the intestinal mucosa following the absorption of the products of fat digestion. These large lipoproteins enter the blood via the lymphatic ducts. Lipoprotein lipase (LPL), an enzyme present on the capillary endothelium in, for

example, muscle and adipose tissue, hydrolyses triglycerides of chylomicrons generating fatty acids that partly is taken up by adipose and muscle tissue, where they can be stored or oxidized (Coppack 1989; Eckel 1989). Alternatively, the FFAs circulate in blood mainly bound to albumin. Chylomicrons depleted of their triglycerides remain in blood as cholesterol-rich lipoproteins called chylomicron remnants. They are carried to the liver where they are internalized by receptor-mediated endocytosis and degraded in lysosomes. LPL also removes triglycerides from circulating very low-density lipoproteins (VLDL). VLDL particles are formed in liver and transport triglycerides derived from FFAs and glycerol to extrahepatic tissues. When the triglyceride content is hydrolysed by LPL, the VLDLs become intermediate-density lipoproteins (IDL). Two other families of lipoproteins are the low-density lipoproteins (LDL) and the high-density lipoproteins (HDL), which are mainly involved in cholesterol transport. Meal-stimulated insulin release is followed by an activation of adipose tissue LPL leading to clearance of triglyceride-rich lipoproteins (TRLs) from blood (Pykalisto 1975; Lithell 1978). In contrast, skeletal muscle LPL activity is decreased in the postprandial state, possibly by the increase in glucose and/or insulin (Farese 1991). The opposite directions of meal-induced regulation of LPL activity in fat and muscle, respectively, probably serves to divert FFAs derived from triglyceride-rich lipoproteins away from muscle and to adipose tissue for lipid storage.

Table 1.
Properties of major human lipoprotein classes. From Mathews (1990).

		Composition (wt %)				
Lipoprotein	Density (g/ml)	Protein	Free cholesterol	Cholesterol ester	Phospho -lipid	Triacyl-glycerol
Chylomicron	0.92-0.96	1-2	1-3	2-4	3-8	90-95
VLDL	0.95-1.006	11	6	14	15	54
IDL	1.006-1.019	18	7	23	22	31
LDL	1.019-1.063	25	9	42	21	3
HDL	1.063-1.21	49	4	18	27	2

Insulin signalling

Fig. 2 schematically depicts cellular insulin signal transduction leading to activation of glucose transport. Insulin signal transduction is initiated by insulin binding to the extracellular domain of the insulin receptor. The cell surface insulin receptor is composed of two extracellular α subunits and two transmembrane β subunits that are disulfide-linked into a heterotetramer. Following insulin binding to the α subunits a transmembrane conformational change is generated that activates the β subunit tyrosine kinase domain.

Subsequently, the β subunits undergo a series of autophosphorylation reactions at specific tyrosine sites. However, in contrast to many other receptor tyrosine kinases that directly recruit effector molecules to the phosphorylated receptor, the insulin receptor phosphorylates several proximal intracellular target molecules that serve as docking sites for effector proteins. These proximal target molecules are tyrosine phosphorylated and then operate as multisite docking proteins. They include members of the insulin receptor substrate family (i.e. IRS-1, 2, 3 and 4), Shc (SH2 domain-containing oncogenic protein), signal-regulated proteins (SIRPs) and many others (reviewed by Virkamaki 1999). Tyrosine phosphorylation of these proteins creates recognition sites for both src homology (SH2) and phosphotyrosine binding (PTB) domains of downstream effector proteins. The IRS proteins are most extensively characterized and they are well documented as critical components in insulin signal transduction (White 1997).

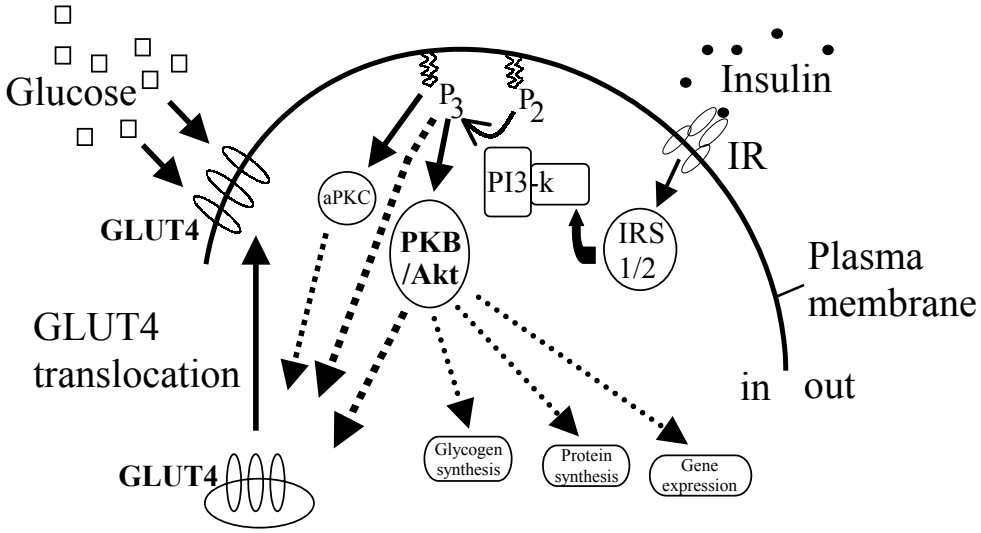

Figure 2.
The major insulin signalling pathway to GLUT4 translocation and glucose uptake. aPKC, atypical protein kinase C (isoforms λ and ζ); P_2, phosphatidyl-inositol-3,4-bisphosphate; P_3, phosphatidylinositol-3,4,5-triphosphate.

IRS-1 is the major tyrosine phosphorylated IRS induced by insulin stimulation in 3T3-L1 adipocytes (Sun 1992) and human adipocytes (Rondinone 1997). The tyrosine-phosphorylated IRSs provide SH2 domain binding sites for the regulatory subunit (p85) of the type 1A phosphatidylinositol 3-kinase (PI3-kinase). PI3-kinase is a lipid and protein serine kinase consisting of a regulatory

subunit that is responsible for binding to IRSs, and a catalytic subunit that promotes phosphorylation of phosphatidylinositols found in cellular membranes. There are several isoforms and splice variants of the Type 1A PI3-kinase. PI3-kinase appears to preferentially phosphorylate PI(3,4)P_2 to PI(3,4,5)P_3 (Domin 1997). The increase in PI(3,4,5)P_3 upon insulin stimulation correlates well with activation of downstream kinases (van der Kaay 1997). PI(3,4,5)P_3 is predominantly located in the plasma membrane (Oatey 1999). Multiple studies have established a critical role of PI3-kinase activation in insulin-stimulated translocation of glucose transporter 4 (GLUT4) to the plasma membrane, which facilitates glucose uptake. For example, inhibition of PI3-kinase activity by pharmacological inhibitors such as wortmannin and LY 294002, microinjection of PI3-kinase blocking antibody, expression of dominant-interfering p85 mutants or expression of PI(3,4,5)P_3 5′ phosphatase (SHIP) and PI(3,4,5)P_3 3′ phosphatase (PTEN) were reported to impair insulin-stimulated GLUT4 translocation and glucose uptake (Cheatham 1994; Okada 1994; Sakaue 1997; Sharma 1998; Vollenweider 1999; Nakashima 2000). The targets of PI3-kinase action, however, are still not fully elucidated.

Two classes of serine/threonine kinases are known to act downstream of PI3-kinase, namely the serine/threonine protein kinase B (PKB), also known as Akt, and the atypical protein kinase C isoforms λ and ζ (PKCλ/ζ). The formation of 3′ phosphoinositides activates the phosphoinositide-dependent protein kinase 1 (PDK1). This kinase phosphorylates PKB on threonine 308. Full activation of PKB also requires phosphorylation on serine 473 (Goransson 2002) that may result from the activation of another putative kinase, PDK2, or through an alteration in substrate recognition of PDK1 following threonine phosphorylation of PKB and/or via autophosphorylation (Balendran 1999; Toker 2000). There are three isoforms of PKB (α, β and γ), and β is the main isoform activated by insulin in adipocytes (Hill 1999). Initial studies expressing a constitutively active PKB mutant was found to increase GLUT4 translocation (Kohn, 1996; Cong, 1997; Hajduch, 1998). Furthermore, introduction of blocking antibodies and expression of dominant interfering mutants were also found to prevent insulin-stimulated GLUT4 translocation (Cong 1997; Wang 1999). Other studies have observed an insulin-dependent association of PI3-kinase and PKB on intracellular GLUT4 containing compartments (Heller-Harrison 1996; Calera 1998; Kupriyanova 1999). Conversely, there are studies suggesting that PKB is not necessary for glucose transport activation (Kitamura 1998; Kotani 1998). Overexpression of a dominant negative form of PKB blocked insulin-stimulated activity of p70S6k phosphorylation but had no effect on insulin-stimulated glucose uptake and GLUT4 translocation in 3T3-L1 adipocytes (Kitamura 1998). Consequently, the exact role for PKB in insulin's action on GLUT4 translocation and glucose uptake is to date not fully understood. In addition to insulin stimulation, there are PI3-kinase-independent pathways that can regulate

GLUT4 translocation in adipocytes and muscle. For instance, hyperosmolarity (Chen 1997; Sakaue 1997), and in muscle also exercise/contraction and hypoxia (Lee 1995; Lund 1995; Yeh 1995) are potent activators of GLUT4 translocation and glucose transport.

Insulin also activates the ras-mitogen-activated protein kinase (ras-MAPK) signalling pathway (Cheatham 1995), but most data do not support a role of the MAPK pathway in the major metabolic actions of insulin (Wiese 1995; Dorrestijn 1996; Shepherd 1997).

Cellular glucose transport

Studies using nuclear magnetic resonance spectroscopy (NMR) have shown that glucose transport across the plasma membrane is a rate limiting step for glucose metabolism in normal as well as in obese and diabetic subjects (Cline 1999; Shulman 2000). However, the glucose molecule is polar by nature and cannot by itself cross the lipid bilayer of the cells. In 1980 it was found that the action of insulin on glucose uptake was mediated through the translocation of "glucose transport activity" from an intracellular site to the plasma membrane independent of *de novo* protein synthesis (Cushman 1980).

It is now established that specific proteins, glucose transporters, facilitate glucose entry into the cells via diffusion along a glucose-concentration gradient (Mueckler 1994). The glucose transporter proteins are integral membrane proteins with a highly conserved 12 transmembrane domain (Olson 1996). The glucose transporters have distinct substrate specificities, kinetic properties and tissue distribution (Shepherd 1999). At least 11 genes for GLUT isoforms have so far been identified in the human genome, and GLUTs 1-5, 8 and 9, respectively, have been shown to transport sugars (Olson 1996; Carayannopoulos 2000; Doege 2000; Ducluzeau 2002) (Table 2).

GLUT1 and GLUT4 seem to be the most important glucose transporters with respect to whole-body glucose disposal (Lienhard 1992). GLUT1 is considered to account for basal glucose uptake (Gulve 1994). It is expressed in most tissues and is relatively insensitive to insulin. GLUT4, on the other hand, is the major insulin-responsive glucose transporter, mainly expressed in muscle and adipose tissue. Theoretically, there are at least three ways in which insulin might modulate GLUT4 function. First, insulin could promote translocation to the cell surface of intracellular GLUT4 (Cushman 1980; Suzuki 1980). Secondly, insulin could upregulate GLUT4 expression by increased synthesis and/or decreased degradation (Yu ZW 2001). Finally, insulin could increase the intrinsic transport activity of GLUT4 proteins in the plasma membrane (Sweeney 1999). It is established that, upon insulin stimulation, GLUT4

containing vesicles are translocated from intracellular compartments to the plasma membrane (Cushman 1980; Birnbaum 1992). A general hypothesis called the SNARE (soluble NSF attachment protein receptors; NSF, N-ethylmaleimide-sensitive fusion protein) hypothesis postulates that the specificity of secretory vesicle targeting is generated by complexes that form between membrane proteins on the transport vesicle (v-SNARE's) and membrane proteins located on the target membrane (t-SNARE's) (St-Denis 1998). Several v- and t-SNARE's have been identified in adipocytes and muscle. The SNARE's are a family of membrane-associated proteins that selectively mediate membrane fusion events via protein-protein interactions (Hay 1997; Pfeffer 1999). Nonetheless, the precise compartment in which the transporter resides in the basal state as well as the exact route it follows to the cell membrane and back to its intracellular compartments remains controversial. One explanation for this is that GLUT4 may be localised to multiple intracellular membrane compartments including the trans-Golgi network and the endosomal system (James 1994). Thus, regulation of GLUT4 trafficking is not yet completely clarified.

Table 2.
Facilitative glucose transporters (GLUTs) in mammals.

Name	Tissue distribution	Functions
GLUT1	Wide distribution	Basal glucose uptake in most cells, including insulin sensitive cells
GLUT2	Pancreatic β-cells, hepatocytes, intestine and kidney	Glucose sensing in β-cells, low affinity glucose transporter
GLUT3	Wide distribution, but primarily in neural cells	Basal transport, uptake from cerebral fluid
GLUT4	Insulin-responsive tissues like muscle and fat	Insulin-stimulated glucose uptake
GLUT5	Intestine, small amounts in fat, muscle, brain and kidney	Absorption of fructose in intestine
GLUT8	High expression in testis, intermediate in brain	Not known
GLUT9	Brain/leukocytes	Not known

Cellular insulin resistance

There is great uncertainty regarding the primary defects and also regarding the relative importance of different tissues in the development of insulin resistance. However, both inherited and acquired factors contribute. The inherited defects responsible for insulin resistance are largely unidentified. Common

polymorphisms in candidate genes that could potentially modulate insulin sensitivity, e.g. β-adrenergic receptors, PPARγ (peroxisome proliferator-activated receptor γ), IRS-1 and glycogen synthase, appear to be associated with human insulin resistance and type 2 diabetes (Groop 2000). However, the quantitative importance of such polymorphisms for an individual's risk to develop type 2 diabetes is limited. Mutations in candidate genes involved in insulin-stimulated glucose transport, e.g. the insulin receptor, glucose transporters and signalling proteins can lead to marked insulin resistance, but these are rare (Fujimoto 2000). For example, defects in the insulin receptor gene are too rare to account for the common forms of insulin resistance (Krook 1996).

In recent years, monogenic and polygenic knockout mouse models as well as tissue-specific knockout models have been created (reviewed by Mauvais-Jarvis 2002). In mice, various degrees of insulin resistance can be created depending on the specific knockout protein and its role in the insulin-signalling cascade. Disruption of IRS-1 in mice does not lead to diabetes. These mice develop β-cell hyperplasia and a mild insulin resistance mainly located in skeletal muscle (Araki 1994; Tamemoto 1994). IRS-2 deficient mice, however, develop overt diabetes in early life because of severe insulin resistance in liver and a lack of compensatory hyperplasia from pancreatic β-cells (Withers 1998; Kubota 2000). IRS-3 and 4 are unlikely to play a major role in glucose homeostasis since knockout of these IRSs leads to either mild glucose intolerance or a normal phenotype (Liu 1999; Fantin 2000). PI3-kinase activation is mediated by the regulatory subunits p85α and its splice variants p50α and p55α. Surprisingly, mice lacking all subunits of PI3-kinase (Fruman 2000) or only the long forms p85α (Terauchi 1999) and p85β (Ueki 2002) have increased insulin sensitivity and hypoglycemia suggesting that PI3-kinase regulatory subunits play a negative regulatory role in insulin-dependent PI3-kinase regulation. However, PKBβ knockout mice, i.e. with a signalling defect downstream of PI3-kinase, are insulin-resistant in skeletal muscle and liver and they develop diabetes (Cho 2001). Mice deficient in GLUT4 exhibit only moderate insulin resistance (Katz 1995). However, male mice heterozygous for the GLUT4 gene knockout have a 50 % reduction in GLUT4 expression and they exhibit insulin resistance and also develop diabetes (Stenbit 1997).

Polygenic knockout models have also been generated, and they support that there is a complex interaction between different genes in the development of type 2 diabetes. For example, mice that are double-heterozygous for the insulin receptor and IRS-1 exhibit a synergistic impairment of insulin action and develop severe insulin resistance in muscle and liver together with β–cell hyperplasia to compensate for insulin resistance, a phenotype much stronger

than the individual heterozygous insulin receptor and IRS-1 knockouts. These "double heterozygous" mice develop diabetes later on in life (Bruning 1997).

Tissue-specific knockouts have revealed that primary genetic defects of glucose or lipid metabolism in a given tissue can lead to acquired insulin resistance in other insulin-sensitive organs. In muscle insulin receptor knockout (MIRKO) mice, muscle insulin resistance leads to a shift of glucose utilization from muscle towards adipose tissue resulting in an increased fat mass (Kim JK 2000), thereby contributing to increased adiposity and development of a prediabetic syndrome. Moreover, severe impairment of glucose transport in white adipose tissue by tissue-specific knockout of the GLUT4 gene leads to acquired insulin resistance in muscle and liver by a mechanism independent of glucose toxicity or lipotoxicity (Abel 2001).

In humans, tissue-specific alterations in gene expression have been described. In adipocytes from obese humans with type 2 diabetes, IRS-1 expression is reduced, resulting in decreased IRS-1 associated PI3-kinase activity, and IRS-2 is instead the main docking protein for PI3-kinase (Rondinone 1997). In contrast, in skeletal muscle of obese subjects with type 2 diabetes, IRS-1 and 2 protein levels are normal but PI3-kinase activity associated with both IRSs is impaired (Kim YB 1999a). Moreover, GLUT4 is decreased in adipocytes (Garvey 1991) but not in skeletal muscle (Handberg 1990; Pedersen 1990) from subjects with type 2 diabetes.

Lipid metabolism and the adipose tissue in insulin resistance

Adipose tissue is the main site for long-term storage of energy in the form of triglycerides. The main lipids of biological importantance are the fatty acids and their derivatives, i.e. triglycerides, phospholipids (constituents of all cell membranes) and the sterols (e.g. steroid hormones and cholesterol).

Insulin is critical in the regulation of adipocyte biology and is one of several factors responsible for preadipocyte differentiation into adipocytes (Gamou 1986). Preadipocyte differentiation also involves transcription factors, and one important family of transcription factors in this respect is probably the PPAR family (Hwang 1997). A new class of anti-diabetic drugs called thiazolidinediones (TZDs) (Henry 1997) enhance target-tissue insulin sensitivity in vivo where they function as high-affinity ligands for the nuclear receptor PPAR-γ, that is particularly abundant in fat cells (Lehmann 1995).

In mature adipocytes insulin promotes triglyceride storage by stimulating glucose uptake and conversion of acetyl-CoA into triglycerides as well as by inhibiting lipolysis. Insulin also increases the cellular uptake of fatty acids

derived from circulating lipoproteins by stimulating lipoprotein lipase activity in the vasculature of adipose tissue.

One of insulin's most potent metabolic actions is the suppression of adipose tissue lipolysis (Jensen 1989; Bonadonna 1990; Campbell 1992). The antilipolytic effect of insulin is mediated through inhibition of hormone-sensitive lipase (HSL). Upon insulin stimulation, phosphodiesterase 3B (PDE3B) is activated leading to a reduction of the intracellular cAMP level and this in turn attenuates the activity of cAMP-dependent protein kinase A (PKA) responsible for phosphorylation and activation of HSL.

The postprandial levels of triglyceride-rich lipoproteins and their remnants are elevated in type 2 diabetes (Syvanne 1994; De Man 1996). There are data suggesting that abnormal clearance of triglyceride-rich lipoproteins in the postprandial phase is an early defect in type 2 diabetes, since healthy first-degree relatives of patients with type 2 diabetes exhibit postprandial hypertriglyceridaemia despite having normal fasting triglyceride levels (Axelsen 1999). In states of marked insulin deficiency, such as uncontrolled diabetes, LPL-mediated clearance of chylomicrons is grossly impaired and this can result in profound hypertriglyceridemia. Improved diabetes control appears to reverse the defect in LPL activity and reduce triglyceride levels (Simsolo 1992). Potential mechanisms for postprandial hypertriglyceridaemia are shown in Fig. 3.

Besides being the main organ for storage of energy, adipose tissue also functions as an important endocrine organ. Adipocytes release a number of peptide hormones, cytokines and other biologically active molecules including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), plasminogen-activator inhibitor-1 (PAI-1), angiotensinogen, leptin, adiponectin and resistin. Adipose tissue can also convert and/or activate steroid hormones, i.e. estrogen and cortisone/cortisol (Deslypere 1985; Bujalska 1997) and it also contributes to lactate, amino acid and, of course, free fatty acid production (DiGirolamo 1992). Certainly, these secreted products can exert biological effects both locally in the adipocyte tissue and also in other tissues of importance for whole-body metabolism and endocrine function, i.e. muscle, liver, β-cells, brain, gonads and the vascular system. When the amount of adipose tissue is incresed, as seen in obesity, the production of many of these secreted products is altered and it is likely that some of them might be involved in the development in insulin resistance associated with obesity (Mohamed-Ali 1998).

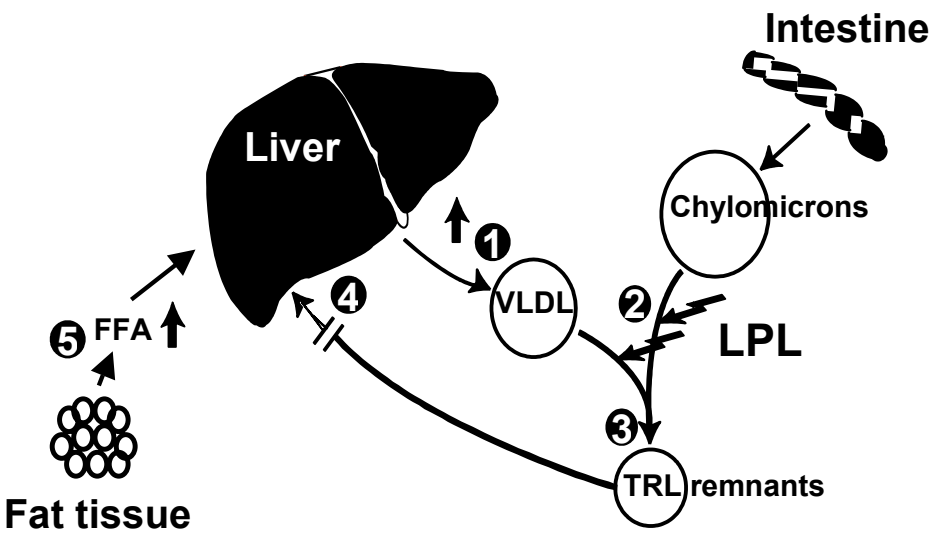

Fig. 3.
Possible mechanisms behind postprandial lipemia in type 2 diabetes, e.g. increased VLDL production from the liver (1), impaired LPL activity (2), enhanced dissociation of LPL from TRL particles (3), impaired interaction of remnant particles with remnant receptors in the liver (4) or elevated FFA release from fat tissue (5). Adapted from Taskinen (2001).

Evidence from knockout animal models suggest that the adipose tissue, in spite of being a minor site for glucose uptake in vivo, may play a major role in controlling overall glucose metabolism. Transgenic ablation of white adipose tissue in mice leads to severe insulin resistance, elevated lipid levels, undetectable leptin levels and diabetes (Moitra 1998). In this model and in other mouse models with reduced adipose tissue, infusions of leptin (Shimomura 1999), transgenic overexpression of leptin (Ebihara 2001) or surgical implantation of white adipose tissue (Gavrilova 2000) can reverse the diabetic phenotype. Of course, one must bear in mind that there are huge differences between knockout as well as other animal models for diabetes and human diabetes.

Human insulin resistance and type 2 diabetes

There are several types of diabetes and the two most common are type 1 and type 2 diabetes. Type 1 diabetes is the result of an autoimmune destruction of the pancreatic β-cells, leading to a deficiency of insulin secretion (Eisenbarth 1987). Type 2 diabetes, accounting for the majority of all diabetes cases, is commonly caused by a combination of insulin deficiency and insulin resistance. Environmental factors like physical inactivity, a high energy and high fat diet,

smoking and stress strongly interact with a genetic predisposition to promote development of the disease. Defects in insulin action generally precedes the clinical manifestation of diabetes and they can be demonstrated in non-diabetic relatives of type 2 diabetic patients. Initially, increased insulin secretion compensates for insulin resistance but, eventually, overt diabetes develops as β-cell compensation becomes insufficient. In spite of an enormous research effort during many years, the primary factors causing insulin resistance and type 2 diabetes remain unclear.

Type 2 diabetes is a progressive metabolic disorder characterized by a continuous loss of β-cell function with time. Thus, treatments that require endogenous insulin secretion become less effective with longer duration of the disease. Treatment for hyperglycemia in type 2 diabetes is usually intensified in a stepwise manner, from lifestyle intervention, e.g. dietary management and increased physical activity, via the addition of one or more oral antidiabetic agents to insulin injections.

Neuroendocrine and humoral factors causing insulin resistance in vivo

Insulin-antagonistic hormones

Glucocorticoids are produced in the adrenal cortex and the production is governed by the hypothalamic-pituitary-adrenal (HPA) axis via adrenocorticotropic hormone (ACTH) that is released by the pituitary (Fig. 4). Glucocorticoids play a key role in regulating salt and water homeostasis, blood pressure, immune function and metabolism. The main glucocorticoid in man is cortisol. The clinical syndrome of glucocorticoid excess, Cushing's syndrome, is associated with insulin resistance, glucose intolerance, central obesity and hypertension. Pharmacological treatment with high doses of glucocorticoids also leads to an impairment of insulin sensitivity. In clinical obesity, there are alterations in cortisol metabolism, and local activation of cortisol in the adipose tissue may be an important link between glucocorticoids and development of the so-called metabolic syndrome (Rask 2001). The metabolic effects of cortisol are partly explained by its effects to oppose the actions of insulin, i.e. to induce a state of insulin resistance. The effects of glucocorticoids in vivo appear to include both an impairment of insulin-dependent glucose uptake in peripheral tissues and a stimulation of gluconeogenesis in the liver (Rizza 1982; Rooney 1993). In addition to their effects on insulin sensitivity, glucocorticoids may also inhibit insulin secretion from pancreatic β-cells (Delaunay 1997; Lambillotte 1997). Glucocorticoid-induced insulin resistance has also been demonstrated in cultured cells in vitro (Olefsky 1975a; Olefsky 1975b; Caro 1982). For a long time it has been known that glucocorticoids inhibit insulin-stimulated glucose metabolism in adipocytes (Fain 1963; Munck 1971) and muscle (Riddick 1962).

This appears to be mediated primarily by an impairment of glucose transport, and dexamethasone-induced insulin resistance in 3T3-L1 adipocytes probably involves the GLUT4 translocation machinery (Sakoda 2000). Previously, it has been reported that insulin-stimulated recruitment of GLUT4 to the cell surface is inhibited in rat skeletal muscle following dexamethasone treatment (Weinstein 1998). Glucocorticoids are reported to activate adipose tissue lipolysis, and this is probably also an important factor in promoting insulin resistance, since insulin sensitivity was normalised when lipolysis (Ekstrand 1992) or lipid oxidation (Guillaume-Gentil 1993) was inhibited.

There are several synthetic cortisol analogues available for research purposes and in clinical practice. The glucocorticoid activity of dexamethasone is approximately 25 times stronger compared to that of cortisol (Ganong 1995). A simplified overview of the regulation of cortisol production via the HPA-axis is shown in Fig. 4.

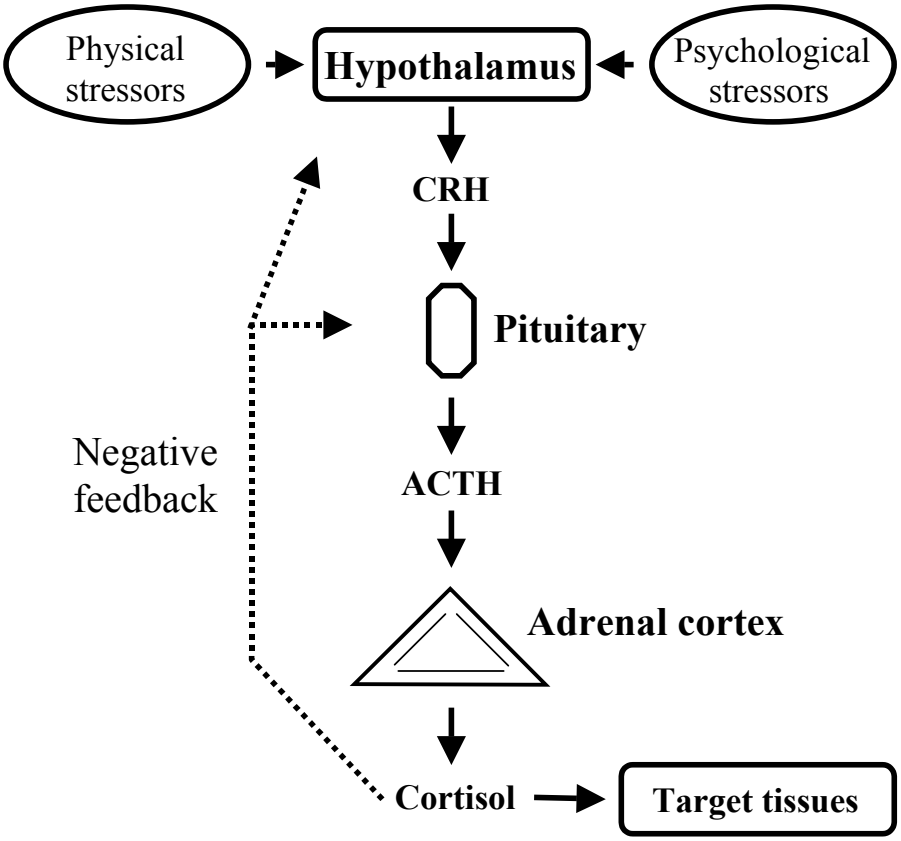

Figure 4.
Schematic illustration of the hypothalamic-pituitary-adrenal axis.

Catecholamines, mainly adrenaline and noradrenaline, are secreted by the adrenal medulla and sympathetic nerve endings, respectively, and this is stimulated by physical and mental stress. An acute injection of catecholamines decreases the sensitivity to insulin's effect on glucose utilization, and leads to elevation of blood glucose (Rizza 1980). This is also mediated by an enhanced rate of glycogen breakdown in liver and an increased rate of fatty acid mobilisation (Cherrington 1984), but also by inhibition of insulin secretion and stimulation of glucagon release. Some physiological situations with a long-term increase in catecholamine-levels, however, are in fact associated with an increase in the sensitivity of glucose metabolism to insulin (e.g. physical exercise).

In fat, catecholamines increase lipolysis by stimulating plasma membrane adenylyl cyclase activity through β-adrenergic receptors, which leads to increased intracellular levels of cyclic adenosine monophosphate (cAMP) and then activation of cAMP-dependent protein kinase A (PKA). Hormone-sensitive lipase, the rate-limiting enzyme in lipolysis, is in turn phosphorylated and activated by PKA.

Growth hormone (GH) is secreted from the anterior pituitary. GH impairs insulin binding and glucose uptake in some tissues, increases hepatic glucose output and mobilises FFA from adipose tissue (Ganong 1995). Moreover, GH treatment in adults with growth hormone deficiency was associated with a measurable increase in insulin and glucose levels, indicating of mild insulin resistance (Fowelin 1993). However, GH may also reduce adiposity, since GH-deficient individuals have an increased fat mass (Rosén 1993). This might also be of importance in humans with visceral obesity, since multiple endocrine perturbations are found, including low GH and elevated cortisol and androgens in women, as well as low testosterone secretion in men (Bjorntorp 1996). However, the role of GH in insulin resistance is not yet fully understood.

The autonomic nervous system could potentially contribute to insulin resistance in type 2 diabetes, and this would mainly be mediated via release of catecholamines. Studies in animal models of type 2 diabetes as well as patients with type 2 diabetes have revealed an altered sympathetic activity and, moreover, their carbohydrate metabolism seems abnormally sensitive to sympathetic stimulation (Surwit 1984; Bruce 1992; Chan 1995). In healthy humans with or without a family history of type 2 diabetes, recent data suggest that insulin resistance is associated with an altered balance in the autonomic nervous system with a relative increase in sympathetic vs parasympathetic activity following standardised stress (S. Lindmark, U. Wiklund, P. Bjerle, J.W. Eriksson, unpublished data) or following hyperinsulinemia (Laitinen 1999).

Thus, dysregulation of the autonomic nervous system might be a potential mechanism for early insulin resistance in the development of type 2 diabetes.

Hyperglycemia and hyperinsulinemia

In diabetes, glucose levels are chronically elevated, and insulin levels are naturally often abnormal, e.g. high in early type 2 diabetes but low in later type 2 diabetes and in type 1 diabetes. Experimental hyperinsulinemia has been shown to cause insulin resistance both in vitro (Garvey 1986; Henry 1996) and in vivo (Rizza 1985; Bonadonna 1993; Iozzo 2001). In isolated rat adipocytes, long-term exposure (24 h) to high glucose in the presence of insulin downregulates subsequent basal and acutely insulin-stimulated glucose transport. The effects of insulin and glucose during cell culture were reported to be synergistic (Garvey 1987) and appeared to be associated with a post-insulin receptor defect (Lima 1991). Hyperglycemia alone exerts detrimental effects on insulin secretion and insulin action (Unger 1985), a phenomenon commonly referred to as glucose toxicity (Rossetti 1990), and in muscle from patients with type 2 diabetes, insulin-stimulated glucose transport is impaired under hyperglycemic conditions (Zierath 1994). Moreover, glucose transport capacity of isolated muscle strips can be restored in vitro following incubation at a physiological glucose level, supporting that glucose levels per se have regulatory effects on the glucose transport machinery and that these effects are reversible (Zierath 1994). Accordingly, reversal of hyperglycemia in rats by phlorizin treatment improves insulin sensitivity (Rossetti 1987; Kahn 1991). Several studies in rats have suggested that increased hexosamine biosynthesis leads to skeletal muscle insulin resistance in vivo and in vitro and that this may be a mechanism involved in glucotoxicity (Baron 1995; Rossetti 1995; Hawkins 1996). Moreover, glucose-induced activation of different PKC isoforms has been shown to interfere with insulin receptor signalling and produce insulin resistance (Muller 1991; Berti 1994; Kawano 1999). However, the mechanisms by which hyperglycemia causes insulin resistance still remain incompletely understood.

Free fatty acids

Elevated FFAs might promote accumulation of fat depots in muscle, liver and/or β-cells, and the accumulated triglycerides might provide an environment that could interfere with metabolic signalling and thus action in these different tissues (Nyholm 1999). A link between insulin resistance and triglyceride content in muscle biopsies has been established (Storlien 1991; Phillips 1996; Pan 1997). Moreover, it was shown that elevations in plasma free fatty acid concentrations can lead to an attenuated effect of insulin to stimulate IRS-1-associated PI-3 kinase activity in muscle (Dresner 1999). The reduced PI-3

kinase activity may be due to a direct effect of intracellular free fatty acids or some fatty acid metabolite, or it may be secondary to alterations in upstream signalling events. Recent data have suggested that fatty acid metabolites activate a kinase that phosphorylates serine/threonine sites on IRSs, which in turn may reduce the ability of the IRSs to activate PI-3 kinase and glucose transport (Griffin 1999).

It is well known that FFAs are important substrates for skeletal muscle energy production (Andres 1956). In the fasting state skeletal muscle has a high fractional extraction of plasma FFAs, and lipid oxidation accounts for the majority of energy production. The capacity of skeletal muscle to utilize lipid or carbohydrate fuels, as well as the potential for substrate competition between fatty acids and glucose, is of interest in insulin resistance. A potential implication of the glucose-fatty acid cycle, originally postulated by Randle and colleagues (Randle 1963), is that increased lipid availability could interfere with muscle glucose metabolism and contribute to insulin resistance for example in obesity and type 2 diabetes. Several studies support the concept that elevated free fatty acids produce an impairment of insulin-stimulated glucose metabolism (Kelley 1993; Boden 1995; Roden 1996). Another concept is that of metabolic inflexibility in insulin resistance. In the fasting condition, skeletal muscle predominantly utilizes lipid oxidation for energy production (Andres 1956). Upon insulin stimulation in the fed condition, healthy skeletal muscle rapidly switches to increased uptake, oxidation and storage of glucose and, moreover, lipid oxidation is suppressed (Kelley 1990). Obese individuals and those with type 2 diabetes manifest higher lipid oxidation during insulin-stimulated conditions as compared to control subjects (Felber 1987), despite lower rates of lipid oxidation during fasting conditions. This suggests that a key feature in insulin resistance of skeletal muscle is an impaired ability to switch between fuels.

Other humoral factors

As mentioned before, the adipose tissue is an endocrine organ, secreting several hormones and other substances into the blood, some of which might be involved in human insulin resistance. Cytokines, e.g. TNF-α and IL-6, has been proposed as candidates in this respect. For example, TNF-α is overexpressed in adipocytes from obese individuals, and can potentially induce insulin resistance through effects on insulin signalling pathways (Hotamisligil 1994).

By the secretion of leptin, adipose tissue can influence appetite and energy expenditure by signalling the body's state of adiposity to the brain (Havel 1996). Leptin also has potent effects on insulin action (Remesar 1997). In *ob/ob* mice, inherited leptin deficiency causes both severe insulin resistance and obesity, but

insulin resistance can be reversed by leptin replacement (Muzzin 1996). However, leptin levels were reported to be elevated in obese and insulin-resistant humans (Considine 1996), and also in subjects with a genetic predisposition for type 2 diabetes (Jansson 2002).

Moreover, the recent discoveries of the adipocyte-secreted hormones resistin and adiponectin have received great attention. They are reported to modulate insulin sensitivity in mice (Steppan 2001; Yamauchi 2001), but the role of resistin in humans has been questioned (Nagaev 2001). The roles of many of the various adipocyte-derived signals are still not fully understood (Bradley RL 2001).

AIMS

The aim of the present study was to investigate mechanisms for cellular insulin resistance that potentially may be of importance in human type 2 diabetes.

Specific aims:

1. To investigate the cellular mechanisms involved in glucocorticoid–induced insulin resistance in rat adipocytes. (study I)

2. To elucidate the cellular mechanisms involved in insulin resistance caused by high levels of insulin and glucose in rat adipocytes. (study II)

3. To investigate whether fat cell insulin resistance in human type 2 diabetes is related to the glycemic level in vivo and whether it is reversible. (study III)

4. To explore a possible dysregulation of fat tissue lipoprotein lipase as a mechanism for postprandial hypertriglyceridemia in type 2 diabetes. (study IV)

METHODS

Animals (study I, II)

Outbred male Sprague-Dawley rats were housed at the Animal Department of Umeå University Hospital. The animals had free access to standard rat chow and water. Rats weighing 150-200 g (age ~4-6 weeks) were killed by decapitation and epididymal fat pads were immediately excised, transported to the laboratory and minced. The Umeå Ethical Committee for Animal Research approved the protocol for study I and II.

Patients and healthy volunteers (study III, IV)

The subjects in study III and IV were recruited among patients at the Diabetes Unit of Umeå University Hospital and by advertisement in a local newspaper. They came to the laboratory, following an overnight fast, and at 08.00 h blood samples were obtained and a needle biopsy of subcutaneous fat was taken from the lower part of the abdomen after local dermal anaesthesia. The Umeå University Ethics Committee approved the studies and all participating subjects gave their informed consent.

In study III, there were three study groups each consisting of ten individuals and they were matched for age, BMI and sex. Two groups consisted of type 2 diabetes patients, classified in accordance with to the 1998 WHO criteria (Alberti 1998), and they had either good (HbA1c <7 %, reference value <5.3 %, group G) or poor (HbA1c >7.5 %, group P) metabolic control. The third group consisted of non-diabetic control subjects (group C). Eight subjects in P and five subjects in G were on oral anti-diabetic medication and the other diabetes patients had no medication.

In study IV, there were eight patients with type 2 diabetes and eight age-, BMI- and sex-matched control subjects. One diabetes patient was treated with insulin, five were treated with oral anti-diabetic agents and two subjects were treated with non-pharmacological measures alone.

Cell preparation

Adipocytes were isolated by treatment of small (~1 mm^3) pieces of adipose tissue with collagenase at a low concentration (0.6 mg/ml). The fat tissue was shaken (at ~150 rpm) at 37 °C in medium 199 containing 5.6 mM glucose with 40 mg/ml BSA for ~50 min. Since collagenase may affect membrane function (Kono 1971) and consequently adipocyte metabolism, several batches of collagenase were obtained from the manufacturer and the one displaying the

least interference with insulin action on glucose uptake was purchased in a large amount, and it was then used throughout the experiments. After cell isolation, the adipocytes were filtered through a nylon mesh and washed four times with fresh medium.

Cell diameter was measured in isolated adipocytes by microscopic examination of 100 adipocytes in each experimental situation. Cell volume and weight were calculated and the cell number per sample was obtained by dividing the triglyceride amount of the sample by the mean cell weight as previously described (Smith U 1972). There was no significant change in cell size following any of the employed culture conditions. Cell morphology was intact according to microscopic examination and cell viability was verified by trypan-blue exclusion tests.

Cell culture

Isolated adipocytes were cultured during gentle shaking (at ~30 rpm) in Teflon flasks containing DMEM with 10 % fetal calf serum, penicillin, streptomycin and with D-glucose and other additions, i.e. insulin, dexamethasone or none, as indicated. Cells were incubated at 37 °C for 24 h unless otherwise specified under a gas phase of 95 % O_2 and 5 % CO_2.

Glucose uptake

Isolated adipocytes were washed four times and glucose uptake was assessed as previously described (Yu ZW 1997; Eriksson 1999). In brief, adipocytes (lipocrit 3-5 %) were incubated in medium 199 with 4 % BSA, ADA and PIA, but without glucose, for 15 min at 37 °C in the presence or absence of insulin. After that, ^{14}C-U-D-glucose (0.86 μM) was added. The cells were then separated from the incubation medium after 1 h by centrifugation through silicone oil and the radioactivity associated with the cells were measured by scintillation counting. Under these experimental conditions glucose uptake is mainly determined by the rate of transmembrane glucose transport (Kashiwagi 1983). The cellular clearance of glucose from the medium was calculated according to the following formula and taken as an index of the rate of glucose uptake:

$$\text{Cellular clearance of medium glucose} = \frac{\text{Cell-associated radioactivity} \times \text{volume}}{\text{Radioactivity of medium} \times \text{cell number} \times \text{time}}$$

Insulin binding

Before assessment of insulin binding, isolated fat cells were washed four times and thus >90 % of any bound insulin remaining from the culture period was removed (Eriksson 1992). Thereafter, the cells (lipocrit 5-10 %) were incubated with ADA, PIA and KCN for 5 min at 37 °C to deplete the cells of ATP and stop receptor internalisation and recycling (Eriksson 1992). Subsequently, cell surface binding of ^{125}I-insulin (0.2 ng/ml) was carried out for 60 min at 16 °C. After the incubation period, cells and medium were separated by centrifugation through dinonyl phtalate and ^{125}I-insulin binding to cells was measured.

Lipolysis

Isolated adipocytes were washed four times and they were incubated at lipocrit 1-3 % in medium 199 containing 5.6 mM glucose, 4 % BSA, ADA, PIA, with and without 8-bromo-cAMP and various insulin concentrations for 60 min at 37 °C. After 1 h the adipocytes were separated from the medium by centrifugation through silicone oil. The rate of lipolysis was then assessed by measurement of the glycerol content in the medium according to Bradley and Kaslow (Bradley DC 1989). In brief, glycerol was phosphorylated in the presence of glycerokinase and [γ-^{32}P]ATP for 30 min at 37 °C and radioactivity reflecting phosphorylated glycerol was measured.

Western blot analysis of proteins in cell lysates and membranes

To obtain cellular lysates, the cells were washed four times with PBS and treated with lysis buffer. The detergent insoluble material was sedimented by centrifugation and the supernatants collected. Protein concentrations were determined with the bicinchoninic acid method (Smith PK 1985).

Total cellular membranes were prepared as previously described (Kitzman 1993). Cells were washed twice with PBS and homogenized in TES homogenization buffer. The homogenate was then centrifuged at 1000 g for 30 min at 4 °C and the supernatant collected and centrifuged at 212000 g for 1 h at 4°C. The resulting pelleted total membrane fraction was resuspended in TES and frozen at –70 °C. Protein determination was based on the method of Bradford (Bradford 1976).

For western blot analyses, equal amounts of proteins were applied to each lane in each set of experiments, generally 40 μg. Protein separation was performed by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked overnight at 4 °C with 5 % dry milk in TBST.

Immunological detection of proteins of interest was carried out with specific polyclonal antibodies, and immunoreactive bands were visualized using the ECL Western blotting protocol (Amersham Pharmacia Biotech, Freiburg, Germany). The corresponding bands were quantified by densitometry (Molecular Analyst^{TM}, Bio-Rad Laboratories, Hercules, CA, USA). Films from each set of experiments were scanned at the same time and the background signal from each single film was subtracted. To confirm equal loading of protein and gel and to exclude general treatment effects on protein expression, β-actin was used as an internal control in some experiments. β-actin is an established housekeeping gene product used for this purpose (Liao 2000), and β-actin content was not consistently altered by the employed culture conditions (i.e. addition of dexamethasone, high glucose, high insulin or high glucose + high insulin, Fig. 5).

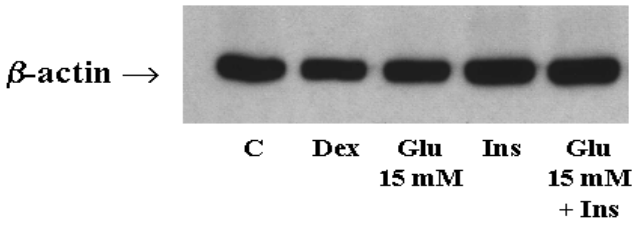

Figure 5. Western blot showing β-actin content in cell lysates following 24 h culture under different culture conditions. C: control situation, 5 mM glucose, no insulin; Dex: 0.3 μM dexamethasone; Glu 15 mM: 15 mM glucose; Ins: 10^4 μU/ml insulin; Glu 15 mM + Ins: 15 mM glucose + 10^4 μU/ml insulin.

PKB phosphorylation

After washing four times, adipocytes (lipocrit ~15 %) were incubated at 37 °C in medium 199 with 5.6 mM glucose, 4 % BSA, ADA, PIA with and without insulin as indicated. After 10 min incubation, which is enough time to elicit maximal phosphorylation of PKB, adipocytes were immediately separated from the medium by centrifugation through silicone oil. Cell lysis and protein determination was performed as described above. Western blotting procedures were performed as described above with a pSer^{473}-PKBα antibody.

Lipoprotein lipase (LPL) and hepatic lipase (HL)

The measurement of LPL and HL mass and activity are described in detail in paper IV. In brief, the fat biopsy was filtered and dissected free of blood and connective tissue. One fraction of adipose tissue was homogenized in ice-cold

buffer with detergents and proteinase inhibitors. Another fraction of adipose tissue was used for incubation in cell culture wells with the addition of heparin. Heparin-releasable activity in the medium as well as activity in the remaining fraction of adipose tissue was measured. For assay of LPL activity a lipid emulsion was used (Holm 2001), but with addition of a trace amount of ^{3}H-oleic acid-labelled triolein. The fatty acids were extracted according to Dole's method (Bengtsson-Olivecrona 1992). LPL protein mass was determined by an enzyme immunosorbent assay (ELISA) as previously described (Holm 2001), and the lipase activity was expressed in mU/ml plasma or mU/g adipose tissue, where 1 mU corresponds to release of 1 nmol fatty acid per minute.

Blood chemistry

Blood glucose was measured using the HemoCue glucose system (HemoCue AB, Ängelholm, Sweden). All other blood chemistry (e.g. HbA1c and insulin) was, unless otherwise specified, analysed by routine methods at the Department of Clinical Chemistry, Umeå University Hospital as previously described (Svensson 2002).

Insulin sensitivity in vivo

In study III, the hyperinsulinemic euglycemic clamp technique was utilized to assess insulin sensitivity (DeFronzo 1979) and it was performed essentially as previously described (Svensson 2002). Subjects arrived to the laboratory after fasting overnight since 22.00 h. A 2 h hyperinsulinemic euglycemic clamp was started at 08.00, and after initial priming, a constant infusion of short acting insulin (Actrapid®) was administered at 56 mU/m^2 body surface/min. A glucose infusion was started and it was adjusted to maintain blood glucose at 5.0 mM. Insulin sensitivity was assessed as the glucose infusion rate at steady state during the time period 60-120 min, i.e. glucose uptake expressed as the so-called M-value (mg glucose infused/kg lean body mass/min).

In study IV, insulin resistance was estimated by the homeostasis model assessment insulin resistance index (HOMA-IR) derived from fasting plasma insulin and glucose concentrations (Matthews 1985).

Standardized meal test

In study IV, a subcutaneous abdominal fat tissue biopsy and blood samples were taken in the fasting state and also 3.5 h following a standardized lipid-enriched meal (Boquist 1998) that was ingested after the first biopsy. The total energy amount was 1000 kJ. 60.2 % of the energy was from fat (soybean oil), 26.5 % of the energy was from carbohydrates and 13.3 % of the energy was from protein.

The time-point 3.5 h after the meal was chosen to represent maximal postprandial lipaemia because the peak level of postprandial triglycerides occurs at 3-4 h after a fat-rich meal (Axelsen 1999; Boquist 1999). On a separate occasion, the subjects again came to the laboratory after an overnight fast. Blood samples were taken for analysis of plasma lipoprotein activity and mass. This was repeated 10 min after an intravenous injection of heparin (Heparin Leo®, Leo Pharma AB, Malmö, Sweden), 100 U/kg body weight.

Statistical analyses

Statistical analyses were performed using the SPSS package (SPSS Inc., Chicago, IL, USA). Results are given as mean ± SEM and conventional statistical methods with appropriate post hoc test were used as indicated. Associations between variables were analysed with simple regression or stepwise multiple linear regression. P-values less than 0.05 were considered as statistically significant.

SUMMARY OF RESULTS

Paper I

The aim of study I was to investigate the mechanisms involved in glucocorticoid–induced insulin resistance in primary cultured rat adipocytes. Treatment with the cortisol analogue dexamethasone for 24 h markedly decreased both basal and insulin-stimulated glucose uptake by ~40-50 % at both a physiological, 5 mM, and an elevated, 15 mM, glucose concentration. The impairment of glucose uptake was time-dependent and the maximal decrease in basal glucose uptake was achieved after 2 h, while the maximal decrease in insulin-stimulated glucose uptake was achieved following 24 h of dexamethasone treatment. Combined long-term (24 h) treatment with insulin and dexamethasone exerted additive effects in reducing basal and, to a lesser extent, insulin-stimulated glucose uptake capacity compared to dexamethasone alone, but this was seen only at the high glucose level. In dexamethasone-treated cells, insulin binding was decreased (by ~40 %) independent of the surrounding glucose concentration. This was due to a reduction in available cell-surface binding sites according to Scatchard analysis (Scatchard 1949; de Meyts 1973).

Dexamethasone induced alterations in insulin signalling proteins. IRS-1 and PKB content was reduced by ~75 % and ~45 %, respectively. In parallel, serine-phosphorylated PKB following insulin stimulation was decreased by ~40 % in dexamethasone-treated cells. Dexamethasone also induced a subtle decrease in PI3-kinase by ~20 % and an increase in IRS-2 by ~150 %. However, dexamethasone did not alter the amount of total cellular membrane-associated GLUT4 protein. The effects of dexamethasone *per se* on insulin signalling proteins and glucose transport were mainly unaffected by the surrounding glucose and insulin levels.

Dexamethasone increased the basal lipolytic rate ~4-fold, but maximal cAMP-stimulated lipolysis was somewhat lower compared to control cells that were cultured without dexamethasone. The effect of insulin to counteract cAMP-stimulated lipolysis was intact following dexamethasone treatment.

Main conclusions: The data in paper I show that the cortisol analogue dexamethasone induces alterations in important mechanisms regulating cellular glucose and lipid metabolism. Independent of the surrounding glucose and insulin concentrations, dexamethasone impairs glucose transport capacity in fat cells. This is not due to alterations in GLUT4 abundance. Instead glucocorticoid-induced insulin resistance may be mediated via reduced cellular content of IRS-1 and PKB accompanied by a parallel reduction in insulin-stimulated activation of PKB.

Paper II

The aim of study II was to further elucidate the mechanisms for insulin resistance elicited by high levels of glucose and insulin in primary cultured rat adipocytes. Long-term (24 h) pretreatment with high glucose (≥ 15 mM) in the culture medium decreases both basal and insulin-stimulated glucose uptake capacity by ~20 % compared to cells cultured at a normal glucose level (5 mM). Long-term insulin treatment in combination with high glucose reduced glucose uptake rate by ~30-50 %. In contrast, a high insulin level alone did not affect glucose uptake capacity.

Independent of the prevailing glucose concentration, insulin treatment for 24 h induced a decrease in insulin binding by ~40 % and shifted the dose-response curve for insulin's effect on glucose uptake 2-3 fold to the right.

The cellular content of IRS-1 was downregulated, by ~20-50 %, at glucose levels of 15 mM or more, whereas IRS-2 was strongly upregulated. At low glucose, long-term insulin did not alter IRS-1 but induced a decrease in IRS-2 content by ~50 %. Insulin treatment in combination with high glucose amplified the suppression of IRS-1 and, moreover, IRS-2 expression was markedly reduced. There were no consistent changes in PI3-K and PKB content following any of the various incubation conditions. The rapid effect of a high concentration of insulin (1000 μU/ml) to induce serine phosphorylation of PKB was intact following 24 h pretreatment with high glucose and/or insulin. However, the dose-response curve for insulin's effect to phosphorylate PKB was shifted to the right. A high glucose level, 15 mM, in the culture medium increased GLUT4 in cellular membranes (by ~140 %) compared to 5 mM glucose but this effect was prevented by a high insulin concentration.

Main conclusions: Long-term exposure to a high glucose level *per se* decreases IRS-1 but increases IRS-2 content in rat adipocytes and it impairs glucose transport capacity. Treatment with high insulin downregulates insulin binding capacity and, when combined with high glucose, it produces a marked depletion of IRS-1 and 2 content together with an impaired sensitivity to insulin-induced activation of PKB activity. These mechanisms may potentially contribute to insulin resistance in different stages of the development and progression of type 2 diabetes in humans.

Paper III

The aim of study III was to investigate whether fat cell insulin resistance in human type 2 diabetes is related to the glycemic level in vivo and whether it is reversible. Three groups of subjects, i.e. type 2 diabetes patients in poor and

good metabolic control, respectively, and non-diabetic subjects were included as described in Methods. Type 2 diabetes patients were insulin-resistant in vivo as measured with the hyperinsulinemic, euglycemic clamp technique, and this was associated with the glycemic level (HbA1c.). In addition, freshly obtained subcutaneous adipocytes from type 2 diabetes patients were insulin-resistant in vitro, and they displayed a ~40-50 % reduction in the maximal effect of insulin on glucose uptake when compared to cells from control subjects. Adipocytes from diabetes patients with poor metabolic control (HbA1c >7.5%, reference value <5.3%) were significantly more insulin-resistant than adipocytes from patients with good metabolic control (HbA1c <7%) when glucose uptake was measured at a low (5 μU/ml) but not at a high (1000 μU/ml) insulin concentration. However, following 24 h incubation at a physiological glucose level (6 mM) no differences in insulin-stimulated glucose uptake were found between the three groups, and a reasonable interpretation is that insulin resistance was completely reversed in the diabetic cells.

Insulin sensitivity in vivo assessed with hyperinsulinemic, euglycemic clamp (M-value) was significantly associated with the insulin-induced increase in glucose uptake in fresh adipocytes in vitro. Fasting blood glucose at the time of biopsy and HbA1c, but not serum insulin, were negatively correlated to insulin's effect to stimulate glucose uptake in vitro in all groups taken together. Furthermore, fasting blood glucose, HbA1c and serum insulin were all negatively correlated to insulin sensitivity in vivo. Cell size, WHR and BMI correlated negatively with insulin's effect to stimulate glucose uptake both in vitro and in vivo.

Multiple regression analyses revealed that adipocyte cell size and WHR independently predicted insulin resistance in vitro. On the other hand, insulin sensitivity in vivo was significantly associated with fasting blood glucose and serum insulin levels.

No significant alterations in basal and cAMP-stimulated lipolysis were found between control cells and cells from diabetes patients, and insulin's ability to inhibit cAMP-stimulated lipolysis in vitro did not differ significantly between the groups. In the in vivo situation there was a tendency to elevated plasma FFA levels, both in the fasting situation as well as during clamp, in the diabetes group with poor metabolic control compared with the two other groups.

Main conclusions: Cellular insulin resistance in subcutaneous adipocytes from type 2 diabetes patients appears to be fully reversible following incubation at a physiological glucose concentration for 24 h. Fat cell insulin resistance may be mainly secondary to the diabetic milieu in vivo, e.g. via glucotoxicity.

Paper IV

The aim of study IV was to explore whether dysregulation of fat tissue LPL may be a mechanism for postprandial hypertriglyceridemia in type 2 diabetes. Subcutaneous adipose tissue biopsies were obtained from type 2 diabetes patients and non-diabetic control subjects, both in the fasting state and 3.5 h after a standardised lipid-enriched meal. Postprandial, but not fasting, triglyceride levels were significantly higher in the diabetic subjects. Following the test meal, total adipose tissue LPL activity increased by ~35-55 %, and this was similar in the type 2 diabetic and non-diabetic group. There was an essentially parallel increase in LPL mass. Thus, the specific activity of LPL was unchanged. Although the type 2 diabetes patients overall tended to have a slightly lower adipose tissue LPL activity, the levels of fasting or postprandial LPL activity did not differ significantly between the two groups. In addition, post-heparin plasma LPL activity, mass and specific activity did not display any consistent differences between the two groups.

Insulin resistance, estimated as the HOMA-IR index, was positively correlated with fasting and postprandial triglyceride levels and it was near-significantly and negatively associated with post-heparin plasma LPL activity. However, HOMA-IR displayed no association with adipose tissue LPL activity.

Basal as well as insulin-stimulated glucose uptake capacity in vitro was decreased by ~30 % in adipocytes from type 2 diabetes patients compared to the control group. Furthermore, there was a positive association between the insulin effect to stimulate glucose uptake in vitro vs fasting as well as postprandial adipose tissue LPL activity and this may support a role for insulin in the regulation of adipose tissue LPL.

Main conclusions: LPL activity in human subcutaneous adipose tissue is upregulated following food intake and this occurs to a similar extent in type 2 diabetic as well as non-diabetic individuals. Thus, the postprandial hypertriglyceridemia found in type 2 diabetes patients does not seem to be explained by gross alterations in the nutritional regulation of LPL activity in subcutaneous fat.

DISCUSSION

Perhaps the most interesting and challenging task in the research field of insulin resistance is to unravel the early underlying mechanisms. The development of insulin resistance in humans is considered to be a gradual process usually occurring over many years. Potential factors that may be involved in the early development of insulin resistance include cellular defects in insulin signalling or glucose transport as well as humoral factors, e.g. metabolic substrates, cytokines and insulin-antagonistic hormones. Neuroendocrine pathways regulate the release of such hormones, e.g. growth hormone, catecholamines and glucocorticoids in the circulation. They can oppose insulin action on carbohydrate and lipid metabolism in many tissues.

Effects of glucocorticoids

The negative effect of dexamethasone treatment in vitro on glucose metabolism has been established since many years (Fain 1963). The data on isolated rat adipocytes in the present work provide evidence of novel cellular mechanisms for glucocorticoid-induced alterations in carbohydrate metabolism that may potentially contribute to the development of human insulin resistance. However, the impact of endogenous cortisol production in type 2 diabetes is not yet established and future studies should address this topic.

Dexamethasone treatment for 24 h induced a ~40 % decrease in insulin binding capacity that seemed to be due to a reduction in the number of available cell-surface binding sites. However, we found a general reduction in glucose uptake capacity which is not likely to be explained by the reduction in insulin binding since fat cells have a large proportion of spare receptors (Kono 1971).

In our study, dexamethasone produced alterations in insulin signalling proteins in primary adipocytes that were not previously reported. The dexamethasone-induced decline in IRS-1 and PKB content is of great interest since these insulin signalling proteins are considered to play an important role in propagating the insulin signal from the insulin receptor to GLUT4 translocation. The amount of phosphorylated PKB in response to insulin stimulation decreased in parallel to total cellular PKB content. There is substantial evidence supporting a role for PKB in insulin's regulation of glucose metabolism. Insulin activates PKB via PI3-kinase-dependent phosphorylation in the insulin responsive adipocyte cell line 3T3-L1 (Kohn 1996), adipose tissue (Tanti 1997) and skeletal muscle (Brozinick 1998). However, the exact role of PKB in the regulation of glucose uptake and metabolism remains unclear and should be further investigated. Recent data on PKBβ knockout mice have suggested an important role for the β isoform of PKB since these animals acquire a phenotype similar to type 2

diabetes with elevated blood glucose levels accompanied by an increase in serum insulin levels (Cho 2001).

The insulin-responsive glucose transporter GLUT4 has been suggested as potential target for dexamethasone-induced insulin resistance and a reduction in GLUT4 was previously reported in adipocytes from type 2 diabetes patients and could thus be linked to insulin resistance (Garvey 1991). However, no suppression of GLUT4 was seen in our dexamethasone study and the negative effect of dexamethasone on glucose transport capacity could not be explained by depletion of GLUT4 content. Nevertheless, dexamethasone might alter GLUT4 function and it is possible that dexamethasone somehow interacts with the intrinsic activity or the translocation of GLUT4, as suggested previously (Garvey 1989b). Our findings that dexamethasone impairs both basal and insulin-stimulated glucose uptake would be compatible with alterations in GLUT1 content or function since GLUT1 is considered to be responsible for basal non-stimulated glucose transport (Gulve 1994). However, there were no consistent alterations in GLUT1 content following dexamethasone treatment. The time-course for dexamethasone's effect to inhibit glucose uptake differed with respect to basal and maximally insulin-stimulated glucose uptake as full inhibition was seen after 2 and 24 h, respectively. One potential explanation for this difference is that dexamethasone impairs basal glucose uptake by alterations in glucose transporter activity or function, and that insulin-stimulated glucose transport is altered by depletion of insulin signalling proteins like IRS-1 and PKB. This concept is supported by our findings that dexamethasone treatment for 8 h or less did not change IRS-1 or PKB content, whereas 24 h treatment suppressed IRS-1 and PKB expression considerably.

Since FFAs have been implicated in the development of insulin resistance (Boden 1997), the effects of dexamethasone on lipolysis and on the antilipolytic action of insulin were investigated. In man, elevated cortisol increases the rate of lipolysis (Divertie 1991) and data from rats suggests that the glucose-FFA cycle contributes to dexamethasone-induced insulin resistance and that it can be prevented when the FFA level is decreased by nicotinic acid (Venkatesan 1987). In the present work, dexamethasone pretreatment raised the basal lipolysis rate ~4-fold. In contrast, insulin's antilipolytic effect was left intact following dexamethasone. The enhanced lipolysis rate may very well be a critical mechanism for glucocorticoid-induced insulin resistance in fat cells, but further studies should be performed.

Effects of elevated glucose and insulin concentrations

Insulin and glucose levels affect whole-body insulin sensitivity, and during the natural course of insulin resistance the organism may experience different

milieus in this respect. For instance, when insulin resistance in peripheral tissues develops, the pancreas compensate for this by increasing insulin secretion from the β-cells, and hence, insulin levels are elevated. In subjects developing type 2 diabetes, the β-cells eventually fail to compensate for increasing insulin resistance and blood sugar levels start to rise. At later stages, insulin secretion gradually diminishes and, if it is not treated properly, blood glucose will be constantly elevated. To reflect the different stages in the development of type 2 diabetes, long-term effects of high insulin and glucose levels alone or in combination were studied with respect to insulin action in vitro in our cell culture system with isolated rat adipocytes. Pretreatment with a saturating insulin concentration (10,000 μU/ml) induced a downregulation of insulin binding by ~40 %. The reduced insulin binding could possibly explain the demonstrated rightward shift in the dose-response curve for insulin's effect to stimulate glucose uptake. Similarly, 24 h pretreatment with combined high insulin and glucose concentrations induced a rightward shift in the dose-response curve for insulin's acute effect to phosphorylate PKB. Insulin pretreatment reduced glucose uptake capacity, but only in the presence of high glucose. Similarly, IRS-1 and GLUT4 content was reduced by insulin only in the presence of high glucose. Long-term insulin also reduced IRS-2 content, and the most pronounced effect was seen when cells were concomitantly exposed to the highest glucose concentration.

Data from CHO cells and 3T3-L1 adipocytes suggest that insulin promotes IRS-1 degradation by the proteasome degradation pathway (Sun 1999). In 3T3-L1 adipocytes, it was recently reported that chronic insulin treatment induced a reduction of IRS-1 and GLUT4 protein contents with a parallel decrease in acutely insulin-stimulated PKB phosphorylation as well as a reduction in insulin-stimulated glucose transport (Berg 2002). In that study, the signalling protein mTOR (mammalian target of rapamycin) appeared to be involved in the attenuation of IRS-1 protein levels and insulin signalling, but not in the depletion of GLUT4. Taken together, these results suggest that chronic insulin exposure leads to a down-regulation of IRS-1 and GLUT4 through different mechanisms in adipocytes, but alterations in the rate of protein degradation could very well be involved. This is supported by our preliminary finding that alterations in the levels for IRS-1 and GLUT4 protein following treatment with high insulin and/or high glucose were not accompanied by any changes in mRNA levels (Renström F, Burén J, Eriksson JW, unpublished observations).

In our study, a high glucose level (≥ 15 mM) in the medium impaired glucose uptake capacity in isolated adipocytes but had not effect by itself on insulin binding. Cellular IRS-1 protein was downregulated and could be a target for glycemia-induced insulin resistance since IRS-1 is the major tyrosine-phosphorylated IRS in adipocytes (Sun 1992; Rondinone 1997). Since IRS-2

content was upregulated it is possible that IRS-2 becomes the main docking protein in this situation, similar to what was suggested in human type 2 diabetes (Rondinone 1997). Pretreatment with high glucose had no consistent effect on PKB content or maximal insulin-stimulated PKB phosphorylation. It was previously reported that insulin-stimulated tyrosine phosphorylation of IRS-1 is reduced in adipocytes from human type 2 diabetes patients due to a ~70 % reduction in IRS-1 protein content (Rondinone 1997). Moreover, insulin-stimulated PI3-kinase activation as well as down-stream activation of PKB may be impaired (Carvalho 2000) along with a reduction in GLUT4 expression (Garvey 1991). In skeletal muscle from type 2 diabetes patients, the situation appears to be similar with respect to insulin-stimulated tyrosine phosphorylation of IRS-1 and PI3-kinase activation (Bjornholm 1997). However, there was no demonstrable alteration in IRS-1 content. Moreover, downstream insulin-induced activation of PKB in muscle from patients with type 2 diabetes has been reported normal (Kim YB 1999) or slightly reduced (Krook 1998). Defects in the GLUT4 trafficking machinery or the intrinsic activity of glucose transporters have been described in the literature (Vannucci 1992). This may be of relevance for our finding that long-term exposure to a high glucose level produced an impairment in glucose uptake capacity in spite of increased GLUT4 content in the adipocytes.

The present study indicates that combined pretreatment with high glucose and high insulin displays complex interaction effects on the expression of IRS-1 and 2. In animal models that display hyperglycemia combined with hyperinsulinemia, the expression of IRS-1 and 2 in muscle are reduced (Kerouz 1997; Anai 1998). These insulin- and glucose-induced aberrations in the amount of insulin signalling proteins might be of relevance for the development and progression of human type 2 diabetes, as also supported by data from mice with combined heterozygous null mutations suggesting that combined IRS-1 and IRS-2 perturbations may contribute to the development of diabetes (Kido 2000). Data from study II demonstrate that enhanced insulin and/or glucose levels can induce alterations in insulin binding, IRS-1 and 2 content, PKB activation and glucose uptake in isolated rat adipocytes. The data thus suggest that cellular insulin resistance is aggravated by high glucose and insulin levels in vivo.

A common denominator for reduced glucose uptake capacity in the insulin-resistant adipocyte models used in study I and II is a reduction in IRS-1 protein. However, it is very likely that other components of the IRS pathway or completely different signalling pathways are also important for the regulation of insulin-stimulated glucose transport. Nevertheless, our data may suggest that IRS-1 is a critical mechanism, and that a reduced amount of cellular IRS-1 is accompanied by an impaired glucose transport.

An important feature in study I and II is that although pretreatment with dexamethasone or the combination of high glucose and high insulin were potent in reducing glucose uptake capacity in isolated adipocytes, the response to acute insulin stimulation *per se* was generally not affected. Thus, basal, non-stimulated and insulin-stimulated glucose uptake rates were both suppressed to a similar extent. The reason for this is not clear, but different mechanisms may be involved, e.g. a defect in the general capacity of cellular glucose uptake and an impairment in insulin action on GLUT4 trafficking, respectively. A general decrease in basal uptake would be compatible with altered GLUT1 content or function. However, following dexamethasone pretreatment, GLUT1 content was not altered, and in rats made hyperglycemic by streptozotocin treatment GLUT1 was reported to be unaffected (Kahn 1989; Garvey 1989a). A summary of the results from study I and II is presented in table 3.

Table 3.
Summary of results in study I and II.

	24 h pretreatment			
	Dexamethasone	High glucose	High insulin	High glucose & Insulin
Insulin binding	↓	↔	↓	↓
IRS-1	↓↓	↓	↔	↓↓
IRS-2	↑↑	↑↑	↓	↓↓
PI3-K	↓	↔	↔	↔
PKB	↓	↔	↔	↔
pPKB	↓	↔	↔	↔
GLUT4	↔	↑↑	↔	↔
Glucose uptake	↓	↓	↔	↓↓

Arrows indicate alterations in cellular protein content, insulin binding or glucose uptake in rat adipocytes. pPKB, Ser^{473}-phosphorylated PKB following 10 min insulin stimulation.

In vivo insulin resistance in type 2 diabetes – is glucotoxicity critical?

Since glucose and insulin *per se* induced alterations in insulin signalling proteins and glucose uptake capacity, the next goal was to investigate whether transferring the cells from a "diabetic" milieu in vivo to a euglycemic milieu in vitro could reverse insulin resistance in human adipocytes. For this purpose, three groups of subjects were recruited; two groups of type 2 diabetes patients with different degree of metabolic control (good and poor metabolic control, respectively) and, for comparison, one group with non-diabetic subjects. As expected, the diabetes groups were insulin-resistant both with respect to whole-

body glucose uptake in vivo and with respect to glucose uptake in freshly obtained fat cells in vitro. Of interest, it has been reported that subcutaneous abdominal fat cell size is associated with hyperinsulinemia and glucose intolerance even after adjustment for adiposity (Stern 1972; Kissebah 1982; Krotkiewski 1983) and also that enlarged subcutaneous abdominal fat cell size is an independent predictor of type 2 diabetes (Weyer 2000).

The present data revealed that fasting blood glucose, HbA1c, serum insulin levels, adipocyte cell size and also WHR and BMI were negatively correlated to in vivo insulin sensitivity, calculated as M-value. According to stepwise multiple regression calculations, fasting blood glucose and serum insulin levels were significantly associated with insulin sensitivity in vivo.

The ability of insulin to stimulate glucose uptake in fresh adipocytes in vitro, i.e. the cellular insulin response, was strongly and positively correlated with insulin sensitivity in vivo. Fasting blood glucose was negatively and near-significantly correlated with the insulin response in vitro. In addition, long-term glycemia reflected by HbA1c as well as adipocyte cell size, WHR and BMI also correlated negatively to insulin's effect to stimulate glucose uptake in vitro. Stepwise multiple regression analyses indicated that adipocyte cell size and WHR, reflecting abdominal obesity, are important factors determining insulin action in vitro.

In vitro data on glucose uptake suggested that the impairment of insulin responsiveness in the diabetic groups with respect to glucose uptake in fresh adipocytes was reversed following a 24 h cell culture in a euglycemic milieu. These data may indicate that there are in fact no primary defects causing insulin resistance in fat cells in type 2 diabetes, but rather an acquired defect due to factors present in the in vivo milieu. The "prime suspect" is glucose since glucose levels were lower in the 24 h culture compared to the hyperglycemic situation for "diabetic" cells in vivo. The so-called glucotoxic effect is established in rat skeletal muscle (Sasson 1987; Richter 1988). Moreover, the glucotoxic effect in muscle seems reversible since insulin-resistant muscle strips from type 2 diabetes patients incubated at a "euglycemic" milieu recover their insulin sensitivity (Zierath 1994). Thus, our present results on human adipocytes from type 2 diabetes patients are in accordance with previous work on skeletal muscle, and in both tissues, cellular insulin resistance appears to be reversible to a great extent.

The fat cells are taken out from their in vivo environment and they are washed before the culture period. Therefore, other circulating factors present in vivo such as FFAs, interleukins or TNF-α (Hotamisligil 1994; Hunnicutt 1994) are probably removed, and that this may potentially contribute to the normalisation

of cellular insulin action following 24 h cell culture. According to recent work, preincubation of fat cells from type 2 diabetes patients at physiological glucose level for 6 h did not improve the effect of insulin on glucose uptake (Smith U 2002), suggesting that a longer incubation period may be necessary to restore insulin response. In addition, 16 h incubation of fat cells from non-diabetic subjects at 25 mM glucose did not significantly impair insulin action (Smith U 2002). Those finding may challenge the critical role of glucotoxicity, and in addition, our multiple regression analyses in study III could not demonstrate a significant association between glycemia in vivo and insulin response in vitro.

Postprandial blood lipids and lipoprotein lipase

Insulin resistance is of great importance not only in carbohydrate metabolism, but also with respect to lipid metabolism. It has since long been established that there is a strong relationship between insulin resistance, compensatory hyperinsulinemia and hypertriglyceridemia (Reaven 1967; Olefsky 1974; Tobey 1981). The importance of perturbations in lipid metabolism is stressed by the fact that postprandial lipid intolerance in type 2 diabetes appears to be a very early hallmark of the disease, since it was demonstrated in normoglycemic relatives of type 2 diabetes patients who are at high risk of future diabetes (Axelsen 1999). However, the role of insulin in the regulation of postprandial lipemia is not fully understood. The activity of adipose tissue LPL is increased in healthy individuals following food intake (Lithell 1978; Taskinen 1987; Ong 1989) and this may be governed by the insulin levels (Pykalisto 1975). Some studies have indirectly indicated that insulin resistance is an important factor behind accumulation of triglyceride-rich lipoproteins after fat-containing meals (Jeppesen 1995; Couillard 1998), and that the amplitude of postprandial lipemia correlates with the severity of insulin resistance (Jeppesen 1995). A conceivable mechanism for the postprandial hypertriglyceridemia reported in type 2 diabetes could be a reduced clearance of triglyceride-rich particles in the postprandial state. These triglyceride-rich particles are mainly chylomicrons carrying triglycerides of dietary origin from the gastrointestinal tract to the circulation. In study IV, serum triglycerides were significantly higher in type 2 diabetes subjects compared to control subjects following a standardized lipid-enriched meal, whereas fasting triglycerides did not differ significantly. However, LPL activity was increased to a very similar degree (by ~35-55 %) in both groups, and this is comparable to what was previously reported in healthy subjects (Lithell 1978; Taskinen 1987; Ong 1989). From these data it can be concluded that the demonstrated postprandial hypertriglyceridemia in type 2 diabetes subjects is not explained by grossly altered prandial regulation of adipose tissue LPL activity. Hence, if dysregulation of LPL activity following a meal is of relevance for alterations in blood lipid clearance, this would be expected to occur in other tissues than subcutaneous fat, e.g. skeletal muscle which probably

contributes more than adipose tissue to the overall regulation of LPL in the circulation (Ruge 2001). Alternatively, other mechanisms that do not involve LPL could be responsible for postprandial dyslipidemia, and these could include perturbations in FFA turnover and/or hepatic VLDL synthesis.

SUMMARY

1) The cortisol analogue dexamethasone impairs glucose transport capacity in rat adipocytes independent of the prevailing glucose and insulin levels. If this is extrapolated to the in vivo setting, glucocorticoids may have detrimental effects on glucose turnover in subjects with normal glucose tolerance, impaired glucose tolerance as well as overt diabetes, and this may occur at any degree of glycemic control and β–cell function. Glucocorticoids may exert their diabetogenic effect partly by impairing cellular glucose uptake capacity. Reduced amounts of the signalling proteins IRS-1 and PKB and the accompanying decrease in insulin-stimulated PKB phosphorylation can be important mechanisms leading to attenuation of insulin-stimulated glucose transport.

2) Long-term exposure to a high glucose concentration *per se* downregulates IRS-1 but upregulates IRS-2 content in rat adipocytes and this occurs along with an impairment in glucose transport capacity. A 24 h cell incubation at a high insulin level downregulates insulin binding capacity and, when combined with high glucose, it produces a marked depletion of both IRS-1 and 2 content together with an impaired sensitivity to insulin-induced activation of PKB and a further reduction in glucose uptake capacity. These mechanisms may potentially contribute to cellular insulin resistance in human type 2 diabetes and may be of relevance for the so-called glucotoxic effect.

3) Insulin resistance in adipocytes from type 2 diabetes patients can be reversed by a 24 h incubation of the cells at a physiological glucose level. Thus, cellular insulin resistance may be mainly secondary to the diabetic in vivo milieu, e.g. via glucotoxicity.

4) Lipoprotein lipase activity in human adipose tissue is upregulated following food intake and this occurs to a similar extent in type 2 diabetes patients and in non-diabetic control individuals. Thus, postprandial lipid intolerance in type 2 diabetes is probably not explained by an altered nutritional regulation of adipose tissue LPL.

CONCLUDING REMARKS

Although insulin was discovered about 80 years ago, the mechanisms involved in insulin action in the human body are still not completely clarified. The importance of understanding insulin action is emphasized by the fact that insulin resistance is involved, and probably plays an important pathophysiological role, in many common disorders, e.g. type 2 diabetes, obesity, hypertension and dyslipidemia. The primary factors responsible for the development of insulin resistance are so far unknown, although both genetic and environmental factors are likely to be involved. The genetic defects responsible for the common forms of insulin resistance, which are typically present in type 2 diabetes, are largely unidentified. This study shows that glucocorticoids and also other humoral factors like elevated glucose and insulin concentrations can impair cellular glucose uptake capacity and that this might be due to alterations in key proteins involved in insulin's intracellular signalling pathways. The role of these factors in the early development of insulin resistance and type 2 diabetes, however, need to be further addressed in future studies. In overt diabetes, cellular insulin resistance could be, at least partly, secondary to the diabetic in vivo milieu. A hypothetical scheme for the development of insulin resistance in type 2 diabetes is presented below (Fig. 6).

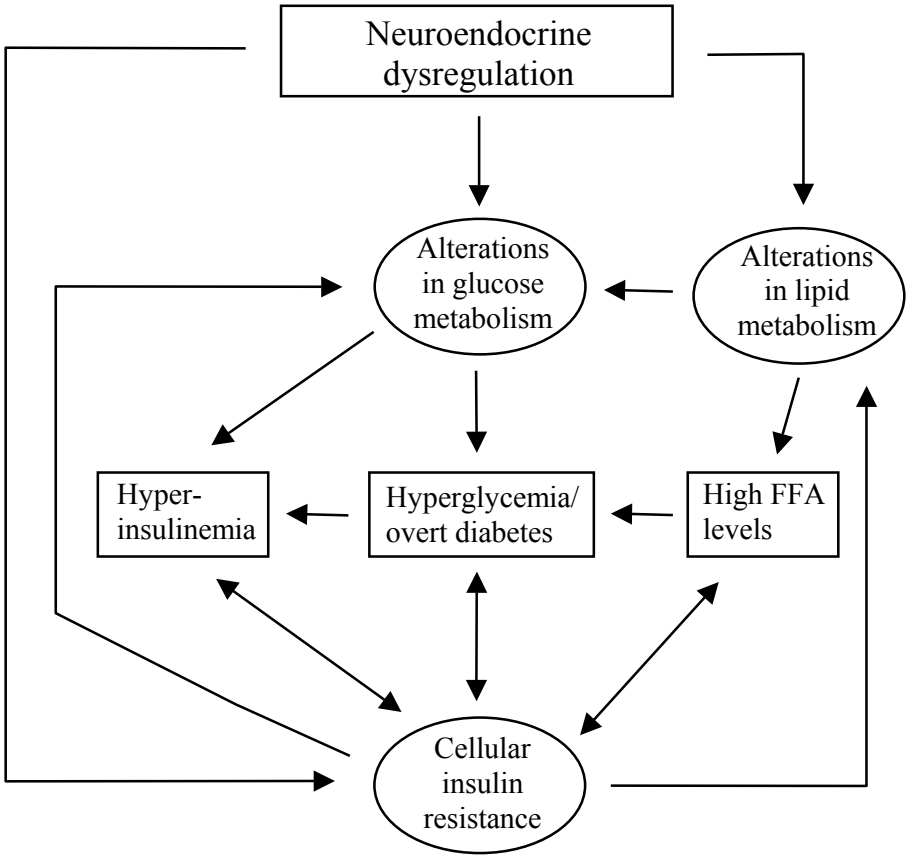

Figure 6.
Hypothetical pathways for the development of insulin resistance in type 2 diabetes.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Typ 2-diabetes är i hög grad en välfärdssjukdom och antalet drabbade individer ökar i Sverige och i stora delar av världen, särskilt utvecklingsländerna. Den globala förekomsten uppskattas till 2-3 % av befolkningen. Både ärftliga och miljöbetingade faktorer är av betydelse vid uppkomst och utveckling av sjukdomen. Diabetes kännetecknas av höga nivåer av glukos (druvsocker) i blodet och vid typ-2 diabetes är både okänslighet för insulin, s.k. insulinresistens, i olika vävnader (lever, muskel och fett) samt en bristande insulinproduktion i bukspottkörteln av betydelse för uppkomst av sjukdomen.

Insulinresistens kan definieras som ett tillstånd med nedsatt effekt av en viss mängd insulin. Insulinresistens och åtföljande höga insulinnivåer i blodet är förknippade med andra sjukdomstillstånd som typ-2 diabetes, högt blodtryck, bukfetma, blodfettsrubbningar samt hjärt-kärlsjukdomar. Insulinresistens förekommer alltså vid en mängd olika sjukdomstillstånd.

Insulin utsöndras till blodet från bukspottkörtelns β-celler. Via blodet transporteras sedan insulinet ut till kroppens alla vävnader och organ. I vävnaden binder insulinmolekylen till specifika insulinreceptorer som sitter på ytan av cellerna. Denna bindning är startsignalen för en mängd olika reaktioner inne i cellen. Fortplantning av insulinsignalen i cellen sker via en lång rad med proteiner som aktiveras bl.a. genom fosfatbindning. I fett- och muskelceller aktiveras bland annat transporten av glukos in i cellerna av denna signal.

Orsakerna bakom insulinresistens är ej fullständingt klarlagda. Insulinresistens på cellnivå kan teoretiskt orsakas av defekter i insulinets bindning till cellerna, i reaktionerna inne i cellen eller hos glukostransportörer. Insulinresistensen medför att insulinets metabola effekter i cellen på bl.a. glukos- och fettomsättning hämmas. Några av insulinets viktigaste uppgifter i detta avseende är att stimulera glukosupptaget i muskel och fettväv, hämma glukostillverkning i levern samt att i fettväven hämma fettnedbrytningen. Insulinresistens på cellnivå kan vara sekundära till cirkulerande faktorer i blodet, t.ex. hormoner som motverkar insulin, såsom adrenalin och kortisol. Långvarigt förhöjda insulin- och/eller glukosnivåer i sig självt leder också till insulinresistens.

Fettväven fungerar inte bara som en enegireserv, utan utsöndrar även hormoner, inflammationssubstanser (s.k. cytokiner) och andra biologiskt aktiva substanser till blodbanan. Störd reglering av fettvävens produktion av dessa substanser kan tänkas leda till insulinresistens och mycket tyder på att insulinkänsligheten i fettväven är viktig för reglering av hela kroppens insulinkänslighet.

I **delarbete I** odlades råttfettceller i närvaro av dexametason, ett kortisolliknande ämne, detta för att studera en endokrin faktor som kan bidra till utvecklingen av insulinresistens och typ 2-diabetes hos människa. Dexametason medför tydliga störningar i fettcellernas insulin-signalering och glukosomsättning, som kan vara relevanta för uppkomsten av insulinresistens. Dexametason minskar cellens förmåga att binda insulin till sig och minskar även glukosupptaget med ~40-50 %. Studien tyder på att dexametason reducerar mängden, och därmed aktiviteten av IRS-1 och PKB, två av nyckelproteinerna inom insulinets signalsystem. Insulinets förmåga att motverka fettnedbrytning är intakt, men den underliggande fettnedbrytningen tycks vara förhöjd efter dexametasonbehandling av fettceller. Effekterna av dexametason är i huvudsak oberoende av omgivande glukos- och insulinkoncentrationer.

I **delarbete II** ville vi återspegla fyra olika stadier i utvecklingen av typ-2 diabetes, nämligen 1) ett förstadium med låga glukos- och insulinnivåer, 2) hög insulinnivå (till följd av insulinresistens), 3) höga insulin- och glukosnivåer, och slutligen 4) hög glukos- men låg insulinnivå. Sammanfattningsvis kan sägas att långvarigt högt glukos nedreglerar IRS-1-mängden och glukostransportkapaciteten i cellerna. Långvarig exposition för högt insulin minskar cellernas förmåga att binda insulin och i kombination med högt glukos så ses en nedreglering av signalmolekylerna IRS-1 och IRS-2, vilket sannolikt kan kopplas till en nedsatt effekt av insulinets förmåga att aktivera PKB och glukostransport. Dessa mekanismer kan bidra till insulinresistens vid typ 2-diabetes, och våra data talar också för att förändringar hos insulinets signalproteiner kan uppträda som en följd av det diabetiska tillståndet.

I **delarbete III** har vi försökt klargöra huruvida insulinresistensen på cellnivå hos typ 2-diabetiker är reversibel eller ej. Vävnadsprover från underhudsfett togs från diabetespatienter, med gott respektive för högt sockerläge och som jämförelse undersöktes fett från fullt friska kontrollindivider. Cellerna hos patienter med typ 2-diabetes är insulinresistenta, och de diabetespatienter som har riktigt höga blodsockernivåer är mest insulinresistenta. Cellerna odlades under 24 tim vid normal glukoskoncentration (5-6 mmol/L) och därefter mättes insulineffekterna på glukosupptag. Efter denna odling fann vi ingen skillnad i insulinkänslighet mellan celler från diabetespatienter och kontrollpersoner och inte heller mellan de två diabetesgrupperna. Dessa data tyder alltså på att insulinresistensen på cellnivå är reversibel hos typ 2-diabetiker, och att den till stor del beror på den omgivande miljön i kroppen, t.ex. höga sockernivåer, och inte på någon inneboende defekt hos cellerna.

I **delarbete IV** jämförde vi aktiviteten av ett enzym, lipoproteinlipas (LPL), i fettväv hos patienter med typ 2-diabetes och kontrollpersoner. Sedan tidigare är det känt att förhöjda nivåer av fett i form av triglycerider i blodet efter måltider

kan uppträda tidigt vid utvecklingen av typ 2-diabetes. Detta är även en riskfaktor för hjärt-kärlsjukdomar. En förändrad mängd eller aktivitet hos enzymet LPL, som spjälkar fetter i blodet, skulle kunna vara en tänkbar mekanism bakom detta. Efter en måltid transporteras fettpartiklar från tarmen i form av s.k. chylomikroner ut i blodbanan där LPL bryter ner triglyceriderna till fria fettsyror som kan tas upp i vävnader och användas, bl.a. för lagring av energi. Insulin underlättar denna transport av fett från blodbanan till fettcellerna genom att stimulera LPL och insulin ökar också upplagring av fett i fettcellerna.

Vävnads- och blodprover togs vid fasta samt 3,5 tim efter en fettberikad måltid. Triglyceridnivåer i blod efter måltid var högre hos typ 2-diabetes patienter jämfört med kontrollpersoner. Analyserna visade att LPL-aktiviteten i fettväv efter måltid ökade med ~35-55 % hos både diabetiker och kontrollpersoner. Dessa fynd talar för att de förhöjda triglyceridernivåerna i blod efter måltid hos typ 2-diabetiker ej orsakas av försämrad måltidsreglering av LPL i fettväven.

Sammanfattningsvis visar denna avhandling på några tänkbara mekanismer vid utvecklingen av insulinresistens och typ 2-diabetes. Förhöjda nivåer av cirkulerande faktorer i blodet i form av t.ex kortisol, insulin och socker kan medverka till utvecklingen eller försämring av insulinresistens. Våra fynd tyder på att insulinresistens i fettceller är reversibel och orsakas av miljön omkring cellerna och inte till följd av någon inneboende defekt hos cellerna.

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