Effects of immunosuppressive drugs on human adipose tissue metabolism

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To my family

Especially to my father,
who taught me the value of learning.

ABSTRACT

The immunosuppressive agents (IAs) rapamycin, cyclosporin A and tacrolimus, as well as glucocorticoids are used to prevent rejection of transplanted organs and to treat autoimmune disorders. Despite their desired action on the immune system, these agents have serious long-term metabolic side-effects, including dyslipidemia and new onset diabetes mellitus after transplantation.
The overall aim is to study the effects of IAs on human adipose tissue glucose and lipid metabolism, and to increase our understanding of the molecular mechanisms underlying the development of insulin resistance during immunosuppressive therapy.

In *Paper I* and *II*, it was shown that rapamycin and the calcineurin inhibitors, cyclosporin A and tacrolimus, at therapeutic concentrations, had a concentration-dependent inhibitory effect on basal and insulin-stimulated glucose uptake in human subcutaneous and omental adipocytes. Rapamycin inhibited mammalian target of rapamycin complex (mTORC) 1 and 2 assembly and phosphorylation of protein kinase B (PKB) at Ser473 and of the PKB substrate AS160, and this leads to impaired insulin signalling (*Paper I*). On the other hand, cyclosporin A and tacrolimus had no effects on expression or phosphorylation of insulin signalling proteins (insulin receptor substrate 1 and 2, PKB, AS160), as well as the glucose transport proteins, GLUT4 and GLUT1 (*Paper II*). Instead, removal of GLUT4 from the cell surface was observed, probably mediated through increased endocytosis, as shown in L6 muscle-derived cells. These studies suggest a different mechanism for cyclosporin A and tacrolimus, in comparison to rapamycin, with respect to impairment of glucose uptake in adipocytes.
In *Paper III*, all three IAs increased isoproterenol-stimulated lipolysis and enhanced phosphorylation of one of the main lipases involved in lipolysis, hormone-sensitive lipase. The agents also inhibited lipid storage, and tacrolimus and rapamycin down-regulated gene expression of lipogenic genes in adipose tissue. All three IAs increased interleukin-6 (IL-6), but not tumor necrosis factor α (TNF-α) or adiponectin, gene expression and secretion.
In *Paper IV*, we proposed that FKBP5 is a novel gene regulated by dexamethasone, a synthetic glucocorticoid, in both subcutaneous and omental adipose tissue. FKBP5 expression in subcutaneous adipose tissue is correlated with clinical and biochemical markers of insulin resistance and adiposity. In addition, the FKBP5 gene product was more abundant in omental than in subcutaneous adipose tissue.

In conclusion, adverse effects of immunosuppressive drugs on human adipose tissue glucose and lipid metabolism can contribute to the development of insulin resistance, type 2 diabetes and dyslipidemia in patients on immunosuppressive therapy. The cellular mechanisms that are described in this thesis should be further explored in order to mitigate the metabolic perturbations caused by current immunosuppressive therapies. The findings in this thesis could potentially also provide novel pharmacological mechanisms for type 2 diabetes as well as other forms of diabetes.

Keywords: Cyclosporin A, tacrolimus, rapamycin, glucocorticoids, new onset diabetes after transplantation, adipocytes, insulin signalling, glucose uptake, lipolysis, lipogenesis

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LIST OF PAPERS

The thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Maria J Pereira, Jenny Palming, Magnus Rizell, Manuel Aureliano, Eugénia Carvalho, Maria K Svensson, Jan W Eriksson
mTOR inhibition with rapamycin causes impaired insulin signalling and glucose uptake in human subcutaneous and omental adipocytes
Mol Cell Endocrinol 355:96-105, 2012

II. Maria J Pereira, Jenny Palming, Magnus Rizell, Manuel Aureliano, Eugénia Carvalho, Maria K Svensson, Jan W Eriksson
Cyclosporin A and tacrolimus reduce cell-surface amount of GLUT4 via increased endocytosis: a potential mechanism for the diabetogenic effects of immunosuppressive agents
Submitted (2012)

III. Maria J Pereira, Jenny Palming, Magnus Rizell, Manuel Aureliano, Eugénia Carvalho, Maria K Svensson, Jan W Eriksson
The immunosuppressive agents rapamycin, cyclosporin A and tacrolimus increase lipolysis, inhibit lipid storage and alter expression of genes involved in lipid metabolism in human adipocytes
Submitted (2012)

IV. Maria J Pereira*, Jenny Palming*, Maria K Svensson, Magnus Rizell, Jan Dalenbäck, Mårten Hammar, Per-Arne Svensson, Jan W Eriksson
Effects of dexamethasone on gene expression in human subcutaneous and omental adipose tissue - is FKBP5 a novel link between insulin resistance and immune modulation?
*these authors contributed equally
Submitted (2012)

LIST OF ABBREVIATIONS

AMPK	5'-AMP-activated protein kinase
AS160	Protein kinase B substrate of 160 kDa
ATGL	Adipose triacylglycerol lipase
BMI	Body mass index
CNR1	Cannabinoid receptor 1
DGAT	Diacylglycerol acyltransferase
ESRD	End-stage renal disease
FABP4/aP2	Fatty acid binding protein 4
FAS	Fatty acid synthase
FFA	Free fatty acid
FKBP5	FK506-binding protein 5
FKBP51	51 kDa FK506-binding protein
GLUT1, 4	Glucose transporter 1, 4
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment - insulin resistance
HSL	Hormone-sensitive lipase
Hsp	Heat shock protein
IA	Immunosuppressive agent
IL-6	Interleukin-6
IR	Insulin receptor
IRS1, 2	Insulin receptor substrate 1, 2
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MGAT	Monoacylglycerol acyltransferase
mTOR	Mammalian target of rapamycin
mTORC1, 2	Mammalian target of rapamycin complex 1, 2
NFAT	Nuclear factor of activated T cells
NODAT	New onset diabetes after transplantation
p70S6K	p70 ribosomal S6 kinase
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKA, B, C	Protein kinase A, B, C
PPARγ	Peroxisome proliferator-activated receptor γ
SEM	Standard error of the mean
SERTM1	Serine-rich and transmembrane domain containing 1
SREBP	Sterol regulatory element-binding proteins
Sin1	Stress-activated protein kinase interacting protein 1
TAG	Triacylglycerol
TIMP4	Metallopeptidase inhibitor 4
TNF-α	Tumor necrosis factor α
TSC	Tuberous sclerosis complex
VLDL	Very-low density lipoprotein

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INTRODUCTION

Insulin resistance and diabetes

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterised by chronic hyperglycaemia, and resulting from defects in insulin secretion, insulin action, or both (1). Diabetes mellitus is classified on the basis of the pathogenic process that results on hyperglycemia or on the circumstances present at the time of diagnosis. There are four main types of diabetes mellitus: type 1, type 2, gestational diabetes and secondary diabetes. Typically type 1 diabetes results from autoimmune destruction of insulin-producing β-cells in the pancreas and type 2 diabetes from insulin resistance combined with insulin deficiency. Gestational diabetes is formally defined as glucose intolerance with onset or first recognition during pregnancy. The last group, so called secondary diabetes, includes genetic defects of β-cell function; diseases of the exocrine pancreas, such as pancreatitis or cystic fibrosis; other endocrinopathies (e.g., acromegaly); and pancreatic dysfunction and/or insulin resistance caused by drugs.

Type 2 diabetes

Type 2 diabetes is the most common form of diabetes and is caused by a combination of genetic and environmental factors (2). Currently, about 200 million people have type 2 diabetes, and the prediction is that by 2030 about 400 million people worldwide will have type 2 diabetes (3). The high prevalence of diabetes and its complications has vast socioeconomic consequences. The development of type 2 diabetes is usually preceded by impaired insulin sensitivity in skeletal muscle, adipose tissue and liver, a metabolic condition called insulin resistance and commonly driven by a sedentary lifestyle and obesity (2). As a consequence the β-cells in the pancreas produce more insulin to overcome the insulin resistance in peripheral tissues. Eventually β-cells fail to produce enough insulin to overcome insulin resistance, leading to hyperglycaemia and development of type 2 diabetes.

Several factors have been proposed to contribute to insulin resistance, including increased circulating non-esterified fatty acids, inflammatory cytokines (e.g. tumor necrosis factor α, TNF-α and interleukin-6, IL-6), adipokines (e.g. resistin) and defects in mitochondrial lipid oxidation (2). On the other hand, the β-cell dysfunction has been attributed to glucose toxicity, lipotoxicity and islet amyloid deposits. Moreover, genome-wide association studies have provided support that genetic factors are also important in predisposing some individuals to type 2 diabetes and insulin resistance (4), including polymorphisms of calpain 10 and peroxisome proliferator-activated receptor γ (PPARγ) (5).

Drug-induced diabetes

It is well known that medications may cause unwanted side-effects, although designed to relieve symptoms and improve quality of life. There are several drugs that may induce diabetes (secondary diabetes) and these include glucocorticoids, other immunosuppressive agents (IAs), diuretics, β-blockers and antipsychotic agents (6). This can be caused by impaired insulin secretion from the pancreatic β-cells or by insulin resistance in peripheral tissues and liver, or, most commonly, by both. In many cases, the underlying mechanisms are not fully understood and warrant further investigation. The focus will be on IAs since this is most relevant in the context of this thesis.

Complications of diabetes

Although, diabetic long-term complications develop gradually, they can eventually be disabling or even life-threatening (7). Generally the diabetic long-term complications are divided into micro- (diabetic nephropathy, neuropathy and retinopathy) and macrovascular complications (coronary artery disease, peripheral arterial disease and stroke) (8). The cardiovascular mortality in type 2 diabetic patients is more than double compared with age-matched subjects, being the major cause of death in people with diabetes.

Diabetic nephropathy is the most common cause of end-stage renal disease (ESRD) in many countries. It accounts for approx 45 to 50% of cases of ESRD in the United States and approximately 25% of cases with ESRD in Sweden. Diabetic nephropathy is the aetiology of ESRD in approximately 25% of kidney transplant recipients transplanted in the United States and in Sweden the proportion is somewhat lower (9-11).

Diabetes presents particular challenges both in the pre-transplant evaluation and after transplantation (12). These challenges are related to the high incidence of cardiovascular disease among patients with diabetes, and an increased risk of bacterial and fungal infections compared with transplant recipients without diabetes. In addition, glycemic control is often more difficult after transplantation. This is because immunosuppressive regimens used after transplantation have detrimental effects on both pancreatic β-cell function and peripheral insulin action as discussed in detail in this thesis. Therefore, IAs makes it difficult to achieve target glucose levels and prevent the recurrence of the diabetic lesions in the transplanted kidney.

Combined kidney-pancreas transplantation is an established treatment for selected patients with type 1 diabetes and ESRD (13). Patients where the potential benefit of a combined transplant outweighs the increased morbidity of the surgical procedure and the use of lifelong immunosuppression are also considered. Combined kidney-pancreas transplantation, if successful, improves both patient survival and quality of life.

A few patients with type 1 diabetes not controlled by exogenous insulin therapy and without substantial renal disease may be considered as candidates for pancreas transplantation alone (14). An alternative to solid organ pancreas transplantation is transplantation of pancreatic islets. In 2009 ~400 individuals have received allogenic isolated pancreatic islets with ~40 centers world-wide actively engaged in further developing this therapy (15). All these modes of transplantation currently require treatment with life-long immunosuppressive therapy.

Immunosuppressive drugs and adverse metabolic effects

In the early/mid-1980s, the discovery and introduction of modern IAs into clinical practice was a major breakthrough to prevent and treat allograft rejection, resulting in an improved long-term patient and graft survival after solid organ transplantation (16). A commonly used basic immunosuppressive protocol uses a higher immunosuppressive load in the early post-transplant phase, commonly a calcineurin inhibitor (cyclosporin A or tacrolimus), glucocorticoids and an antiproliferative agent (e.g. azathioprine or mycophenolate mofetil). During the subsequent maintenance phase, lower levels of a calcineurin inhibitor or a inhibitor of the mammalian target of rapamycin (mTOR) is combined with the lowest dose possible of glucocorticoids and/or one of the anti-proliferative agents. Individualized immunosuppressive protocols aim to combine the most effective drug combination to balance the risk of rejection against long-term adverse drug effects. However, even with these individualized protocols, long-term outcome of solid organ transplantation is hampered by the development of metabolic perturbations, like diabetes, dyslipidemia and hypertension (17-19). Taken together these metabolic complications are associated with an increased risk of non-fatal and fatal cardiovascular events, and other adverse outcomes including infections, malignancies, reduced patient and graft survival (18, 20).

New-onset diabetes after transplantation (NODAT)

Diabetes as a long-term complication after kidney transplantation was first identified in 1964 by Starzl *et al.* (21). Since then, it has become clear that new onset diabetes after transplantation (NODAT) is a common metabolic complication, with reported incidence rates up to 50% during the first year after transplantation (19, 22). Most patients develop NODAT within the first 6 months after transplantation, although the cumulative incidence continues to increase also after this period (17).

Similar to type 2 diabetes, both decreased insulin secretion and increased insulin resistance seem to be principal pathogenic components of NODAT (23, 24). Accordingly, adoption of current American Diabetes Association diagnostic criteria for type 2 diabetes was recommended for the diagnosis of NODAT (25).

In addition to other risk factors (Table 1), immunosuppressive therapy and obesity are reported to be modifiable risk factors for the development of NODAT. Montori *et al* reported that 74% of the variability of the incidence of NODAT could be explained by the immunosuppressive therapy (19). This may be due to the fact that different IAs can produce different metabolic perturbations (Table 2).

Table 1. Non-modifiable and modifiable risk factors for new onset diabetes (NODAT).

Non-modifiable risk factors	Modifiable risk factors
- Age > 40 years	- Obesity
- Ethnicity	- Metabolic syndrome
- Family history of diabetes	- Immunosuppression
- Genetic susceptibility	glucocorticoids
- HLA mismatches	cyclosporin A
- Deceased donor	tacrolimus
- Acute rejection history	rapamycin
- Male donor	- Hyperlipidemia
- Polycystic kidney	- Pre-transplant IGT
	- Hepatitis C and cytomegalovirus infection

HLA, human leukocyte antigen; IGT, impaired glucose tolerance. Adapted from (26).

The association between glucocorticoid therapy and the development of diabetes was established in the 1960s (21). The diabetogenic effect of glucocorticoids is dose-dependent and includes reduction in insulin synthesis and insulin sensitivity, increased hepatic gluconeogenesis and central obesity (27-30) (Table 2). The introduction of more active IAs, including the calcineurin inhibitors cyclosporin A and tacrolimus, made it possible to lower corticosteroid doses and thereby reduce the incidence of NODAT (31). Unfortunately, cyclosporin A and tacrolimus are also diabetogenic, probably due to both reduced insulin secretion from the pancreas and impaired insulin sensitivity in peripheral tissues (32-39) (Table 2). Moreover, treatment with the newer IA rapamycin also increases the risk for NODAT, in a similar manner (40, 41) (Table 2).

Table 2. Drug-induced new onset diabetes (NODAT): potential pathogenic mechanisms.

Immunosuppressive drug	Pathogenic mechanisms
Glucocorticoids	↓ Peripheral insulin sensitivity (27) ↓ Insulin synthesis and secretion (28) ↑ Hepatic gluconeogenesis (29) ↑ Central obesity (30)
Cyclosporin A	↓ Peripheral insulin sensitivity (32-34) ↓ β-cell mass (35) ↓ Insulin synthesis and secretion (CsA<FK) (36)
Tacrolimus	↓ Peripheral insulin sensitivity (37, 38) ↓ β-cell mass (35) ↓ Insulin synthesis and secretion (FK>CsA) (36)
Rapamycin	↓ Peripheral insulin sensitivity (40) ↓ Insulin synthesis and secretion (41)

CsA, cyclosporin A; FK, tacrolimus

Dyslipidemia

Dyslipidemia is a well-recognized metabolic complication of immunosuppressive therapy with reported incidence rates of 20% to 80%, during the first year after transplantation (18, 42, 43). Clinical studies have shown that immunosuppressive therapy, and most markedly rapamycin, may increase serum levels of triacylglycerol (TAG), total cholesterol, low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) cholesterol, free-fatty acids (FFA) and apolipoprotein B, in a dose-dependent manner (44-47).

Although immunosuppressive therapy has been strongly associated with NODAT and dyslipidemia, its effects on glucose and lipid metabolism in human adipose tissue have not been studied in details previously.

Adipose tissue

Adipose tissue is mainly divided into two subtypes, white and brown fat (48). White fat is widely distributed and represents the primary site of lipid storage. Brown fat is relatively scarce and is specialized in thermogenesis, dissipating heat to maintain body temperature. We will focus on white adipose tissue, since it is most relevant to the context of this thesis. White adipose tissue is mostly composed of adipocytes, surrounded by connective tissue that is highly vascularised and innervated. It also contains macrophages, fibroblasts and adipocyte precursor cells.

Storage of triglycerides

The classical view of the white adipose tissue is that it stores and delivers energy substrate, under the control of several regulatory factors, such as insulin, catecholamines and the autonomic nervous system (49). The energy stores in adipocytes are determined mainly by a balance between fatty acid storage as TAG and release of FFA from TAG via lipolysis (Figure 1). Approximately 90% of the lipids in the adipocyte is composed of TAG (50).

In the fed state, the concurrent increase in insulin, glucose and lipid levels in blood drives the storage of TAG in adipocytes (49). TAG synthesis requires glycerol-3 phosphate and acyl-CoA as substrates (Figure 1). Glycerol-3 phosphate is derived via glycolysis from glucose and via glyceroneogenesis from sources other than glucose and glycerol, like lactate (51). Acyl-CoA is derived from uptake of FFA from the circulation and re-utilization of FFA released from the adipocyte. The acyl-CoA is then attached to glycerol, via esterification or so-called re-esterification (51). FFA from circulation is provided by hydrolysis of TAG in VLDL particles and chylomicrons catalysed by lipoprotein lipase (LPL) at the luminal surface of capillary endothelial cells (52). The hydrolysed FFA are taken up by adipocytes via specific fatty acid transporters (CD36 and fatty-acid-binding protein, FABP). The intracellular FFA is then converted to acyl-CoA (53). In humans' adipocytes the endogenous synthesis of acyl-CoA from glucose (known as de novo lipogenesis) seems to be of minor importance (54).

There are two major pathways for TAG synthesis. The glycerol phosphate pathway includes the acylation of glycerol-3-phosphate, through a step-wise addition of acyl groups, catalysed by distinct enzymes (glycerol-3-phosphate acyltransferase; 1-acylglycerol-3-phosphate acyltransferase; lipin 1) (55). The second pathway generates the acylation of diacylglycerol from monoacylglycerol by monoacylglycerol acyltransferase (MGAT) (56). These 2 pathways share the final step in converting diacylglycerol into TAG, which is catalysed by diacylglycerol acyltransferase (DGAT).

Conversely, in the fasting state or when energy expenditure is increased for example during exercise, adipose tissue provides energy via hydrolysis of TAG into glycerol and FFA, a process known as lipolysis (Figure 1) (49). Catecholamines (e.g. epinephrine) stimulate lipolysis through the activation of β-adrenergic receptors, which activates adenylate cyclase, increasing cAMP production. A rise in cAMP activates protein kinase A (PKA), which leads to activation of the lipases adipose triacylglycerol lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (57-59). This process leads to the breakdown of TAG into FFA and glycerol. In addition to lipases other proteins, like perilipin, are important for lipolysis (58). Perilipin is a lipid-droplet coating protein that acts as a barrier to lipases, thereby maintaining a low rate of basal lipolysis.

Insulin is a powerful anti-lipolytic hormone in adipose tissue. Insulin inhibits lipolysis by activation of phosphodiesterase 3B, which degrades cAMP, thus reducing PKA and subsequently HSL activity (Figure 1) (60). Additional, circulation factors, such as, glucocorticoids, TNF-α, IL-6 and adiponectin, can also regulate lipolysis. Disturbances in the pathways regulating the storage and release of energy by adipose tissue may lead to a reduced capacity of adipocytes to keep lipids from the circulation and other tissues. Fatty acids may become elevated in the circulation and TAG may accumulate in pancreas, liver, muscle and heart (61). This so-called ectopic fat deposition may seriously affect the function of these organs and contribute to the pathogenesis of obesity-related conditions such as insulin resistance, diabetes and cardiovascular diseases.

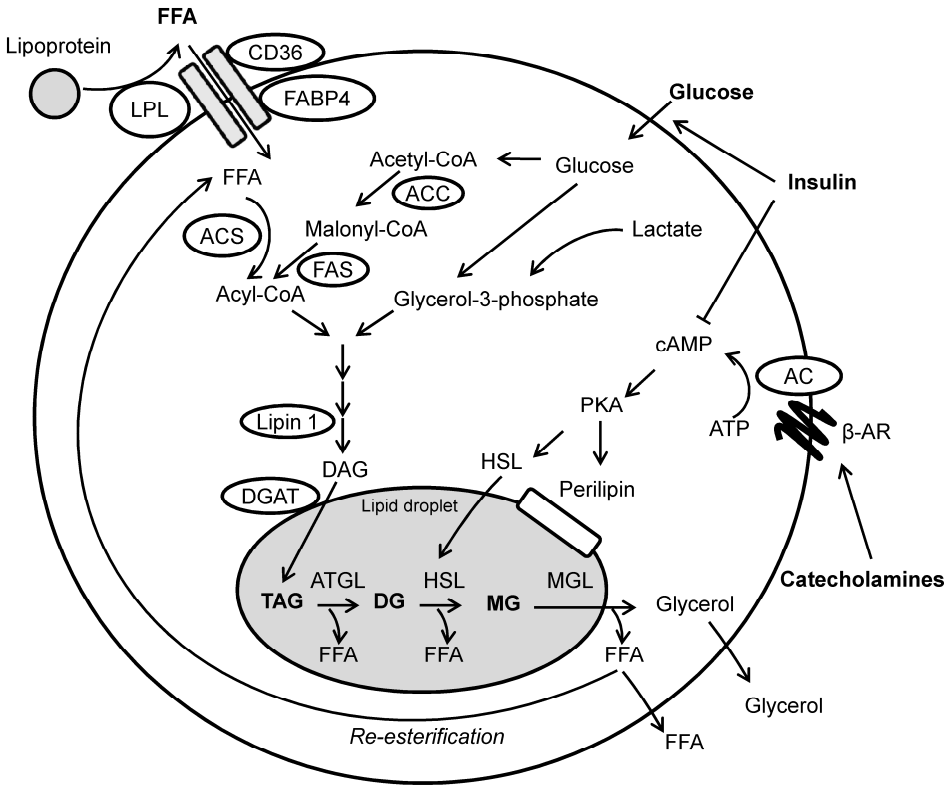

Figure 1 - Overview of the lipid storage and lipolysis in adipocytes. Adapted from (50, 55). Free-fatty acids (FFA); Lipoprotein lipase (LPL); fatty acid binding protein 4 (FABP4/aP2); acyl-CoA synthase (ACS); acetyl-CoA carboxylase (ACC); Fatty acid synthase (FAS); diacylglycerol (DAG); diacylglycerol acyltransferase (DGAT); triacylglycerol (TAG); diacylglycerol (DG); monoacylglycerol (MG); adipose triacylglycerol lipase (ATGL); hormone-sensitive lipase (HSL); monoacylglycerol lipase (MGL); β adrenergic receptor (β-AR); adenylate cyclase (AC); protein kinase A (PKA).

Adipose tissue as an endocrine organ

In addition to its role as storage for TAG, adipose tissue is also well known to secrete a variety of bioactive peptides, collectively called adipokines (62). Adipokines act at both locally (autocrine/paracrine) and systemically (endocrine) levels and may profoundly influence glucose and lipid metabolism. Increased levels of IL-6 and TNF-α, have been proposed to contribute to insulin resistance and dyslipidemia, while adiponectin is an insulin sensitising adipokine (62).

Adipose tissue distribution and metabolic disease

The main white adipose tissue depots are located subcutaneously and intra abdominally, respectively (48). Subcutaneous adipose tissue is located beneath the skin and can be divided into deep and superficial adipose tissue. Intra abdominal adipose tissue is composed of the retroperitoneal and the visceral depot, which is located inside the peritoneal cavity, and includes the omental and the mesenteric fat.

Central obesity, in particular visceral obesity, has been associated with a cluster of metabolic alterations, which include insulin resistance, components of the metabolic syndrome and increased risk of cardiovascular disease (63-65). The physiological basis for these associations may be the anatomical location of the visceral depot in relation to other organs and differences in function and response to regulating signals. Unlike subcutaneous adipose tissue, visceral fat tissue is drained into the portal vein, which has direct contact with the liver (66). Therefore, it can affect hepatic glucose and lipid metabolism directly. Genetic susceptibility combined with environmental factors have major influence on adipose tissue distribution (67). Several endocrine hormones, like growth hormone, sex steroids and cortisol, play fundamental roles in regulating body fat distribution (67).

Body mass index (BMI) is the most commonly used index of body composition in clinical practice (68). In adults, a BMI between 18.5 and 24.9 kg/m^2 corresponds to normal, BMI between 25.0 and 29.9 kg/m^2 to overweight, and BMI of $\geq$30.0 kg/m^2 is defined as obesity. As described above, the common complications of obesity, such as insulin resistance, type 2 diabetes and cardiovascular disease, are more closely related to the distribution of body fat than to the absolute amount of adiposity (63-65). As simple measures, the waist-hip ratio and sagittal abdominal diameter, are often used and reflect the degree of abdominal obesity. Other methods to assess fat distribution include bioelectrical impedance analysis (69), computed tomography and magnetic resonance imaging (68). These last two imaging methods have the advantage of being able to distinguish subcutaneous adipose tissue from visceral adipose tissue, but they are also more expensive and technically complex.

Obesity is associated with a chronic inflammatory response characterised by abnormal adipokine and cytokine production, and the activation of pro-inflammatory signalling pathways (70). However, the precise physiological events leading to the initiation of the inflammatory response and its potential metabolic consequences are not yet completely understood. It is believed that during expansion of adipose tissue, adipocyte hypertrophy and hyperplasia may lead to cell hypoxia and activation of cellular stress pathways (71). This may cause adipose tissue inflammation and release of cytokines and other pro-inflammatory signals. Cytokines, such as TNF-α and IL-6 produced by adipose tissue, can also promote inflammation and insulin resistance. As a part of the chronic inflammatory process, locally secreted chemokines attract pro-inflammatory macrophages, which in turn also release cytokines and further activate the inflammatory program in adipocytes (72).

Insulin action in adipose tissue

Insulin is a pleiotropic hormone which has diverse anabolic and anti-catabolic functions in adipose tissue (73). This includes stimulation of glucose uptake, inhibition of lipolysis and stimulation of de novo fatty acid synthesis in adipocytes. Insulin also stimulates gene expression of several adipokines and transcription factors, which are important for adipose tissue growth and differentiation, including SREBP1c and PPARγ (74, 75).

The major insulin signalling (phosphoinositide 3-kinase (PI3K) dependent) is schematically depicted in Figure 2. Insulin signalling is initiated by the binding of insulin to the extracellular α-subunit of the insulin receptor (IR) on the cell surface, resulting in the autophosphorylation of a number of residues in the transmembrane β-subunits (76). The active tyrosine kinase phosphorylates insulin receptor substrate (IRS) proteins. The IRS proteins function as docking proteins for other signalling proteins containing Src homology 2 domains. IRS1 and IRS2 are best characterised, and are the main docking proteins for the binding of the p85 regulatory subunit of PI3K, which leads to the activation of the catalytic subunit p110 (77). PI3K mediates many of the intracellular effects of insulin, including threonine phosphorylation of PKB and its activation (73). Serine phosphorylation of PKB by the mTOR complex 2 (mTORC2) (78) is also necessary for the full activation of PKB. Moreover, PKB activates AS160 (PKB substrate of 160 kDa) that quickly increases glucose transport by increasing the rate of glucose transporter (GLUT) 4-vesicle exocytosis and slightly decreases the rate of internalization (79). GLUT1 is another glucose transporter present in adipose tissue. GLUT1 is less abundant than GLUT4 and it has been proposed to act as a constitutive transport protein (80). In the basal state, GLUT4 continuously recycles between the plasma membrane and intracellular compartment, with only about 3-10% of the total GLUT4 protein pool localized at the cell surface (81). However, in response to insulin stimulation, up to ~50%

of GLUT4 is rapidly (within 5-10 min) translocated to the cell surface, thus increasing transmembrane glucose transport.

Activation of the described PI3K/PKB pathway is thought to mediate many of the metabolic effects of insulin. Besides glucose transport, it inhibits lipolysis and it promotes mTOR signalling and expression of adipokines and transcription factors. In addition to the PI3K pathway, there are however alternative pathways. Thus, activation of the CAP-Cbl-TC10 signalling pathway has been suggested to play an important role for insulin-stimulated GLUT4 translocation and glucose uptake (73). Moreover, insulin can also stimulate the mitogen-activated protein kinase (MAPK) cascade initiating a transcriptional programme that is involved in cellular proliferation and differentiation.

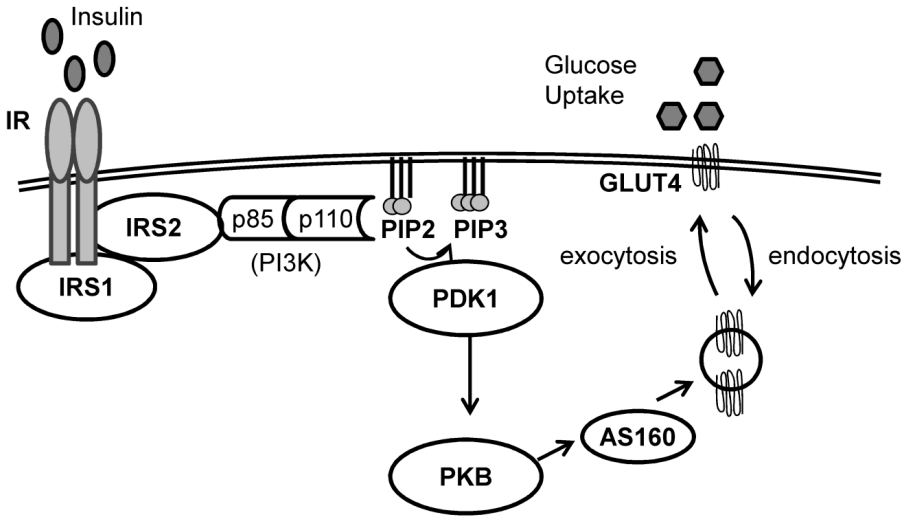

Figure 2 – Overview of the insulin signalling pathway. Adapted from (73).
Insulin receptor (IR); insulin receptor substrate 1, 2 (IRS1, IRS2), phosphoinositide 3-kinase (PI3K), phosphatidylinositol (4,5)-biphosphate (PIP2), phosphatidylinositol (3,4,5)-triphosphate (PIP3), phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (PKB), PKB substrate of 160 kDa (AS160) and glucose transporter 4 (GLUT4).

Glucocorticoids

Glucocorticoids are steroid hormones produced in the adrenal cortex under the control of the hypothalamic-pituitary-adrenal axis (82). The main glucocorticoid in humans is cortisol. The name glucocorticoid (glucose + cortex + steroid) is derived from the role in the regulation of the metabolism of glucose, synthesis in the adrenal cortex and steroidal structure. Various synthetic glucocorticoids are available, e.g. prednisone, prednisolone and dexamethasone (83). These are used to treat diseases caused by an overactive immune system, such as inflammatory diseases and autoimmune diseases or to prevent organ rejection after transplantation.

Glucocorticoids induce their anti-inflammatory and immunosuppressive effects mainly through regulation of gene transcription (84) (Figure 3, pathway A). Glucocorticoids diffuse passively into the cells through the plasma membrane due to their lipophilic structure and form a complex with the glucocorticoid receptor. The glucocorticoid receptor is a multi-protein complex containing co-factors; heat-shock proteins (Hsp, such as Hsp70 and Hsp90) and immunophilins (such as 51 kDa FK506-binding protein, FKBP51) (85). Upon glucocorticoid binding the complex translocates to its site of action in the nucleus. It suppresses the transcription of inflammatory and immune genes (e.g. IL-2) and induces transcription of anti-inflammatory genes (e.g. annexin 1) (84).

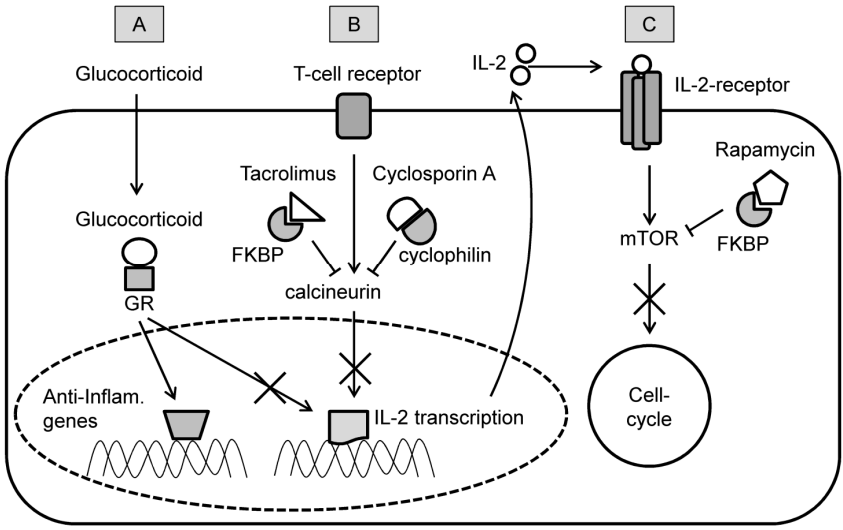

Figure 3 – Pathways for inhibition of the immune system by immunosuppressive drugs (84, 86). (A) Glucocorticoids; (B) calcineurin inhibitors cyclosporin A and tacrolimus; and (C) mTOR inhibitor rapamycin. Glucocorticoid receptor (GR); FK506-binding protein (FKBP); interleukin-2 (IL-2); mammalian target of rapamycin (mTOR).

It is well known that glucocorticoids also produce many serious side-effects that limit their use, including high blood glucose levels, central obesity, depression and anxiety (27, 30), but the metabolic side-effects are the ones most relevant to the context of this thesis.

Cortisol, is a powerful insulin-antagonistic hormone (29) that regulates a variety of important metabolic functions and may promote the development of insulin resistance, hyperglycaemia and features of the metabolic syndrome (82). Cortisol opposes the effects of insulin, including impaired insulin-dependent glucose uptake in peripheral tissues, enhanced gluconeogenesis in liver and impaired insulin secretion from pancreatic β-cells (28, 29). The effects of glucocorticoids is exemplified in clinical syndromes of excess (Cushing's syndrome) or during prolonged corticosteroid therapy (87, 88). Cortisol excess is characterised by central obesity and other components of the metabolic syndrome, such as glucose intolerance and hypertension. Central obesity, in particular visceral adiposity, is associated with insulin resistance, cardiovascular disease and the metabolic syndrome, as previously reported (64). Consequently, it is of interest to further explore the response of adipose tissue to cortisol.

Glucocorticoids cause sex- and depot-specific regulation of processes and/or gene expression. For example, glucocorticoids stimulate adipose tissue lipoprotein lipase production and activity, predominantly in visceral fat tissue, particularly in men (89, 90), possibly contributing to central obesity. In contrast, glucocorticoids in subcutaneous adipose tissue stimulate lipolysis, particularly in women (91), resulting in reduced lipid storage and increased release of fatty acids to the circulation. This may partly explain the excess of visceral obesity observed in conditions with elevated levels of cortisol. Furthermore, the capacity of glucocorticoids to impair glucose uptake appears to be more prominent in the visceral compared to the subcutaneous fat depot (92). Accordingly, dexamethasone has been demonstrated to down regulate critical signalling proteins in the visceral fat depot, e.g. IRS1 and PKB (92, 93). However, the molecular mechanisms for the underlying differential regulation of subcutaneous and visceral adipose tissue by glucocorticoids are still unknown.

Recently, key metabolic pathways regulated by dexamethasone treatment were identified by microarray analysis in human subcutaneous and omental adipose tissue from severely obese individuals (94) and in human skeletal muscle (95). In these studies, dexamethasone increases gene networks that promote carbohydrate and amino acid catabolism and lipid deposition, while genes related to inflammatory pathways were suppressed. Identification of biomarkers and new pharmacological targets would be valuable in the prevention and treatment of glucocorticoid-induced metabolic dysregulation and could potentially also be of relevance for other metabolic conditions.

The calcineurin inhibitors, cyclosporin A and tacrolimus

The introduction of calcineurin inhibitors has significantly improved the outcome of transplantation and autoimmune diseases (96). In addition to the expected beneficial action on the immune system, calcineurin inhibitors are associated with long-term side-effects. These include dose-dependent renal and hepatic toxicity and metabolic effects important for the patient's long-term outcome. Cyclosporin A and tacrolimus are the calcineurin inhibitors currently used in the clinic.

Cyclosporin A is a lipid-soluble cyclic undecapeptide produced by *Tolypocladium inflatum*, a fungus first isolated in 1970, from a soil sample collected in Norway (97). Since its clinical introduction in 1983, cyclosporin A has become the first line treatment for prevention of rejection of transplanted organs and for certain autoimmune diseases. Tacrolimus (also known as FK506) is a lipid-soluble macrocyclic lactone, first isolated in 1987, from the soil fungus *Streptomyces tsukubaensis*, in the Tsukuba region of northern Japan.

Cyclosporin A and tacrolimus mediate its immunosuppressive actions by blocking the expression of the IL-2 gene in activated T-lymphocytes, a growth factor critical for T-cell proliferation (98) (Figure 3, pathway B). Although they are structurally related, upon entering the T-cell cytoplasm they bind distinct intracellular receptors referred to as immunophilins; cyclophilin for cyclosporin A and FK506-binding protein (FKBP) for tacrolimus (99). The drug-immunophilin complexes bind to and inhibit the activity of calcineurin, a calcium dependent serine/threonine phosphatase required for early T-cell activation (99, 100). Furthermore, by preventing the calcineurin-mediated dephosphorylation of the transcription nuclear factor of activated T-cells (NFAT), cyclosporin A and tacrolimus block the translocation of the NFAT from the cytoplasm to the nucleus (100). NFAT is involved in the transcription activation of genes for cytokines such as IL-2. In all parameters of T-cell inhibition, tacrolimus appears to be 10-100 fold more active than cyclosporin A. Moreover, tacrolimus is more potent and the clinical therapeutic plasma concentration is lower than for cyclosporin A, 6-20 nM and 40-120 nM, respectively (101, 102).

Effects of calcineurin inhibitors on glucose and lipid metabolism

Both *in vitro* and biopsy studies in man and animals indicate that calcineurin inhibitors inhibit insulin production and secretion from the β-cells of the islets of Langerhans in a dose dependent manner (35, 36, 103). This effect is mediated through cell death, impaired insulin production, and/or diminished insulin secretion. Clinical studies have mainly confirmed these pre-clinical results (23, 104, 105). However, a recent study with healthy human volunteers treated during 5 h with clinically relevant doses of cyclosporin A and

tacrolimus, reported that both drugs acutely increased insulin sensitivity, while the first phase and pulsatile insulin secretion remained unaffected (106). These contradictory results may be explained by the fact, that a younger and healthier population were exposed short-term to the effect of calcineurin inhibitors, whereas previous studies usually include longer exposures and older populations. The divergent results also highlight the complexity of these drugs and suggest that their diabetogenicity may be time, dose and situation dependent.

Furthermore, studies in both human and mice/rodents have indicated that calcineurin inhibitors are associated with reduced insulin sensitivity in peripheral tissues, as well as, impaired endothelial function (23, 32-34). In humans, the simultaneous use of glucocorticoids has made it difficult to define the underlying mechanism of the calcineurin inhibitors in insulin resistance, since glucocorticoids per se, are associated with increased insulin resistance (107). Direct effects of cyclosporin A and tacrolimus on human adipocytes have never been studied previously and such results are now reported in the present thesis.

The calcineurin inhibitors, cyclosporin A and tacrolimus, also increase serum levels of cholesterol, TAG, LDL and VLDL, in a dose-dependent manner (44-47). In addition, cyclosporin A and tacrolimus increase LDL oxidation and fatty acid content in LDL (108). Although, calcineurin inhibitor therapy has been associated with reduced LPL (109) and hepatic triglyceride lipase (110) activity, little is known about the effects of calcineurin inhibitors on adipocyte lipid metabolism and on adipose tissue expression of genes involved in regulation of lipid metabolism.

Several randomised clinical trials and meta-analyses, have suggested that tacrolimus has a more diabetogenic profile than cyclosporin A (96, 111-113), although in other reports the differences between the two agents were not significant (114, 115). In contrast, cyclosporin A has been associated with greater adverse effects on the patient's lipid profile (44, 108, 111).

The mTOR inhibitor rapamycin

Rapamycin (also known as sirolimus) is a lipophilic macrocyclic lactone, produced by the bacterium *Streptomyces hygroscopicus*, which was isolated in 1970 on the Easter Island *Rapa Nui*, and from here the name rapamycin is derived (116). Rapamycin was developed initially as an antifungal agent, but was abandoned because of its immunosuppressive effect. In the early 1990s rapamycin-related research renewed the clinical interest, and in 1997 rapamycin was approved as an IA in kidney transplantation. For the time being the role of rapamycin in transplant immunosuppression remains undecided (117). The

prevailing belief is that the calcineurin inhibitors, mycophenolate mofetil and glucocorticoids should be standard therapy in renal transplantation both in terms of tolerability and outcomes. In addition, rapamycin displays a wide range of organ and tissue toxicity owing to the critical role of its site of action (the mTOR) in the signal transduction pathways of numerous cytokines, growth factors, hormones and nutrients.

Rapamycin is structurally similar to tacrolimus, and it binds the same FKBP immunophilin species (Figure 3, pathway C) (118). However, the resulting complex does not affect calcineurin activity, and therefore it does not block the calcineurin-dependent activation of cytokine genes (118). Instead, rapamycin renders the T-cells unresponsive to IL-2. The molecular target of the rapamycin-FKBP complex in T-cells is mTOR. mTOR controls proteins that regulate mRNA translation initiation and progression from the G1 to the S phase of the cell cycle, and thus its suppression inhibits T-cells proliferation (119). The therapeutic concentration in plasma for rapamycin is 10 to 26 nM (120).

mTOR signalling

mTOR is a conserved serine-threonine kinase that controls protein synthesis, cell survival and proliferation (121). mTOR is a large protein (289 kDa) that belongs to the phosphatidylinositol kinase-related kinase family and is detected in nearly all eukaryotic organisms and cell types (122). mTOR forms two physically and functionally distinct multi-protein complexes, the mTOR complex 1 (mTORC1) and 2 (mTORC2). Several components of mTORC1 and mTORC2 have been identified. Raptor is a unique component of mTORC1, while rictor and stress-activated map kinase-interacting protein 1 (Sin1) are unique components of mTORC2. mTOR signalling is regulated by growth factors, insulin, nutrients (e.g. amino-acids), stress, oxygen and energy status. The mTOR signalling pathway is shown in Figure 4.

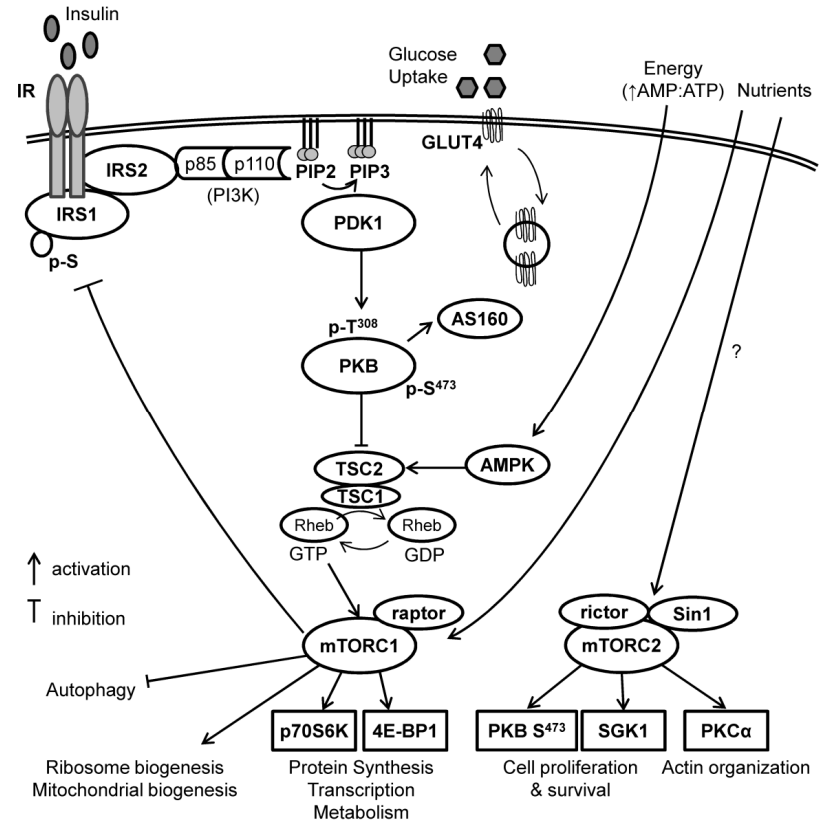

Figure 4 – Overview of the mTOR signalling pathway. Adapted from (122).
Insulin receptor (IR); insulin receptor substrate 1 and 2 (IRS1, IRS2), phosphoinositide 3-kinase (PI3K), phosphatidylinositol (4,5)-biphosphate (PIP2), phosphatidylinositol (3,4,5)-triphosphate (PIP3), phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (PKB), PKB substrate of 160 kDa (AS160), glucose transporter 4 (GLUT4) tuberous sclerosis proteins 1 and 2 (TSC1, TSC2), mammalian target of rapamycin complex 1 and 2 (mTORC1, mTORC2), serum- and glucocorticoid-induced protein kinase 1 (SGK1), protein kinase C α (PKCα), p70 ribosomal S6 kinase (p70S6K), 4E binding proteins 1 (4B-BP1), 5'-AMP-activated protein kinase (AMPK), stress-activated map kinase-interacting protein 1 (Sin1).

The mTOR pathway responds to growth factors through the PI3K-PKB pathway. mTOR and PI3K signalling are connected through the tuberous sclerosis proteins (TSC) 1 and 2. TSC1 and TSC2 compose a heterodimer that negatively regulates mTORC1 (123). Nutrients, especially amino acids, also activate mTORC1 and 2 independently of the TSC1/2 complex (124), but the exact mechanism is not yet identified. In addition mTORC1 mediates protein synthesis, and consequently requires higher level of cellular energy. mTORC1 detects the cellular energy level ratio by the 5'-AMP-activated protein

kinase (AMPK) (125). Upon activation, mTORC1 regulates protein synthesis and transcription of many genes involved in glucose and lipid metabolism by activation of p70 ribosomal S6 kinase (p70S6K) and 4E binding proteins 1 (126). The phosphorylation of p70S6K and 4E binding proteins 1 are standard markers of mTORC1 activity.

A crucial element of mTORC1 signalling is its inhibitory feedback loop on insulin-induced glucose uptake through inhibitory phosphorylation of IRS1 on multiple serine residues (127). Phosphorylation of IRS1 on serine residues interferes with PI3K/PKB pathway, inhibiting insulin action on glucose uptake (128). mTORC1 is also thought to modulate protein synthesis through stimulation and transcription of ribosomal RNA and ribosomal biogenesis (129) and to play a role in mitochondrial biosynthesis and autophagy (130).

On the other hand, mTORC2 regulates cell proliferation and survival, metabolism and actin organization (122). mTORC2 promote serine phosphorylation of PKB, which is required for its maximal activation (78). Often, mTORC2 activation is determined by measuring the serine phosphorylation of PKB.

Rapamycin is known to be an mTORC1 inhibitor (131). However, it has been proposed, that prolonged rapamycin treatment of 3T3-L1 adipocytes and other cell types, can also disrupt the mTORC2 complex and thereby decrease serine phosphorylation of PKB (132).

Effects of mTOR signalling in adipose tissue: adipogenesis and lipid storage

mTORC1 is essential for differentiation and maintenance of adipocytes by regulating transcription factors critical for the early commitment of embryonic stem cells to adipogenic progenitors (133). It also regulates the transcription of factors for terminal differentiation, including the master regulators PPARγ and SREBP1 (134, 135). In addition, mTORC1 activates lipin 1 (136). Lipin 1 is involved in diacylglycerol synthesis and also a coactivator of transcription factors involved in lipid metabolism, including PPARγ and SREBP1.

Effects of rapamycin on glucose and lipid metabolism

The diabetogenic effect of rapamycin is well-documented. Clinical studies have demonstrated that treatment with rapamycin alone or in combination with calcineurin inhibitors (137, 138) is associated with insulin resistance, higher incidence of NODAT and dyslipidemia.

However, the observed *in vitro* effects of rapamycin on glucose metabolism are conflicting and its mechanisms of actions have not yet been clarified. In fact, some *in vitro* studies have shown that rapamycin relieves the mTORC1 repression of IRS-

1/PI3K/PKB signalling, leading to enhanced insulin stimulated glucose uptake in different cell types, including in L6 cells, 3T3-L1 cells and differentiated human adipocytes (128, 139, 140). In contrast, other studies have suggested that long-term treatment of 3T3-L1 adipocytes (141, 142) and L6 cells (143) with rapamycin reduce their insulin dependent glucose uptake capacity. The reason for the differences between these studies is not clear but different cell lines, drug concentrations and/or experimental conditions may partially explain the differences found.

Rapamycin increases serum levels of total, LDL and VLDL cholesterol, TAG, and FFA (46, 47). Consistent with its effects in humans, chronic rapamycin treatment in rats causes hyperlipidemia and inhibits adipogenesis (144, 145). In addition, these studies show that mTOR inhibition with rapamycin impairs lipid deposition in adipose tissue by inhibiting the PPARγ activation and expression of downstream target genes involved in lipid storage. In addition to effects on lipogenic pathways, *in vitro* studies have also shown that rapamycin stimulates lipolysis via ATGL expression (146) and phosphorylation of HSL (147) in 3T3-L1 cells.

Since rapamycin can affect the activity of both mTORC1 and mTORC2, several studies have been performed to determine which of the complexes that is involved in the effects of rapamycin on the dysregulation of lipid metabolism. Mice lacking the mTORC1 substrate, p70S6K, as well as mice with adipose-specific knockout of raptor, an mTORC1 essential component, are lean and are prevented from diet-induced obesity and dyslipidemia (146, 148-150). On the other hand, mice with fat cell ablation of rictor, a mTORC2 essential component, have normal adipose tissue mass, are unable to suppress lipolysis in response to insulin, and have elevated circulating FFA and glycerol (151). Thus both mTORC1 and mTORC2 in fat cells seems to have an important role in whole-body energy homeostasis and inhibition with rapamycin can disrupt lipid homeostasis.

Although the effects of mTOR inhibition with rapamycin on adipocyte lipid metabolism have been investigated before, particularly in rodents and *in vitro* in 3T3-L1 cells, to our knowledge, the effects of rapamycin on human adipocytes have not been studied so far.

AIMS

The overall aim was to study the effects of immunosuppressive agents on human adipose tissue glucose and lipid metabolism. These studies could contribute to increase our understanding of the molecular mechanism for the metabolic adverse effects of such drugs.

The specific aims were to:

Investigate the direct effects of the mTOR inhibitor rapamycin on glucose uptake in human subcutaneous and omental adipocytes, and to characterise its interaction with the insulin signalling pathway (*Paper I*).

Investigate the effects of the calcineurin inhibitors, cyclosporin A and tacrolimus, on glucose uptake in human adipocytes and their impact on insulin action and on the glucose transport machinery (*Paper II*).

Explore the effects of rapamycin, cyclosporin A and tacrolimus on lipolysis, lipid storage and expression of genes involved in lipid metabolism in human subcutaneous and omental adipocytes and adipose tissue (*Paper III*).

Study the effects of dexamethasone on gene expression in human subcutaneous and omental adipose tissues, aiming to identify novel mechanisms explaining glucocorticoid-induced insulin resistance (*Paper IV*).

METHODS

Detailed description of material and methods is given in each individual paper.

Subjects and samples

Subjects were recruited via advertisement or before elective abdominal surgery, mainly kidney donation. Subjects with diabetes, endocrine disorders and cancer or other major illnesses, as well as ongoing medication with systemic glucocorticoids and immune-modulating therapies were excluded from the studies. Anthropometric measurements including body composition assessed by bioimpedance were obtained in all subjects (69). Subjects were fasted overnight (>10 h) and venous blood samples were collected for analysis of glucose, insulin and lipids by routine methods at the Department of Clinical Chemistry, Sahlgrenska University Hospital. A more detailed description of adipose tissue donors is found in *Papers I-IV*. The studies were approved by the Regional Ethics Review Board in Gothenburg. All participants gave their written informed consent. Due to limited amount of tissue, not all experiments were performed in the adipose sample from each subject. The clinical characteristics of adipose tissue donors and samples used in *Papers I-IV* are shown in Table 3.

Table 3 – Clinical characteristics of adipose tissue donors and samples in *Papers I-IV*.

	Paper I	***Paper II***	***Paper III***	***Paper IV***
Male/Female (n)	25M/39F	19M/25F	26M/34F	10M/15F
Age (years)	23-72	23-70	18-72	28-60
BMI (kg/m^2)	21-37	20-36	21-36	21-31
Fat mass (%)	14-45	18-49	14-49	13-39
Incubations	Rapamycin	CsA/FK	CsA/FK/Rap	Dexamethasone
Adipocytes				
Subcutaneous	Glucose uptake Insulin signalling	Glucose uptake Insulin signalling	Lipolysis Lipid storage Lipolytic proteins	Glucose uptake
Omental	Glucose uptake	Glucose uptake	-	Glucose uptake
Adipose tissue				
Subcutaneous	IRS1/2 (PCR)	-	Lipogenic genes and proteins (PCR, WB)	Gene expression (Microarray and PCR) and WB
Omental	-	-	Lipogenic genes and proteins (PCR, WB)	Gene expression (Microarray and PCR) and WB

CsA, cyclosporin A; FK, tacrolimus; Rap, rapamycin; PCR, real-time polymerase chain reaction; WB, western blot

Subcutaneous adipose tissue biopsies were obtained by needle aspiration from the lower part of the abdomen after local dermal anaesthesia with lidocaine. Alternatively, subcutaneous and omental biopsies were obtained during elective abdominal surgery, after induction of general anaesthesia. Biopsies were transferred to the laboratory for immediate processing and biopsies obtained from surgery were cut into smaller pieces (approximately 1-2 mm^3).

Adipocyte isolation and fat cell size

Adipocytes were isolated from adipose tissue (*Paper I-IV*) according to methods previously reported (152). Briefly, adipose tissue was digested with collagenase type II (from *Clostridium histolyticum*) in Hank's medium supplemented with 6 mM glucose, 4% BSA, 0.15 µM adenosine, pH 7.4, in a gently shaking water-bath at 37ºC for 60 min. Isolated adipocytes were filtered through a 250 µm nylon mesh and were washed four times and suspended in Hank's medium. The first media collected from the isolated adipocytes contains the stromal vascular fraction, and was used for culture and differentiation of the pre-adipocytes (*Paper II*).

The average cell diameter was measured in subcutaneous and omental adipocytes isolated from all subjects (*Paper I-IV*), as previously reported (153) and is described in *Paper I*.

Adipocyte and adipose tissue incubation

In *Paper I* and *II*, isolated subcutaneous and omental adipocytes were pre-incubated for short- (15 min and/or 3h) or long-term (20 h) with either rapamycin (0.001–10 µM), cyclosporin A (0.001–1 µM) or tacrolimus (0.001–1 µM) to study effects on glucose uptake and on relevant proteins of the insulin signalling pathway. For short-term incubations, adipocytes were diluted to a lipocrit of 5% in Hank's medium (4% bovine serum albumin, 0.15 µM adenosine and pH 7.4) without glucose, while for long-term incubations adipocytes were placed in polystyrene flasks containing DMEM (6 mM glucose, 10% foetal bovine serum, 1% penicillin-streptomycin) at 37ºC, 5% CO_2 with gentle rotation (~30 rpm) in a culture chamber.

In *Paper III*, subcutaneous adipocytes were incubated with or without rapamycin (0.01 µM), cyclosporin A (0.1 µM) or tacrolimus (0.1 µM) in a gently shaking water bath at 37ºC for 2 h, to study the effects of the IAs on lipolysis and lipid storage. In addition, to study the effects of the IAs on lipolytic proteins, subcutaneous adipocytes were pre-incubated for 15 minutes, with or without rapamycin (0.01 µM), cyclosporin A (0.1 µM)

or tacrolimus (0.1 µM) in a shaking water-bath at 37ºC, before isoproterenol (1 µM) was added for an additional 60 min.

Abdominal subcutaneous and omental adipose tissue was incubated in DMEM (6 mM glucose, 10% foetal bovine serum and 1% penicillin-streptomycin) with or without rapamycin (0.01 µM, *Paper I and III*), cyclosporin A (0.1 µM, *Paper III*) or tacrolimus (0.1 µM, *Paper III*) for 20 h and dexamethasone (0.003-3 µM, *Paper IV*) for 24 h at 37ºC and 5% CO_2. Adipose tissue was thereafter snap-frozen for gene expression studies (*Paper I, III and IV*) and protein (*Paper III and IV*) analysis, or treated with collagenase for adipocyte isolation and glucose uptake studies (*Paper IV*). Incubation medium was used to measure adiponectin and IL-6 released in the medium (*Paper III*). Simplified schematic figure on adipose tissue and adipocytes sample experiments is shown in Figure 5.

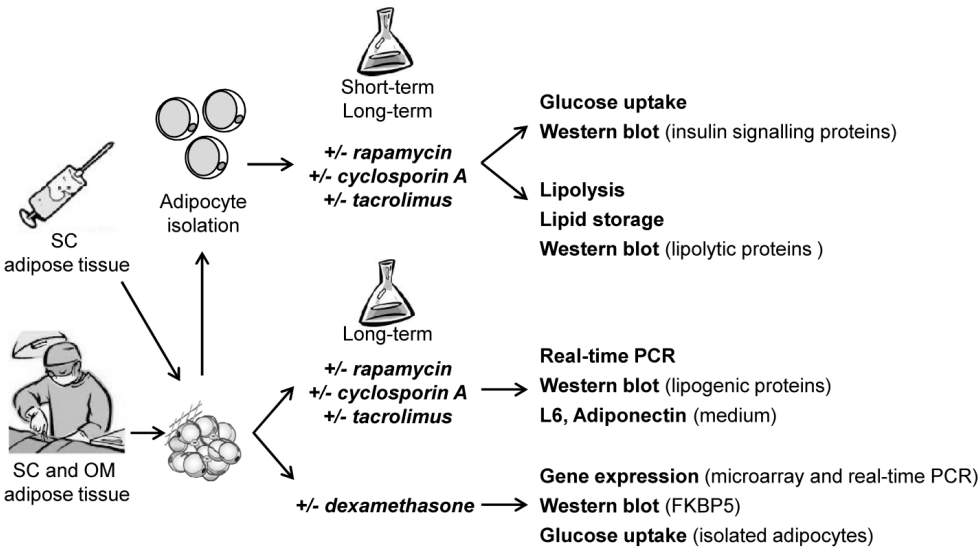

Figure 5 – Simplified schematic figure on adipose tissue and adipocytes sample experiments. SC, subcutaneous; OM, omental; Short-term incubation: 15 min - 3 h; Long-term incubation: 20 h - 24 h.

Glucose uptake assay

Glucose uptake in subcutaneous and omental adipocytes was assessed according to a previously reported technique (154) and is described in *Paper I*. Briefly, following short-term incubation (15 min) at 37ºC with either rapamycin, cyclosporin A or tacrolimus (*Paper I-II*) in glucose-free medium, adipocytes were incubated for a further 15 min with or without human insulin (6 nM). Then D-[U-^{14}C] glucose was added and the incubation

continued for another 45 min. The cells were then separated from the medium by centrifugation through silicone oil and the cell-associated radioactivity was determined by scintillation counting. Under these experimental conditions, glucose uptake is mainly determined by the rate of transmembrane glucose transport (155) and calculated according to the following formula: cellular clearance of medium glucose = (cell-associated radioactivity × volume)/(radioactivity of medium × cell number × time) (154).

After long-term incubation (20 h) with either rapamycin, cyclosporin A or tacrolimus (*Paper I and II*) in a glucose containing medium, the cells were washed and diluted in a glucose-free medium and glucose uptake was assessed as described above. After incubation of adipose tissue with or without dexamethasone (*Paper IV*), adipocytes were isolated and glucose uptake was performed as described above.

Protein extraction and immunoblotting

After the indicated pre-incubations (*Paper I-III*), adipocytes were stimulated with or without a maximal insulin concentration (6 nM) for an additional 15 min. Thereafter, the cells were lysed in ice-cold buffer at 4ºC during 2 h and the insoluble substances were sedimented through centrifugation. The protein content of adipocytes lysate was measured with the bicinchoninic acid protein assay kit and stored at -80ºC.

To obtain adipose tissue lysates (*Paper III and IV*), adipose tissue was homogenized in lysis buffer using a Tissue Lyser. Thereafter the protocol continues as previously described for adipocyte lysates.

Immunoblotting (*Paper I-IV*) was performed as described in *Paper I*. Briefly, equal amount of protein from each sample were loaded in polyacrylamide gels and proteins were separated according to their electrophoretic mobility using SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane and detection of the proteins of interest was done with the respective primary antibodies, followed by the corresponding secondary antibody linked to horseradish peroxidase. Detection of the proteins was made with a chemiluminescence reagent and visualized using Chemidoc XRS detection system or on high performance chemiluminescence film.
For mTOR and IRS1 immunoprecipitation (*Paper I*), the primary antibodies were coupled with protein G-coupled Dynabeads, as suggested by the manufacturer. Thereafter, 0.5 mg of cell lysates were incubated overnight at 4ºC with 1 µg of antibody coupled with G-coupled Dynabeads. Samples were subjected to SDS-PAGE and continued, as previously reported, for immunoblotting.

Gene expression

RNA from subcutaneous and omental adipose tissue (*Paper I, III and IV*) was extracted according to the manufacturer's instructions using the Qiagen Lipid Tissue Kit. The extracted RNA was quantified at 260 nm in a spectrophotometer and cDNA was synthesized with the Applied Biosystems High Capacity cDNA Reverse Transcriptase kit.

Microarray

Microarrays provide a powerful research tool for determination of thousands of genes (transcripts) in a single experiment. It allows visualization of which genes are expressed in a particular tissue under a particular set of conditions, and that can be used to test, as well as, generate new hypothesis.
Microarray analysis was performed as described in *Paper IV*. Briefly, we used the Human Exon 1.0 ST Array from Affymetrix that allows monitoring of over 28,000 gene transcripts in the human genome. For gene expression analysis of human adipose tissues that was incubated either with or without dexamethasone (*Paper IV*), total RNA was extracted, amplified and used to generate sense-strand cDNA synthesis. The sense-strand cDNA was fragmented and biotin-labelled before hybridization with the microarray. After scanning, the microarray results were analysed with Array Studio Microanalysis Software. To compare the effects of dexamethasone treatment on subcutaneous and omental adipose tissue the statistical general linear model was used.

Real-time PCR

Real-time polymerase chain reaction (real-time PCR) is one of the most sensitive techniques to verify DNA microarray results and further explore the expression of genes of interest. Real-time PCR was performed as described in *Paper I*, *III* and *IV*.
We used the ABI Prism 7900 HT Sequencing Detection System that takes advantage of the 5' nuclease activity of Taq DNA polymerase to generate a fluorescent signal during the thermal cycling of the PCR. The measured fluorescence reflects the amount of amplified product in each cycle. The cycle number at which enough amplified product accumulates to yield a detectable fluorescent signal, is called the threshold cycle. To adjust for differences between the samples not caused by the incubation, for example dilutions and pipetting errors, the data needs to be normalized. Therefore, it is crucial the use of a reference (housekeeping) gene. The normalization was performed with the housekeeping gene 18S rRNA. In *Paper I and III*, quotient of the expression for each gene was normalized to control, and calculated as a relative fold change.
In *Paper IV*, a standard curve for each primer-probe set of pooled adipose tissue cDNA was used. Therefore, the relative concentrations of the target gene and the housekeeping gene were calculated from the standard curve.

Lipolysis and lipid storage

Lipolysis was performed according to a previous reported technique (156) and as described in *Paper III*. In brief, the isolated adipocytes were incubated with or without rapamycin (0.01 μM), cyclosporin A (0.1 μM) and tacrolimus (0.1 μM) and with or without isoproterenol (0.01 and 1 μM) and insulin (0-100 μU/ml) in a shaking water bath at 37ºC for 2 h. The medium was then separated from the adipocytes, and the glycerol concentration on the medium was measured by colorimetric absorbance in a kinetic enzymatic analyser and used for estimations of the effects of the IAs on the lipolysis.

^{14}C-glucose and ^{14}C-palmitate incorporation into TAG were used to measure adipocyte lipid storage (*Paper III*). Briefly, isolated adipocytes were incubated with or without rapamycin (0.01 μM), cyclosporin A (0.1 μM) or tacrolimus (0.1 μM) in a gentle shaking water bath at 37ºC for 2 h. For ^{14}C-glucose incorporation, the medium was supplemented with or without insulin (1000 μU/ml) and D-[U-^{14}C] glucose (0.26 mCi/L, 0.86 μM). For ^{14}C-palmitate incorporation, the medium was supplemented with [1-^{14}C] palmitic acid (1.0 μCi/ml) and sodium-palmitate/BSA-mix (0.12 mM sodium palmitate, 40% BSA). Adipocytes were then separated from medium, and the triglyceride associated radioactivity was measured by scintillation counting after lipid extraction according to Dole and Meinertz method (157).

GLUT4 trafficking in L6 cells

The L6 cell line was originally isolated in 1968 from primary cultures of rat thigh muscle (158). L6 cell exhibits many of the skeletal muscle characteristics seen *in vivo*, like the ability to differentiate into myotubes, express several proteins typical of skeletal muscle, including GLUT4 glucose transporter, respond to insulin and glucose uptake. In *Paper II*, we used L6 cells that express the GLUT4 protein with a c-myc epitope inserted into the first ectodomain. One advantage of the L6-GLUT4myc cells is that this cell line provides a quantitative colorimetric assay to measure the dynamic availability of the myc-epitope to the extracellular milieu, without the need to permeabilize or fractionate the cells. Differentiation into myotubes is not necessary to measure glucose uptake or GLUT4myc translocation.

L6-GLUT4myc cells were cultured in MEM-α supplemented with 10% FCS, at 37ºC, 5% CO_2. At confluence 2 days after seeding, L6-GLUT4myc cells were used to study the effects of either cyclosporin A (0.1 μM) or tacrolimus (0.1 μM) on glucose uptake, cell surface GLUT4myc and GLUT4myc internalization and externalization, as described in *Paper II*. Cellular transport of 2-deoxy-glucose was measured according to a previously described technique (159). The amount of cell surface GLUT4myc and GLUT4myc

internalization and externalization was determined by an antibody-coupled colorimetric absorbance assay, as previously described (160). The data for the kinetics of GLUT4 endocytosis traffic were fitted by nonlinear regression for a single exponential association (161).

Statistical analyses

Results are given as mean ± standard error of the mean (SEM) unless otherwise stated. Comparisons between treated and untreated cells or tissue were performed within the same individuals to minimize the effects of confounding variables. A p-value <0.05 was considered statistically significant. All variables were tested for normality using the Shapiro-Wilk test. The Student's paired *t*-test was applied to compare means of continuous and normally distributed variables; otherwise, the Wilcoxon test was used. Bivariate correlations were performed with Pearson or Spearman correlation coefficients, as appropriate, and statistically significant ($p<0.05$) variables were log-transformed (if not normally distributed) and subjected to multivariate analysis via step-wise linear regression analysis. Statistical analysis was performed using the SPSS package version 18 (SPSS Inc. Chicago, IL).

SUMMARY OF RESULTS

Details of the results can be found in *Papers I-IV*.

Paper I

Rapamycin impairs glucose uptake and alters mTOR and insulin signalling in human subcutaneous and omental adipocytes

Short- and long-term incubation of human subcutaneous and omental adipocytes with a therapeutic concentration of rapamycin (0.01 μM), reduced basal and insulin stimulated glucose uptake by 20-30%, when compared to untreated cells. This effect was concentration dependent (1 nM-10 μM), with a maximal reduction of glucose uptake by 40%. In addition, the degree by which rapamycin inhibited insulin-stimulated glucose uptake, correlated positively with body fat mass and the subcutaneous fat cell diameter, and negatively with the serum levels of HDL cholesterol. Thus, the inhibition of glucose uptake by rapamycin was more pronounced in adipocytes from lean patients (lower fat mass) with smaller adipocytes, and higher serum levels of HDL-cholesterol.

Rapamycin inhibited mTORC1 and mTORC2 complex assembly and also inhibited insulin-stimulated PKB Ser473 phosphorylation, an mTORC2 substrate. Short- and long-term incubation with rapamycin had no effect on p70S6K protein levels, but as expected, reduced the insulin-stimulated phosphorylation (~80 %) of p70S6K, an mTORC1 substrate. Rapamycin did not change the amount of IRS1 or its tyrosine phosphorylation in subcutaneous adipocytes, but reduced phosphorylation of IRS1 on several serine residues (307, 616 and 636), compared with untreated adipocytes.

Rapamycin incubation induced a time-depended decrease in IRS2 protein levels (up to ~35% reduction after 20 h incubation) in adipocytes, whereas IRS2 mRNA levels were increased by ~50% in adipose tissue. In addition, rapamycin incubation reduced insulin-stimulated phosphorylation of IR by up to 50% after 20 h incubation, but had no effects on IR, PKB, GLUT4/1 and p85-PI3K protein levels.

Paper II

Cyclosporin A and tacrolimus reduce glucose uptake in human subcutaneous and omental adipocytes without effecting expression or activation of insulin signalling proteins. Cyclosporin A and tacrolimus remove GLUT4 from the cell surface via an increased rate constant of endocytosis in L6 cells.

Short- and long-term incubation with either cyclosporin A or tacrolimus reduced both basal and insulin stimulated glucose uptake in subcutaneous and omental adipocytes. The inhibitory effect was concentration-dependent (10 nM-1 μM), by up to 40%, and occurred at therapeutic concentrations used as immunosuppressive therapy.

Incubation of adipocytes with tacrolimus reduced IR phosphorylation by ~30%. However, the proteins levels and phosphorylation of relevant insulin signalling proteins (IRS1, IRS2, PKB, AS160, GLUT4/1, mTOR and p70S6K) was unaffected by both cyclosporin A and tacrolimus. In addition, cyclosporin A and tacrolimus, reduced by 60% the amount of insulin-stimulated GLUT4 at the adipocyte surface, but had no effect on basal amounts of GLUT4 at the cell surface.

The effects of cyclosporin A and tacrolimus on glucose uptake and amount of GLUT4 at the cell surface were also investigated in L6 cells. Cyclosporin A and tacrolimus inhibited insulin-stimulated glucose uptake and the amount of GLUT4myc at the cell surface in L6 cells. No effects of the agents were detected in basal (non-stimulated) conditions. In addition, cyclosporin A and tacrolimus increased the insulin-stimulated GLUT4myc rate for endocytosis by 30 and 47%, respectively. This suggests an increased rate of GLUT4 internalization, which may reduce the exposure of the GLUT4 at the cell surface membrane, and contribute to the reduction in glucose uptake.

Paper III

Rapamycin, cyclosporin A and tacrolimus enhance lipolysis and inhibit lipid storage in human subcutaneous adipocytes. Tacrolimus and rapamycin altered the expression of lipogenic genes in human subcutaneous and omental adipose tissue.

All three IAs, rapamycin, cyclosporin A and tacrolimus, increased isoprenaline-induced lipolysis by 20-35%, and isoprenaline-stimulated phosphorylation of HSL Ser563, one of the main lipases involved in lipolysis. In addition, rapamycin increased basal lipolysis by ~20% in subcutaneous adipocytes, and reduced both gene and protein expression of perilipin. Perilipin is a lipid droplet coating protein that serves important functions in the

regulation of basal and hormonally stimulated lipolysis. Rapamycin reversed the antilipolytic effect of insulin, whereas no effects were observed for cyclosporin A or tacrolimus.

Furthermore, the IAs reduced lipid storage by 20-35%, whereas the insulin-stimulated lipogenesis was down regulated by ~10%. Rapamycin reduced the expression levels of genes involved on fatty acid uptake and storage (FABP4/aP2 and SREBP1, respectively) in subcutaneous adipose tissue. In contrast, the gene expression of LPL was increased by rapamycin in both depots. It was also observed that tacrolimus inhibited the gene expression of genes involved in the uptake and transport of fatty acids (CD36 and FABP4/aP2, respectively) in both subcutaneous and omental adipose tissue. No effects were observed with cyclosporin A in any of the studied lipogenic genes.

The gene expression of IL-6, an adipokine known to have pro-inflammatory effects, was increased in subcutaneous and omental adipose tissue that was incubated with all three IAs, although only significant for the subcutaneous depot. An increase in IL-6 was also observed in the incubation media from the subcutaneous adipose tissue, upon incubation with rapamycin. On the other hand, no changes were observed on the expression of either TNF-α or adiponectin upon exposition of adipose tissue to the three IAs.

Table 3 shows a summary of the effects of the IA on human adipocytes/adipose tissue glucose and lipid metabolism (*Paper I-III*).

Table 3. Summary of IA effects on human adipocytes/adipose tissue. From *Paper I-III*.

	Rapamycin	***Cyclosporin A***	***Tacrolimus***
Glucose uptake	↓	↓	↓
IR-phosphorylation	↓	↔	↓
IRS1 Ser phosphorylation	↓	nd	nd
IRS2 protein	↓	↔	↔
PKB Ser473 phosphorylation	↓	↔	↔
GLUT4 endocytosis	nd	↑	↑
Lipolysis	↑	↑	↑
Lipid storage	↓	↓	↓
Lipogenic genes	↓	↔	↓
IL6 gene expression/production	↑	↑	↑

↓, decreased; ↑, increased; ↔, no change; nd, not done

Paper IV

Dexamethasone increased the expression of FKBP5 is human subcutaneous and omental adipose tissue and the expression of FKBP5 was correlated with clinical and biochemical markers of insulin resistance.

DNA microarray analysis showed that dexamethasone at a supra-physiological and a maximally effective concentration (3 μM) suppressed genes related to immune/inflammatory responses. The two genes with the greatest increase in gene expression after dexamethasone incubation were FKBP5 and cannabinoid receptor 1 (CNR1). In contrast, rapamycin decreased FKBP5 gene expression in both adipose tissue depots. Moreover, dexamethasone increased the FKBP5 gene and its protein (FKBP51) expression in a dose-dependent manner in both fat depots. Basal FKBP51 protein levels were 10-fold higher in omental than in subcutaneous adipose tissue. FKBP5 gene expression in subcutaneous depot positively correlated with serum insulin, homeostasis model assessment-insulin resistance (HOMA-IR) and subcutaneous adipocyte diameter. This suggests that its gene expression in subcutaneous adipose tissue may be increased in states of insulin resistance. In addition, the fold change in gene expression exerted by dexamethasone was negatively correlated with HbA1c, BMI, HOMA-IR and serum insulin, but only in subcutaneous adipose tissue.

In this study only serine-rich and transmembrane domain containing 1 (SERTM1) gene displayed a different response to dexamethasone in the two fat depots. SERTM1 gene expression was clearly down-regulated in omental adipose tissue, but unchanged in subcutaneous adipose tissue. The leptin and metallopeptidase inhibitor 4 (TIMP4) genes, encoding for secreted factors, were also up-regulated by incubation with dexamethasone in both subcutaneous and omental adipose tissue.

In addition, dexamethasone reduced basal and insulin-stimulated glucose uptake in subcutaneous and omental adipocytes, but omental adipocytes had a greater sensitivity to the metabolic effects of dexamethasone.

DISCUSSION

Immunosuppressive agents have remarkably improved the success rate of organ transplantation. Besides the expected increase in infection susceptibility, development of other adverse effects has become evident following long-term use of these drugs. These undesired effects include diabetes, dyslipidemia, cardiovascular diseases and malignancies, and have an adverse effect on patient morbidity and mortality. Thus, studies on the underlying mechanisms by which these drugs affect glucose and lipid metabolism, are warranted in order to develop pharmacological approaches for treatment and prevention that mitigate these unwanted side-effects.

Adipose tissue is a well recognized endocrine organ that participates actively in energy regulation, through a network of endocrine, paracrine and autocrine signals (62). Importantly, dysregulation of adipose tissue metabolism may lead to a cluster of metabolic alterations that may affect total body glucose and lipid metabolism and insulin sensitivity.

Effects of rapamycin, cyclosporin A and tacrolimus on glucose uptake

Similar to type 2 diabetes, new onset diabetes (NODAT) results from an imbalance between insulin sensitivity and insulin secretion (23, 24). It is well established by both *in vitro* and clinical studies that IAs impair pancreatic β-cell proliferation and insulin production in a dose dependent manner (35, 36, 41, 103). The potential impact of the IAs on insulin sensitivity is less clearly defined. Several clinical studies have indicated that treatment with IAs can reduce insulin sensitivity in peripheral tissues (23, 32, 33, 137, 162), but the underlying mechanism(s) are not known.

In *Paper I* and *II* we show, for the first time, that rapamycin, cyclosporin A and tacrolimus, within the range of the clinical therapeutic concentrations, impair both basal and insulin stimulated glucose uptake in human subcutaneous and omental adipocytes. The inhibitory effects of the IAs on glucose uptake could be detected already after a short-term incubation time (75 min) and at concentrations even lower than those recommended during immunosuppressive therapy.

Results from randomized clinical trials and meta-analyses demonstrate a higher incidence of NODAT in patients treated with tacrolimus than with cyclosporin A (96, 111-113), but these findings has not been confirmed by others (114, 115). In our *in vitro* model (*Paper I*

and *II*), rapamycin, cyclosporin A and tacrolimus had similar dose-response effects on glucose uptake, with a maximal inhibition of ~40% in subcutaneous adipocytes.

It is thought that visceral adiposity is more metabolically active than subcutaneous fat and more strongly associated with adverse metabolic risk, including insulin resistance (63, 64). However, in our *in vitro* model we did not find any differences in the IAs effects on glucose uptake between subcutaneous and omental adipocytes. Interestingly, subcutaneous adipocytes from lean subjects and with a normal lipid pattern (higher HDL-cholesterol levels) may be more sensitive to the inhibitory effects of rapamycin on glucose uptake (*Paper I*). On the other hand, adipocytes from obese individuals are usually larger (hypertrophic) and characterised by reduced insulin sensitivity already at baseline (163). This may mask a worsening of glucose uptake by rapamycin incubation.

Newly all transplanted patients need to take at least one of the IAs for the rest of their life, and the long-term effects may take place over a long period of time. Therefore, we investigated whether a longer incubation time (20 h) with the IAs could have a more pronounced inhibitory effect on adipocyte glucose uptake. The degree of inhibition of insulin-stimulated glucose uptake after short- and long-term incubation with either rapamycin, cyclosporin A or tacrolimus (*Paper I* and *II*) appear to be similar. However, we can not directly translate our findings from long-term incubation (20 h) in an *in vitro* model, to long-term clinical use for several years.

Adipose tissue only accounts for ~10% of the insulin-stimulated whole body glucose uptake, whereas skeletal muscle and liver are the major sites for insulin-stimulated glucose uptake (164). A reduction in the relatively small glucose uptake by adipose tissue is unlikely to lead to a marked whole body insulin resistance during immunosuppressive therapy *in vivo*. However, there is strong evidence that dysfunction of the adipose tissue plays a crucial role in the development of insulin resistance (165). In line with this, a mouse with adipose tissue-selective depletion of GLUT4 promotes impaired glucose intolerance, apparently due to secondary insulin resistance in muscle and liver (166). This suggests that besides contributing to whole-body glucose uptake, adipose tissue can also regulate glucose metabolism in other-insulin sensitive tissues. In addition, impaired insulin signalling in adipose tissue, including reduced IRS1 expression, impaired PKB activity and reduced GLUT4 expression, is detected in normoglycemic individuals with genetic predisposition to type 2 diabetes (167). Therefore, impaired insulin action in adipose tissue could represent an early stage before whole body glucose intolerance (165). In addition, all three IAs reduced L6 muscle-derived cells insulin-stimulated glucose uptake. This suggests that these agents may not only reduce glucose uptake in adipocytes, but also in other insulin-sensitive cells such as muscle cells.

Effects of rapamycin, cyclosporin A and tacrolimus on insulin signalling

In our study (*Paper I*), we show that rapamycin reduces mTOR-raptor (components of mTORC1), mTOR-rictor and mTOR-Sin1 (components of mTORC2) interactions. This suggests that rapamycin inhibits both mTORC1 and mTORC2 complex formation in human adipocytes. Rapamycin is known to be an mTORC1 inhibitor. Sarbassov *et al* (132) has previously demonstrated that in several cell types, including 3T3-L1 cells, prolonged rapamycin treatment also inhibits mTORC2 complex formation. mTORC2 assembly is necessary for the serine phosphorylation of PKB (78). An impairment of mTORC2 could therefore contribute to the decreased PKB serine phosphorylation and down-regulation of AS160 phosphorylation observed in adipocytes incubated with rapamycin. Phosphorylation of AS160 is required for GLUT4 translocation to the plasma membrane and subsequent glucose uptake (79).

Several studies have shown that activation of the mTOR pathway is implicated in development of insulin resistance and type 2 diabetes (128, 168). These studies have demonstrated that the mTOR-p70S6K pathway directly increases phosphorylation of IRS1 at several serine residues and thus inhibits its function, promotes its degradation, and, possibly, inhibits insulin signalling (127). In contrast, more recent studies have demonstrated that adipocytes from obese patients with type 2 diabetes have attenuated mTOR signalling (169). In fact, multi-site serine phosphorylation of IRS1, has been shown to modulate both negative and positive feedback signals (170). Surprisingly, in *Paper I*, reduction of phosphorylation of several IRS1 serine residues by rapamycin did not alter the insulin-stimulated tyrosine phosphorylation of IRS1 or its association with the p85 subunit of PI3K. Interestingly, a previous study has demonstrated that long-term treatment with rapamycin reduces insulin-stimulated IRS1 tyrosine phosphorylation in circulating mononuclear cells, and that this reduction is correlated to the increase in the patient's insulin resistance (171).

In addition, rapamycin reduced IRS2 protein expression levels. IRS2 together with IRS1, are the most important IRS proteins in the regulation of glucose uptake (77). IRS2 knockout mice did not only show insulin resistance of muscle, fat and liver, but also manifest diabetes as a result of β-cell failure (172).

mTOR inhibition with rapamycin has often shown discrepant findings, but in our *in vitro* model, mTOR inhibition with rapamycin resulted in inhibition of glucose uptake and impaired insulin signalling. These effects may contribute to insulin resistance during therapy with rapamycin.

In *Paper II*, we show that although phosphorylation of IR was inhibited by tacrolimus, the expression and phosphorylation of insulin signalling proteins (IRS1, IRS2, p85-PI3K and PKB) was unaffected by both cyclosporin A and tacrolimus, as was mTOR and p70S6K. In addition, we demonstrate that cyclosporin A and tacrolimus reduced insulin-stimulated levels of the glucose transporter GLUT4 at the plasma membrane but they did not change total protein levels of GLUT4 or GLUT1. These results suggest that the inhibitory effect of cyclosporin A and tacrolimus on cell surface GLUT4 and glucose uptake are not mediated thought effects on insulin signalling proteins. Other alternatives to PKB signalling also need to be considered in future studies. For example, the atypical PKC isoforms, PKCλ and PKCζ, increase GLUT4-dependent glucose uptake when activated by PI3K (173). Conversely, overexpression of dominant-negative forms of these PKC isoforms inhibits insulin-stimulated glucose transport and translocation of the GLUT4 to the plasma membrane (174).

The intracellular trafficking that regulates the amount of GLUT4 at the cell surface is a dynamic complex itinerary that involves different steps (175). The translocation of GLUT4 to the plasma membrane, a process called exocytosis, is initiated by the release of GLUT4 storage vesicles from intracellular retention and GLUT4 translocation to the cell surface (which likely involves transport of GLUT4 storage vesicles along cytoskeleton structures). Subsequent tethering, docking and fusion of GLUT4 storage vesicles at the plasma membrane are also required for an efficient GLUT4 surface delivery. Finally, GLUT4 is internalized from the plasma membrane through a process called endocytosis.

In *Paper II*, we used L6 muscle derived cells to determine the exocytic and endocytic rates of the GLUT4 transporter (160). We show, for the first time, that in L6 cells, cyclosporin A and tacrolimus inhibit insulin-stimulated glucose uptake and affect GLUT4 trafficking by increasing the insulin-stimulated rate for endocytosis. In contrast to the effects on human adipocytes, cyclosporin A and tacrolimus did not affect non-stimulated glucose uptake and GLUT4 trafficking in muscle cells. In both muscle and adipose cells GLUT4 internalization occurs through both clathrin-dependent and clathrin-independent endocytosis, but different mechanisms may operate in muscle and adipocyte cells (175). In addition, internalization is also distinctly regulated in the two cell types, since insulin reduces the rate of endocytosis in adipocytes (176), but not in muscle (177) cells.

A key question is how cyclosporin A and tacrolimus alter intracellular trafficking of GLUT4? Additional studies, evaluating the effects on protein expression or co-localization experiments with other proteins involved in GLUT4 trafficking (e.g. clathrin and transferrin) are necessary to confirm that cyclosporin A and tacrolimus can affect the GLUT4 endocytosis rate.

Given the fact that IR tyrosine phosphorylation was reduced by tacrolimus, alternative possibilities not involving the PI3K-PKB-AS160 pathway should also be considered. For example, IR directly phosphorylates Munc18c (178) and signals through TC10α (179) which are required for normal insulin-stimulated GLUT4 uptake. Inhibition of IR tyrosine phosphorylation by tacrolimus may impair activation of these pathways and contribute to the reduced glucose uptake and this should be further investigated.

The results in *Paper I* and *II* suggest that pharmacological concentrations of the anti-mTOR rapamycin and the calcineurin inhibitors, cyclosporin A and tacrolimus, have similar inhibitory effects on glucose uptake in human adipocytes, but through effects on different signalling mechanisms (Figure 6). In contrast to rapamycin, cyclosporin A and tacrolimus seem to reduce cellular glucose uptake without affecting expression or activation of insulin signalling and seemingly by increasing the rate for endocytosis, as demonstrated in L6 cells.

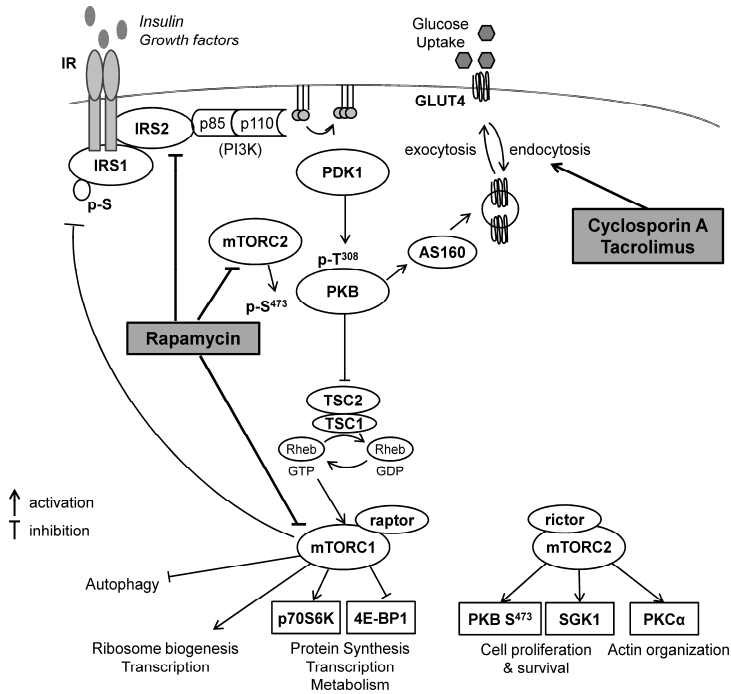

Figure 6 – Schematic overview of sites of action for rapamycin, cyclosporin A and tacrolimus interference with insulin action. Insulin receptor (IR); insulin receptor substrate 1, 2 (IRS1,2), phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (PKB), PKB substrate of 160 kDa (AS160), glucose transporter 4 (GLUT4) tuberous sclerosis proteins 1, 2 (TSC1, 2), mammalian target of rapamycin complex 1, 2 (mTORC1, 2), serum- and glucocorticoid-induced protein kinase 1 (SGK1), protein kinase C α (PKC), p70 ribosomal S6 kinase (p70S6K), 4E binding proteins 1 (4B-BP1).

Effects of rapamycin, cyclosporin A and tacrolimus on lipid metabolism

In *Paper III*, we show that rapamycin, cyclosporin A and tacrolimus increase isoproterenol-stimulated lipolysis in human subcutaneous adipocytes and that rapamycin also increase basal lipolysis. The mechanism underlying these effects on lipolysis is not known, but our results show that the three IAs increased phosphorylation of HSL on Ser552, one of the major sites controlling HSL activity (180). Soliman *et al* (147), also demonstrated that rapamycin increases lipolysis and phosphorylation of HSL, without affecting cellular cAMP levels, or PKA phosphorylation. Besides PKA, HSL phosphorylation on Ser552 is also regulated by glycogen synthase kinase-4 (181). Other protein kinases (e.g. extracellular signal-regulated kinase and AMPK) have also been shown to regulate HSL enzyme activity by phosphorylation of other serine sites. Thus assessment of activation of other regulators of HSL will be important in further work evaluating the effects of IAs on lipolysis.

In addition, rapamycin also reduced perilipin gene and protein expression. Isolated adipocytes of perilipin null mice exhibit elevated basal lipolysis because of the loss of the protective function of perilipin (58). Therefore reduced perilipin protein levels may contribute to increase basal lipolysis during rapamycin incubation. Notably, rapamycin impaired the antilipolytic effect of insulin, while cyclosporin A and tacrolimus did not (*Paper III*). PKB is critical for the ability of insulin to activate phosphodiesterase 3B, and thereby its antilipolytic effect (182). Thus, the inhibitory effect of rapamycin on PKB serine phosphorylation (*Paper I*) could also have contributed to this finding.

In *Paper III* we also show that rapamycin, cyclosporin A and tacrolimus reduce the synthesis of TAG from fatty acids (esterification) and glucose (lipogenesis). The inhibition on glucose uptake by rapamycin (*Paper I*) and by cyclosporin A and tacrolimus (*Paper II*) could also contribute to the reduced lipogenesis.

Surprisingly in *Paper III*, we show that LPL gene expression was increased by rapamycin in both subcutaneous and omental adipose tissue. LPL mediated hydrolysis of circulating lipoprotein-TAG provides the adipocytes with fatty acids (52). Thus increased LPL gene expression by rapamycin would contribute to adipose tissue clearance of circulating lipoprotein-TAG. Previous studies have shown that rapamycin reduces LPL gene expression and activity in the retroperitoneal adipose tissue of rats (144, 145) and LPL activity in human plasma (183). When considering the conflicting results between our and previous studies regarding LPL expression in adipose tissue, it should be emphasized that activity, rather than gene expression, is most relevant to measure, and this has been not measured so far.

We also observed an inhibition of the expression of two PPARγ target genes, the CD36 and FABP4/aP2 in adipose tissue incubated with tacrolimus. PPARγ is a "master regulator" of adipogenesis and adipocyte lipid metabolism (184). In addition, it has been implicated in playing an important role in obesity-related metabolic diseases such as hyperlipidemia, insulin resistance, and coronary artery disease. A co-ordinated down-regulation of CD36 and PPARγ by tacrolimus has been shown in a human THP-1 macrophage model (185). Therefore it is important to further investigate effects of tacrolimus on PPARγ in human adipose tissue in future studies.

Recent studies have demonstrated that mTORC1 plays an active role in lipid synthesis by promoting activation of SREBP1, a fundamental transcription factor involved in insulin mediated fatty acid synthesis (186). The SREBP1 gene gives rise to two proteins, SREBP1a and SREBP1c, by alternative splicing. The transcription potency of SREBP1a is higher than that of SREBP1c, but SREBP1c is the dominant isoform involved in insulin-mediated fatty acid synthesis. In *Paper III*, we show that rapamycin, during non-stimulated conditions, inhibits SREBP1 gene expression (both isoforms), as well as it co-activator lipin 1 (186) in subcutaneous adipose tissue. In contrast, FAS gene expression was not changed by rapamycin, indicating that SREBP1c activity was not affected. Consequently, the effects of rapamycin on SREBP1a and SREBP1c in human adipose tissue should be further investigated and especially insulin-stimulated conditions should be evaluated.

In addition to effects on lipid storage, treatment of subcutaneous and omental adipose tissue with either rapamycin, cyclosporin A or tacrolimus (*Paper III*), lead to an increase in IL-6 gene expression and secretion. IL-6 is a circulating cytokine that is involved in different cellular processes and has both pro-inflammatory and anti-inflammatory properties (62). Adipose tissue IL-6 expression and circulating IL-6 concentrations are positively correlated with obesity, dyslipidemia, impaired glucose tolerance and insulin resistance (187). In addition, high levels of IL-6 in the circulation are associated with reduced adipose tissue glucose uptake (188) and increased lipolysis (189). Thus, we can not exclude that reduction of glucose uptake or stimulation of lipolysis after incubation with the IAs, could be an indirect effect of secreted IL-6 in the incubation media.

In *Paper I, II* and *III*, incubation with either rapamycin, cyclosporin A or tacrolimus reduced both basal and insulin stimulated glucose uptake in human adipocytes, enhanced lipolysis stimulation and impaired lipid storage partially via down-regulation of lipogenic genes in adipose tissue. Hence, these findings may contribute to higher circulating levels of glucose, glycerol and FFA production, and a reduced plasma lipid clearance, promoting insulin resistance in skeletal muscle, liver and pancreatic β-cells (61). Plasma FFA are recognized as one of the major substrates for hepatic VLDL production (190). Therefore, simultaneous increase in FFA delivery and reduced VLDL catabolism by adipocytes

during immunosuppressive therapy can contribute to increased hepatic VLDL secretion. This can provide one explanation for the increased plasma VLDL levels observed during immunosuppressive therapy (44, 46). Fatty acid flux into muscle can also increase the intramuscular levels of lipid metabolites, such as acyl CoA and diacylglycerol (191) and contribute to insulin resistance in skeletal muscle by defects in insulin signalling (192).

Effects of dexamethasone on adipose tissue gene expression

Endogenous glucocorticoid excess (e.g. Cushing's syndrome) or during prolonged corticosteroid therapy, induces hyperglycemia, insulin resistance, central obesity and other components of the metabolic syndrome including dyslipidemia and hypertension (82, 193). Central obesity, in particular visceral obesity, is associated with increased risk of cardiovascular diseases (194). It also contributes to the development of the components of the metabolic syndrome and inflammation, as well as activation of the immune system. Thus, identification of genes regulated by glucocorticoids in adipose tissue, that may be associated with insulin resistance and activation of the inflammatory/immune system, are of interest and may indicate novel biomarkers for adipose tissue insulin resistance induced by glucocorticoids.

In *Paper IV*, we identified an expected down-regulation of expression of genes related to immune/inflammatory responses in both subcutaneous and omental adipose tissue incubated with dexamethasone, according to microarray analysis. The genes with the greatest increase in gene expression after dexamethasone incubation were CNR1 and FKBP5. CNR1 is mostly expressed in central nervous system, but it is also found in other peripheral tissues, including adipose tissue (195). Recently, it has been suggested that CNR1 modulation alters cytokine production (e.g. adiponectin) in human omental adipose tissue (196). CNR1 has not previously been identified as a gene regulated by glucocorticoids in adipose tissue and our findings thus warrant further investigations.

FKBP5 is a member of the family of immunophilins with peptidyl cis-trans isomerase activity (197). The protein encoded by this gene, FKBP51 was firstly shown to mediate T-cell inhibition by forming a complex with tacrolimus and rapamycin (198, 199). Subsequently, the protein was shown to be involved in the modulation of glucocorticoid receptor function, by forming a complex with the heat shock proteins Hsp70/Hsp90 (200). It is well established that FKBP5 reduces glucocorticoid sensitivity and several studies have presented data supporting the induction of FKBP5 by glucocorticoids (85, 201). To the best of our knowledge, this is the first study reporting that FKBP5 gene and protein levels are regulated by dexamethasone in both human subcutaneous and omental adipose tissue. In contrast, rapamycin reduced FKBP5 gene expression (*Paper IV*). This suggests

that the dexamethasone-mediated increase in FKBP5 gene expression is not a general effect related to immunosuppressive therapy.

A recent discovery suggests that FKBP5 has functions extending beyond its role in glucocorticoid receptor signalling. It was shown that FKBP51 acts as a scaffolding protein for PKB and to its phosphatase, PH domain leucine-rich repeat protein phosphatase, and it promotes dephosphorylation of PKB (202). This has potentially important implications for PKB activity and down-stream effects.

Interestingly, basal FKBP5 gene expression in subcutaneous adipose tissue was correlated positively with HOMA-IR, subcutaneous adipocyte diameter and serum insulin. This suggests that FKBP5 gene expression is increased in states of insulin resistance. Some studies have shown a correlation between plasma cortisol concentration and components of the metabolic syndrome and insulin resistance (calculated as HOMA-IR) (203, 204), although this has not been found by others (205). In addition, obesity and insulin resistance are associated with increase expression of adipose tissue 11-beta hydroxysteroid dehydrogenase type 1, that converts inactive cortisone into active cortisol locally (206). Thus we can not exclude the possibility that the observed high basal levels of FKBP5 in subcutaneous adipose tissue in individuals displaying insulin resistance may be due to increased circulating and/or tissue levels of cortisol in these individuals. Unfortunately plasma cortisol levels and other parameters of glucocorticoid metabolism were not measured in the blood samples from the subjects in *Paper IV*.

Therefore, FKBP5 gene expression seems to be correlated with insulin resistance and adiposity and is altered by dexamethasone and rapamycin. These findings suggest that FKBP5 can be one important link between the insulin resistance and the immune modulation caused by these drugs and awaits further confirmation.

In an attempt to identify potential links between genes induced by dexamethasone and secreted factors, we also identified leptin and TIMP4. Leptin appear to be regulated by dexamethasone in both subcutaneous and omental adipose tissue, as reported by microarray studies (94). However, plasma leptin levels increase with adiposity (207), thus, it may not be an optimal biomarker to study effects of glucocorticoids. TIMP4 is a metalloproteinase inhibitor and it is up-regulated in human cardiovascular disorders, suggesting its use as a marker for vascular inflammation (208). TIMP4 has not previously been identified as a gene regulated by glucocorticoids in adipose tissue, and our findings may warrant further investigations on its potential utility as a biomarker.

Previous studies have demonstrated sex- and depot-specific differences in sensitivity and responsiveness to glucocorticoid effects. Our group has previously reported that glucocorticoids reduce the glucose uptake capacity in omental but not in subcutaneous

human adipocytes (92). In parallel, a down-regulation of the cellular levels of the insulin signalling proteins IRS1 and PKB were demonstrated in omental adipocytes. Thus, identification of genes differentially regulated by dexamethasone may help to identify mechanisms by which glucocorticoids contribute to depot differences in adipose tissue function. Surprisingly, in our study (*Paper IV*) only one gene, SERTM1, was differently regulated by dexamethasone at supra-physiological concentrations in the two fat depots. SERTM1 displayed a clear change only in the omental adipose tissue. However, SRETM1 is a gene with unknown function, thus further studies are needed to determine whether this gene can contribute to the depot differences in sensitivity and responsiveness to glucocorticoid effects.

The primary advantages of *in vitro* studies are that they give rapid readouts, are relatively inexpensive and permit simplification, so that a specific mechanism in tissues or cells can be tested. But, even though we have used human adipose tissue in our studies, we need to be cautions when extrapolating *in vitro* results to the *in vivo* situation. Nonetheless, impairment of glucose uptake and dysregulated lipolysis and lipid storage in adipocytes may be mechanisms underlying impaired glucose and lipid metabolism observed in patients treated with immunosuppressive agents. The perturbations found in adipose tissue, may also have an impact in other insulin sensitive tissue such as skeletal muscle and liver. Firstly, similar mechanisms could be relevant in those tissues and, secondly, dysregulated adipose metabolism can lead to altered inter-tissue signalling, i.e. via adipokines that in turn affect metabolism in other tissues.

We have elucidated effects of immunosuppressive agents on human adipose metabolism and we propose links to inflammatory mechanisms. Figure 7 gives an overview of the demonstrated drug effects and the hypothetical interactions between pathways.

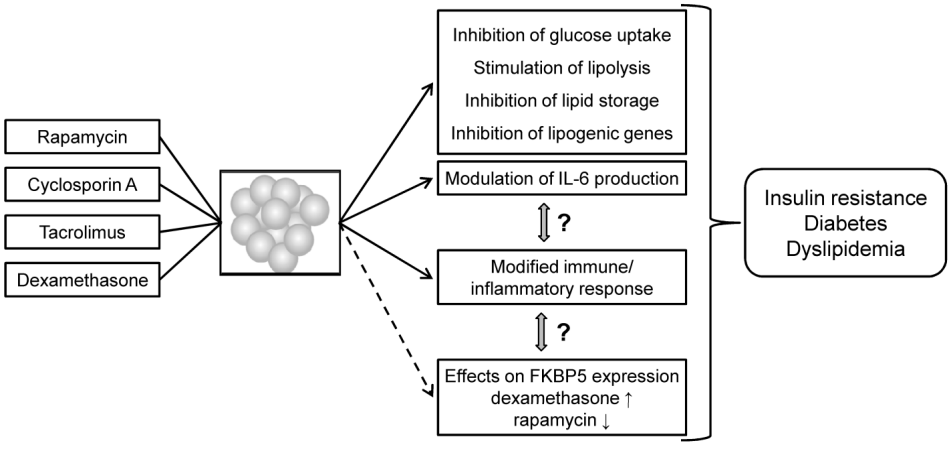

Figure 7 – Hypothetically schematic overview of effects of rapamycin, cyclosporin A and tacrolimus and dexamethasone on adipose tissue metabolism.

CONCLUDING REMARKS

Taken together, our results suggest that therapeutic concentrations of rapamycin, cyclosporin A and tacrolimus impair glucose uptake in human subcutaneous and omental adipocytes, via partly different mechanisms. The effects of rapamycin on glucose uptake may be explained by the decreased mTORC1 and mTORC2 assembly and by alterations in the insulin signalling pathway. Tacrolimus and cyclosporin A, on the other hand, seem to reduce cellular glucose uptake without affecting expression or activity of insulin signalling components. Instead, cyclosporin A and tacrolimus may enhance the rate of endocytosis of the glucose transporter GLUT4, thus leading to a redistribution from the surface to the interior of the cell. In addition, rapamycin, cyclosporin A and tacrolimus enhance lipolysis in adipocytes and they inhibit lipid storage and the expression of related genes. This may contribute to dyslipidemia and also to insulin resistance found in patients on immunosuppressive therapy.

Furthermore, we show that dexamethasone influences gene expression similarly in the two studied fat depots, i.e. subcutaneous and omental adipose tissue. FKBP5 was identified as being strongly regulated by dexamethasone, in both subcutaneous and omental adipose tissue. In addition, its expression appears to be correlated with markers of insulin resistance and adiposity. FKBP5 may be a mechanism involved in glucocorticoid-induced insulin resistance and it could potentially also provide a link between regulation of nutrient metabolism and immune response.

It should be acknowledged that the molecular mechanisms associated with the development of insulin resistance and dyslipidemia during immunosuppressive therapy need to be explored in more detail, and tissues other than adipose should also be addressed. Importantly, the clinical relevance of the present findings needs to be demonstrated in controlled trials in patients treated with immunosuppressive agents.

In conclusion, adverse effects of immunosuppressive agents on human adipose tissue glucose and lipid metabolism may contribute to the development of insulin resistance, diabetes and dyslipidemia in patients receiving such drugs. The cellular mechanisms described in this thesis may provide novel pharmacological approaches for prevention and treatment of metabolic disorders associated with immunosuppressive therapy. Such mechanisms could potentially also be of relevance for future treatments for type 2 as well as other forms of diabetes.

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mTOR inhibition with rapamycin causes impaired insulin signalling and glucose uptake in human subcutaneous and omental adipocytes

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ABSTRACT

Rapamycin is an immunosuppressive agent used after organ transplantation, but its molecular effects on glucose metabolism needs further evaluation. We explored rapamycin effects on glucose uptake and insulin signalling proteins in adipocytes obtained via subcutaneous (n = 62) and omental (n = 10) fat biopsies in human donors.

At therapeutic concentration (0.01 μM) rapamycin reduced basal and insulin-stimulated glucose uptake by 20–30%, after short-term (15 min) or long-term (20 h) culture of subcutaneous (n = 23 and n = 10) and omental adipocytes (n = 6 and n = 7). Rapamycin reduced PKB Ser473 and AS160 Thr642 phosphorylation, and IRS2 protein levels in subcutaneous adipocytes. Additionally, it reduced mTOR–raptor, mTOR–rictor and mTOR–Sin1 interactions, suggesting decreased mTORC1 and mTORC2 formation. Rapamycin also reduced IR Tyr1146 and IRS1 Ser307/Ser616/Ser636 phosphorylation, whereas no effects were observed on the insulin stimulated IRS1–Tyr and TSC2 Thr1462 phosphorylation.

This is the first study to show that rapamycin reduces glucose uptake in human adipocytes through impaired insulin signalling and this may contribute to the development of insulin resistance associated with rapamycin therapy.

1. Introduction

Rapamycin (or sirolimus) is a macrolide antibiotic and immunosuppressive agent used as a non-nephrotoxic alternative to calcineurin inhibitors in organ transplantation, but adverse effects including development of proteinuria, diabetes, dyslipidemia and impaired wound healing in the post-operative period have caused concern (Cravedi et al., 2010; Cutler et al., 1999).

Rapamycin inhibits the mammalian target of rapamycin (mTOR). mTOR exists in two physically and functionally distinct multiprotein complexes located in the cytoplasm and in the nucleus; the mTOR complex 1 (mTORC1: mTOR, raptor, mLTS8, deptor and the regulatory component PRAS40) and the mTOR complex 2 (mTORC2: mTOR, rictor, stress-activated protein kinase interacting protein 1 (Sin1), protor, mLST8 and deptor) (Dowling et al., 2010; Rosner and Hengstschlager, 2008). mTORC1 can be activated by insulin via the insulin signalling pathway, the insulin receptor (IR), insulin receptor substrate (IRS1), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB) and tuberous sclerosis complex 2 (TSC2)/TSC1 (Howell and Manning, 2011; Manning et al., 2002; Polak and Hall, 2009). mTORC1 is rapamycin sensitive and regulates cell growth, proliferation, gene transcription, protein synthesis and ribosomal biogenesis (Dowling et al., 2010) by phosphorylation of two downstream effectors, the p70 S6 kinase (p70S6K) and the eukaryotic initiation factor 4E-binding protein

Abbreviations: BCA, bicinchoninic acid protein; ECL, enhanced chemiluminescence; IR, insulin receptor; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; NODAT, new onset diabetes after transplantation; p70S6K1, p70 ribosomal S6 kinase; PDK1, phosphoinositide-dependent kinase 1; PEST, penicillin–streptomycin; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; sc, subcutaneous abdominal; Sin1, stress-activated protein kinase interacting protein 1; TSC, tuberous sclerosis complex.

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1 (Polak and Hall, 2009). mTORC2 controls cytoskeleton regulation and cell survival by phosphorylation of PKB, and is considered as rapamycin insensitive (Polak and Hall, 2009). However, long-term treatment with rapamycin inhibits the formation of mTORC2 in some cell types, including 3T3-L1 adipocytes (Hagan et al., 2008; Sarbassov et al., 2006).

Several *in vitro* studies have shown that rapamycin relieves the repression of IRS-1/PI3K/PKB signalling in states of increased activity of mTOR/p70S6K and lead to enhanced insulin stimulated glucose uptake and AKT phosphorylation in L6-muscle and 3T3-L1 cells and in differentiated human adipocytes (Berg et al., 2002; Tremblay et al., 2005; Tremblay and Marette, 2001). Contradictory to the *in vitro* findings, treatment with rapamycin in combination with calcineurin inhibitors (Johnston et al., 2008), or conversion to rapamycin from calcineurin inhibitors (Teutonico et al., 2005) is associated with insulin resistance and an increased risk of new onset diabetes after transplantation (NODAT). Other studies have shown that treatment with rapamycin does not increase the incidence of NODAT and even may improve glucose metabolism *in vivo* (Egidi et al., 2003). The reasons for these conflicting findings are not yet clear.

Despite considerable interest in the metabolic effects of rapamycin on glucose uptake in human insulin-sensitive tissues, studies on the effects on freshly isolated mature human adipocytes are absent. In this study we aim to explore potential metabolic effects of rapamycin on glucose uptake in freshly isolated mature human subcutaneous and omental adipocytes and to elucidate possible underlying mechanisms related to insulin signalling.

2. Material and methods

2.1. Study population

Human abdominal subcutaneous (sc, $n = 62$) and omental ($n = 10$) adipose tissues biopsies were obtained from 64 non-diabetic subjects (25 men/39 women; body mass index (BMI) 21–37 kg/m^2; age 23–72 years) by needle aspiration from the lower part of the abdomen ($n = 39$) after dermal local anaesthesia with lidocaine (Xylocain; AstraZeneca, Södertälje, Sweden), or during elective abdominal surgery ($n = 25$) after induction of general anaesthesia. Anthropometric measurements including body composition assessed by bioimpedance were obtained in all subjects (Lukaski et al., 1986). Fasting venous blood samples were collected for analysis of glucose, insulin and lipids by routine methods at the Department of Clinical Chemistry, Sahlgrenska University Hospital. Clinical and biochemical characteristics of adipose tissues donors are shown in Table 1. Subjects with diabetes, other endocrine disorders, cancer or other major illnesses, as well as ongoing medication with systemic glucocorticoids and immune modulating therapies, were excluded from the study. Three subjects used local inhalation treatment with β2-agonist and glucocorticoids for asthma, two subjects had ongoing treatment with low-dose antidepressants (venlafaxine and sertraline, respectively) and one patient had ongoing antihypertensive treatment with amlodipine. The study was approved by the Regional Ethical Review Board in Gothenburg and all participants gave their written informed consent.

Table 1
Anthropometry and fasting blood chemistry of the donors of subcutaneous and omental adipose tissues.

Variable	Subcutaneous	Omental
N (male/female)[a]	24/38	4/6
Age (years)	50 ± 12	44 ± 15
Body mass index (kg/m^2)	27.2 ± 4.1	26.5 ± 2.3
Waist-hip ratio (WHR)	0.92 ± 0.10	0.94 ± 0.07
Systolic blood pressure (mmHg)	131 ± 17	130 ± 11
Diastolic blood pressure (mmHg)	82 ± 11	75 ± 6[b]
Fat cell diameter (μm)	99.8 ± 12.0	92.3 ± 12.0
HbA_{1c} (mmol/mol, IFCC)[c]	34.1 ± 2.9	33.0 ± 2.2
Glucose (mM)	5.1 ± 0.5	5.0 ± 0.6
Insulin (mU/L)	7.5 ± 3.9	7.7 ± 3.5
HOMA-IR index[d]	1.7 ± 1.0	1.7 ± 0.7
Body fat mass (%)	30.0 ± 7.6	30.3 ± 6.8
Triglycerides (mM)	1.1 ± 0.4	1.4 ± 0.7
Cholesterol (mM)	5.7 ± 1.2	5.5 ± 1.8
LDL-cholesterol (mM)	3.6 ± 1.0	3.6 ± 1.5
HDL-cholesterol (mM)	1.7 ± 0.6	1.7 ± 0.6

Data are means ± SD.
HbA_{1c}, glycosylated haemoglobin; LDL, low-density lipoprotein; HDL, high density lipoprotein.
[a] Paired subcutaneous and omental surgical biopsies obtained from 8 subjects.
[b] $p < 0.05$, subcutaneous versus omental.
[c] Normal range 27–46 mmol/mol (IFCC standard).
[d] Calculated as: fasting insulin (mU/L) × fasting glucose (mM)/22.5.

2.2. Chemicals

Dulbecco's modified Eagle's medium (DMEM), Hank's medium 199, foetal bovine serum (FBS), penicillin–streptomycin (PEST), Nupage Novex Bis–Tris Mini-Gels and Protein G-coupled Dynabeads were obtained from Invitrogen Corporation (Paisley, UK). Collagenase, type II from *Clostridium histolyticum*, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), adenosine (adenine riboside) and rapamycin were obtained from Sigma Chemical Co. (St. Louise, MO, USA). D-[U-^{14}C] glucose (specific activity, 200–300 mCi/mM) and enhanced chemiluminescence (ECL)™ Western Blotting Detection Reagents were purchased from Amersham Biosciences GE Healthcare (Buckinghamshire, UK). Human Insulin Actrapid, 100 U/ml was purchased from Novo Nordisk A/S (Copenhagen, Denmark). Down corning 200/100cS fluid (silicon oil) was obtained from Silicone Products, VWR International (Leicestershire, UK). Bicinchoninic acid protein (BCA) assay kit was purchased from Thermo Scientific (Rockford, IL, USA). Okadaic acid was from Alexis Biochemicals (Lausen, Switzerland). RNeasy lipid tissue mini-kit was purchased from Qiagen (Hilden, Germany). High capacity cDNA reverse transcriptase kit, Human 18S rRNA, Universal PCR Master Mix, primers and probes were purchased from Applied Biosystems (Foster City, CA, USA). Anti-IRS1, anti-phospho-IRS1 (Ser307), anti-IRS2, anti-insulin-receptor β-subunit (IR), p85 subunit of phosphoinositide 3-kinase (PI3-kinase) and anti-stress-activated protein kinase-interacting protein 1 (Sin1) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti AS160, anti-phospho-AS160 Thr642, anti-PKB1, anti-phospho-PKB Thr308, anti-phospho-PKB Ser473, anti-phospho-IRS1 (Ser616), anti-mTOR, anti-raptor, anti-rictor, anti-p70S6 Kinase, anti-phospho p70S6K (Thr421/Ser424), anti-insulin receptor β (Tyr1146), anti-GLUT4, anti-GLUT1, anti-tuberous sclerosis complex-2 (TSC2), anti-phospho TSC2 (Thr1462), anti-rabbit and anti-mouse HRP linked secondary antibodies were obtained from the Cell Signaling Technologies (Beverly, MA, USA). Anti-phospho-IRS1 (Ser636), anti-actin, anti-glyceralhehyde-3-phosphate dehydrogenase (GAPHD) and anti-phospho-Tyr was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Adipocyte isolation and incubation

Surgical biopsies were cut into small pieces. Thereafter, the adipose tissue from both needle and surgical biopsies was digested with collagenase type II in Hank's medium containing 6 mM glucose, 4% BSA, 0.15 μM adenosine, pH 7.4 (adjusted with NaOH) for 60 min at 37 °C in a shaking water bath. After filtration through a 250 μm nylon mesh the isolated adipocytes were washed four times in

Hank's medium without glucose (4% BSA, 0.15 μM adenosine and pH 7.4, adjusted with NaOH). Due to limited amount of adipose tissue, all experiments could not be performed in all subjects and in both adipose tissues (fresh cells experiments: glucose uptake (sc, $n = 23$; omental, $n = 6$); lysates (sc, $n = 18$) and/or 20 h incubation experiments: glucose uptake (sc, $n = 10$; omental, $n = 7$); lysates (sc, $n = 8$); mRNA expression (sc, $n = 21$)). The average cell diameter was measured in isolated sc and omental adipocytes from all subjects. In brief, 0.2 ml of cell suspension was placed on a siliconized glass slide and the diameters of 100 consecutive cells from each subject were determined by light microscopy using a calibrated ocular (Lundgren et al., 2007). For the 20 h incubations the adipocytes were placed in polystyrene flasks containing DMEM with 6 mM glucose, 10% FBS, 1% PEST in absence or presence of rapamycin (0.01 μM), and incubated at 37 °C under a gas phase of 5% CO_2 with gentle agitation (~30 rpm) in a culture cupboard (Lundgren et al., 2004). After incubation the cells were washed in fresh Hank's medium without glucose before further experiments.

For the experiments 0.01 μM (10 nM) rapamycin was selected as the standard total concentration, unless otherwise specified, since it is within the therapeutic range (10–26 nM) recommended and used in clinical practice in patients after organ-transplantation (Wyeth, 1999).

2.4. Glucose uptake

The isolated adipocytes were diluted ten times in Hank's medium without glucose and pre-incubated for 15 min in absence or presence of rapamycin (sc: 0–10 μM; omental: 0.01 μM) in a shaking water-bath, and thereafter stimulated or not with insulin (25 or 1000 μU/ml) and/or okadaic acid (1 μM). After an additional 15 min, D-[U-^{14}C] glucose (0.26 mCi/L, 0.86 μM) was added to the medium and the accumulation of glucose followed for 45 min. The glucose uptake in sc and omental adipocytes freshly isolated and after 20 h incubation with rapamycin, was performed as previous reported (Yu et al., 1997). Under these experimental conditions, glucose uptake is mainly determined by the rate of transmembrane glucose transport (Kashiwagi et al., 1983) and calculated according to the following formula: cellular clearance of medium glucose = (c.p.m. cells × volume)/(c.p.m. medium × cell number × time) (Yu et al., 1997). For adipocytes incubated for 20 h the incubation continued in absence or presence of rapamycin (0.01 μM) with or without addition of insulin (1000 μU/ml), and thereafter the procedure continued as described above. All experiments were performed in triplicates.

2.5. Adipocyte lysates, immunoprecipitation and immunoblotting

After adipocyte incubation in the absence or presence of rapamycin (0.01 μM) for 15 min, 3 h or 20 h, and thereafter with or without a maximal insulin concentration (1000 μU/ml) for an additional 15 min, the cells were separated from medium and lysed at 4 °C for 2 h in lysis buffer (25 mM Tris–HCl, pH 7.4; 0.5 mM EGTA; 25 mM NaCl; 1% Nonidet P-40; 1 mM Na_3VO_4; 10 mM NaF; 0.2 mM leupeptin; 1 mM benzamidine; 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochlorine; 0.1 μM okadaic acid) as previously described (Renstrom et al., 2005). Immunoprecipitation with IRS1 and mTOR was performed only in adipocytes incubated 3 h with rapamycin. For mTOR immunoprecipitation experiments the cell lysis buffer contained 0.3% of CHAPS to achieve maximum mTOR complex recovery, as previously described (Kim et al., 2002). The insoluble substances were sedimented through centrifugation at 12 000g at 4 °C, during 15 min, and protein content was measured with the BCA protein assay kit. For immunoprecipitation, 0.5 mg of cell lysates incubated overnight at 4 °C with 1 μg of anti-mTOR or anti-IRS1 antibody coupled with G-coupled Dynabeads. Total lysate (15 μg/lane) or immunoprecipitated protein samples were subjected to SDS–PAGE, transferred to nitrocellulose membrane and immunoblotted with primary antibody diluted according to the manufacturer's instructions and thereafter with appropriate secondary antibody. Detection was made by ECL with high performance film.

2.6. Real time-PCR

Sc adipose tissue ($n = 21$) incubated for 20 h with or without rapamycin (0.01 μM), as described above, was used for IRS1 and IRS2 mRNA quantification. RNA was isolated with RNeasy lipid tissue mini-kit and used for cDNA synthesis using High Capacity cDNA Reverse Transcriptase kit. The gene expression samples was analysed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) applying gene-specific custom-designed primers and probes (sequences used are available on request). Relative quantification of mRNA levels was plotted as the fold change compared with the basal state, using 18S rRNA as endogenous control.

2.7. Statistical analysis

Results are given as mean ± standard error of the mean (SEM), or as indicated. Results for protein levels and/or phosphorylation (immunoblotting) of adipocytes incubated 3 h with rapamycin were shown only if rapamycin effects differed from that of 15 min and 20 h incubations. Differences between treatments for glucose uptake, protein content or phosphorylation level (parameters not normally distributed were log transformed) was determined using paired *t*-test or ANOVA, as appropriate; and Wilcoxon signed-rank test was used to analyse differences between treatments for mRNA expression. Bivariate linear regressions were used to assess correlations between change in insulin stimulated glucose uptake after rapamycin treatment and anthropometric and biochemical variables (age, BMI, WHR, systolic and diastolic blood pressure, sc adipocyte diameter, HbA_{1c}, HOMA-IR, body fat mass (%), cholesterol, triglycerides, HDL-cholesterol and maximum insulin stimulated glucose uptake (% of basal)). Only significant bivariate linear regressions are shown. Variables displaying associations in the bivariate regressions ($p < 0.05$) were subsequently included in the multivariate step-wise regression analysis. Comparisons between treated and untreated cells were performed with cells from the same individual to minimise confounding variables. A $p < 0.05$ was considered statistically significant. Statistical analyses were performed using the SPSS package version 18 (SPSS Inc., Chicago, IL).

3. Results

The anthropometric and biochemical characteristics of the sc and omental adipocyte donors are shown in Table 1. The characteristics were similar between sc and omental adipocyte donors with exception of the diastolic blood pressure ($p < 0.05$). The study population had a wide range of age (23–72 years) and BMI (21–37 kg/m^2), but on average normal insulin sensitivity (assessed by the HOMA-IR index) and lipid profile (Table 1).

3.1. Glucose uptake in sc and omental adipocytes

The maximum relative response to insulin stimulation was approximately 2- to 3-fold in fresh isolated adipocytes (173 ± 9% of basal, $p < 0.05$, in sc; 285 ± 31% of basal, $p < 0.05$, in omental; Fig. 1A). After a 20 h incubation the insulin stimulated glucose uptake was similar between sc and omental adipocytes (Fig. 1B), but

the relative insulin response tended to be slightly lower than in freshly isolated adipocytes (164 ± 21% of basal, $p < 0.05$, in sc; 203 ± 22% of basal, $p < 0.05$, in omental).

3.2. Effect of rapamycin on glucose uptake in sc and omental adipocytes

Abdominal obesity, and in particular increased visceral fat accumulation, is associated with the metabolic syndrome including insulin resistance and type II diabetes (Kissebah and Krakower, 1994). For this reason the effects of rapamycin on glucose uptake was addressed in omental adipocytes besides the more readily accessible sc adipocytes. After a short-term pre-incubation (15 min) with 0.01 μM rapamycin, both basal and insulin stimulated glucose uptake (1000 μU/ml) was impaired in sc and omental adipocytes, as compared with untreated adipocytes (sc, basal 20% and insulin 27%, $p < 0.05$; omental, basal 21% and insulin 24%, $p < 0.05$; Fig. 1A). After long-term incubation (20 h) with rapamycin, basal glucose uptake was inhibited by ~10%, while insulin stimulated glucose uptake was inhibited by ~30% ($p < 0.05$) in mature sc and omental adipose cells, when compared with untreated adipocytes (Fig. 1B).

A dose–response relationship for rapamycin is found in sc adipocytes (1 nM–10 μM) with a maximal inhibition of ~40% on basal and insulin stimulated glucose uptake at 1 and 10 μM (Fig. 1C). Similarly, rapamycin inhibited the sub-maximal and maximally insulin stimulated glucose uptake (25 and 1000 μU/ml), with IC_{50} values of 0.01 μM (Fig. 1C).

Incubation of human sc adipocytes with okadaic acid (1 μM) produces a similar effect on glucose uptake as the maximally effective insulin concentration, and when combined with insulin, a partial additive effect was seen (Fig. 1D). Short-term pre-incubation (15 min) of the adipocytes with rapamycin impaired both the effect of okadaic acid and the effects of combined stimulation with okadaic acid and insulin on glucose uptake, as compared with untreated adipocytes ($p < 0.05$, Fig. 1D).

3.3. Impact of body fat mass, sc adipocyte diameter and serum-HDL on rapamycin inhibition of insulin stimulated glucose uptake

Using a bivariate linear model, the proportion of rapamycin inhibition of insulin-stimulated glucose uptake, calculated as a percentage change from pre-treatment values, correlated positively with the body fat mass, but this correlation was only significant in females (female, $r = 0.604$, $p < 0.05$; male, $r = 0.528$, Fig. 2A). Rapamycin change also correlated positively with the sc adipocyte diameter ($r = 0.547$, $p < 0.01$, Fig. 2B). In addition, the serum HDL-cholesterol of the sc adipocytes donors, negatively correlates with the rapamycin change of insulin-stimulated glucose uptake ($r = -0.538$, $p < 0.01$). When entering these variables in a step-wise multivariate analysis, adjusting for gender, only HDL-cholesterol remained a significant predictor of rapamycin inhibition of insulin stimulated glucose uptake ($r^2 = 0.33$, $p < 0.01$ for model).

3.4. Effect of rapamycin on p70S6K, mTOR complexes, PKB, TSC2 and AS160 protein levels and phosphorylation

To study the effect of rapamycin on the mTOR pathway, the phosphorylation of the mTOR downstream substrate p70S6K was assessed (Fig. 3A). Rapamycin did not affect total p70S6K protein levels under basal or insulin stimulated conditions (15 min and

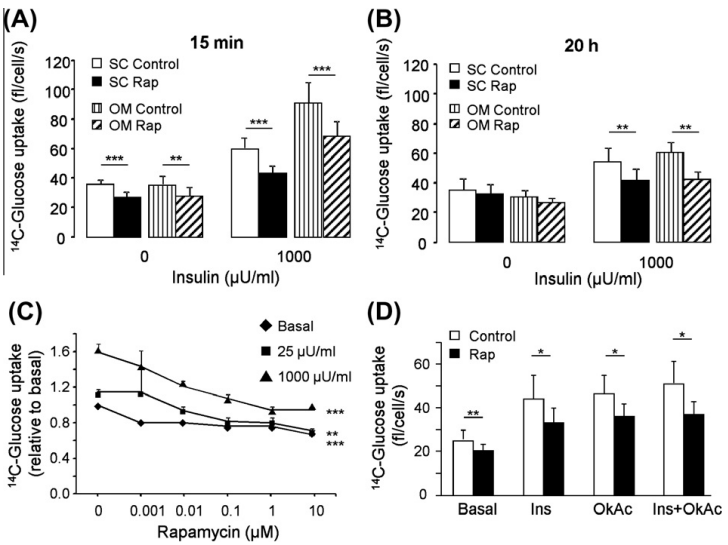

Fig. 1. Rapamycin reduces sc and omental adipose tissues glucose uptake. Freshly isolated human sc and omental adipocytes were incubated (A) short-term (15 min pre-incubation) and (B) long-term (20 h pre-incubation) with 0.01 μM rapamycin. Thereafter, the incubation continued in absence or presence of insulin (1000 μU/ml) for an additional 15 min, before D-[U-^{14}C]-glucose was added, and the glucose uptake was assessed during the following 45 min. (C) Freshly-isolated human sc adipocytes pre-incubate during 15 min with different rapamycin concentrations (0–10 μM) and the incubation continued in absence or presence of insulin (25 and 1000 μU/ml) before glucose uptake was measured as previously described. (D) Freshly-isolated human sc adipocytes pre-incubate during 15 min with or without rapamycin (0.01 μM) and thereafter incubation continued for 15 min without or with insulin (1000 μU/ml) and/or okadaic acid (1 μM) before glucose uptake was measured as previously described. Experiments were performed in triplicates and expressed as cellular glucose clearance (fl per cell per second) (A, B and D), or a ratio relative to basal (C). Data are presented as mean ± SEM, (A, $n = 23$ and $n = 6$ for sc and omental, respectively; B, $n = 10$ and $n = 7$ for sc and omental, respectively; C, $n = 6$ and D, $n = 5$). Rap, rapamycin; SC, subcutaneous adipocytes; OM, omental adipocytes; OkAc, okadaic acid; Ins, insulin. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

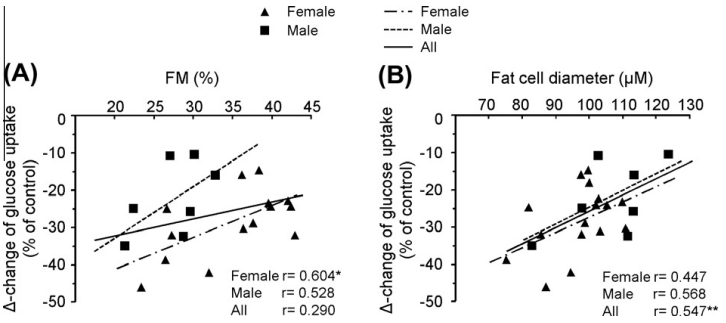

Fig. 2. Rapamycin induced change in insulin stimulated glucose uptake correlates with body fat mass and adipocyte diameter. Linear regression analysis between the percentage changes (Δ-change) of insulin stimulated glucose uptake after rapamycin incubation with (A) adipocyte donors body fat mass and (B) sc adipocytes diameter. Freshly isolated sc adipocytes were pre-incubated for 15 min with 0.01 μM rapamycin in a glucose-free medium. Thereafter, the incubation continued in presence of insulin (1000 μU/ml) for an additional 15 min, before D-[U-^{14}C]-glucose was added, and the glucose uptake was assessed during the following 45 min. Experiments were performed in triplicates and expressed as insulin stimulated glucose uptake after rapamycin incubation compared with the control situation (no rapamycin incubation). Female, $n = 16$; male, $n = 7$. FM, body fat mass (%).

20 h, Fig. 3A). Instead, rapamycin inhibited basal and insulin stimulated phosphorylation of p70S6K on Thr421/Ser424 at both 15 min and 20 h incubation (80% reduction, $p < 0.05$; Fig. 3A).

To determine if the impaired glucose uptake after rapamycin treatment could be explained by changes in PKB protein amounts or phosphorylation, Western blot analyses were performed. Incubation with rapamycin did not affect the PKB protein levels or insulin stimulated PKB phosphorylation on Thr308, whereas insulin stimulated PKB phosphorylation on Ser473 was significantly decreased both short-term and long-term (15 min and 20 h: $p < 0.01$; Fig. 3B). In addition, rapamycin inhibited insulin stimulated Thr642 phosphorylation of AS160, at both incubation times (15 min and 20 h, $p < 0.05$; Fig. 3C). Since rapamycin inhibits the PKB phosphorylation on Ser473, we studied whether the phosphorylation of the PKB substrate TSC2 was potentially inhibited (Fig. 3C). Rapamycin did not change the protein expression of TSC2 or phosphorylation of Thr1462, which regulates its activity (Fig. 3C). These results suggest that TSC2 phosphorylation is not an important mechanism explaining rapamycin-induced mTORC1 inhibition.

mTOR immunoprecipitates were analysed to determine the mTORC1 unique partner raptor and the mTORC2 unique partners rictor and Sin1 protein interaction (Fig. 3D). Rapamycin did not affect the protein levels of mTOR, raptor, rictor or Sin1 in total lysates from sc adipocytes after 3 h incubation (Fig. 3D). Instead, rapamycin reduced the amount of raptor recovered with the mTOR immunoprecipitate (by ~60% and 80% upon incubation with 0.01 and 0.1 μM rapamycin, respectively, $p < 0.05$, Fig. 3D). In addition, 3 h incubation with 0.01 and 0.1 μM rapamycin, also inhibited the amount of rictor and Sin1 recovered with the mTOR immunoprecipitate, although this effect was only significant for the higher rapamycin concentration (0.1 μM rapamycin: ~70% for rictor and ~40% for Sin1, $p < 0.05$, Fig. 3D).

3.5. GLUT4, GLUT1, p85 subunit of PI3K and IRS1 protein levels, gene expression and phosphorylation

Total protein expression of GLUT4, GLUT1 and p85 subunit of PI3K was similar between adipocytes incubated with or without rapamycin (Fig. 4A).

The total IR protein levels were similar in adipocytes incubated with or without rapamycin (0.01 μM) (Fig. 4B), but the insulin-stimulated phosphorylation of IR Tyr1146, one of the autophosphorylations sites in the β subunit (Wilden et al., 1990), was decreased in the rapamycin treated adipocytes (40% reduction after 15 min incubation, $p < 0.05$; 50% reduction after 20 h incubation, $p < 0.01$; Fig. 4B).

Rapamycin did not change IRS1 protein amount in sc adipocytes or mRNA expression in human sc adipose tissue (Fig. 4C and D, respectively). Compared to basal, insulin stimulated phosphorylation of IRS1 on Ser616 was increased by 900% ($p < 0.001$) and Ser636 was increased by 100% ($p < 0.05$), and although not significant, Ser307 phosphorylation was increased by 50% after 15 min incubation (Fig. 4E). Moreover, rapamycin reduced the phosphorylation of the IRS1 serine residues 307, 616 and 636, after short-term (15 min) as well as long-term incubation (20 h) (Fig. 4E).

IRS1 immunoprecipitates were analysed to determine insulin stimulated IRS1 tyrosine phosphorylation and the IRS1 associated p85 subunit of PI3K (Fig. 4F). Insulin caused a 20-fold increase in IRS1–Tyr phosphorylation and IRS1–p85 association ($p < 0.001$, Fig. 4F), that was not changed by rapamycin treatment (Fig. 4F).

3.6. IRS2 protein and gene expression

The IRS2 protein levels were reduced by 25% and 35% in sc adipocytes after incubation with 0.01 μM rapamycin during 3 and 20 h, respectively ($p < 0.05$, Fig. 5A). But, the IRS2 mRNA levels of sc adipose tissue were increased by ~50% after 20 h incubation with 0.01 μM rapamycin, when compared with untreated adipose tissue ($p < 0.001$, Fig. 5B).

4. Discussion

To our knowledge this is the first study reporting effects of rapamycin in freshly isolated mature human sc and omental adipocytes. This study demonstrated that rapamycin, within the range of the therapeutic concentrations used in organ transplantation, impaired both basal and acute insulin stimulated glucose uptake in human sc and omental adipocytes to a similar extent. We also showed that short-term incubation with rapamycin reduced the insulin stimulated glucose uptake in sc adipocytes by up to 40% in a concentration dependent manner (10 nM–10 μM). Previous studies have suggested that long-term treatment of 3T3-L1 adipocytes (Cho et al., 2004; Veilleux et al., 2010) and L6 myotubes cells (Sipula et al., 2006) with rapamycin reduces their insulin dependent glucose uptake capacity. In contrast, others (Tremblay et al., 2005) have reported that short-term rapamycin treatment (1 h) of 3T3-L1 cells and differentiated human preadipocytes in states

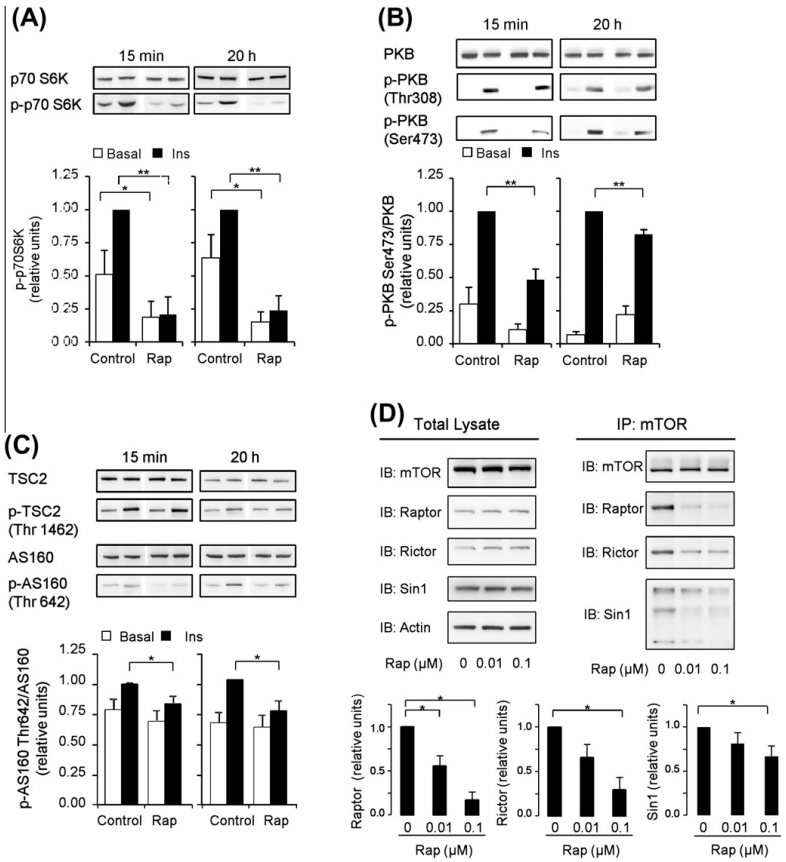

Fig. 3. Rapamycin inhibits insulin stimulated p70S6K, PKB Ser473 and AS160 Thr642 phosphorylation and mTORC1 and mTORC2 assembly in human sc adipocytes. Isolated human sc adipocytes were exposed for 15 min and 20 h in absence or presence of rapamycin (0.01 μM) and thereafter stimulated with or without insulin (1000 μU/ml) for additional 15 min, before lysis and subsequent immunoblotting analysis of (A) p70S6K protein levels and phosphospecific antibody against p70S6K (Thr421/Ser424) (n = 3–5) and (B) PKB protein levels and phosphospecific antibody against PKB Thr308 and PKB Ser473 (n = 5–7) and (C) TSC2 and AS160 protein levels and phosphospecific antibody against TSC2 Thr1462 and AS160 Thr642 (n = 5–6). (D) Total lysates and mTOR immunoprecipitates prepared from isolated sc adipocytes incubated for 3 h, in absence and presence of rapamycin (0.01 and 0.1 μM) were immunoblotted for levels of mTOR, raptor, rictor and Sin1 (n = 3–6). Control and rapamycin treated conditions for protein levels and phosphorylation of p70S6K, PKB, TSC2 and AS160 are different parts of the same gel. Results show representative gels and mean ± SEM of densitometry analysis. Densitometry measures of phosphorylation were normalised for the respectively protein levels. Ins, insulin; Rap, rapamycin. $^{*}p < 0.05$; $^{**}p < 0.01$.

of increased activity of mTOR/S6K pathway, relieve the repression of IRS1/PI3–K/PKB signalling leading to increased insulin stimulated glucose transport. The reasons for the differences between these findings and the current study are unclear, but suggest that rapamycin may have different effects on glucose uptake dependent on whether the adipocytes have elevated basal mTOR/S6K activation or not. Although rapamycin seems to improve glucose uptake in insulin resistant states induced by prolonged insulin exposure and excess nutrient activation of mTOR (Berg et al., 2002; Tremblay et al., 2005; Tremblay and Marette, 2001; Ueno et al., 2005), its inhibition in non-mTOR activation states rather seems to contributes to impairment in glucose uptake (Cho et al., 2004; Sipula et al., 2006; Veilleux et al., 2010).

Okadaic acid is an inhibitor of protein phosphatases type 1 (PP1) and 2A (PP2A), and stimulates glucose transport and PKB kinase activity independent of PI3K activation, in human adipocytes (Rondinone et al., 1999; Rondinone and Smith, 1996). Rapamycin inhibited okadaic acid stimulated glucose uptake, suggesting that PKB or a downstream target has a critical role on rapamycin glucose inhibitory effect.

The proportion of the inhibition of glucose uptake by rapamycin correlated positively with body fat mass and adipocyte diameter, and negatively with HDL-levels suggesting that adipocytes from lean subjects with a normal lipid pattern might be more sensitive to the inhibitory effects of rapamycin on glucose uptake. However, our study does not allow us to rule out the possibility that the proportionally lower reduction of insulin stimulated glucose uptake upon rapamycin treatment in obese subjects might be due to pre-existing insulin resistance in these adipocytes. This condition may mask any further worsening of insulin resistance, and it must be acknowledged that the number of subjects is limited.

Treating human adipocytes in culture with rapamycin do not directly reflect the systemic effects of rapamycin in humans, but the rapamycin-induced reduction in glucose uptake in adipocytes

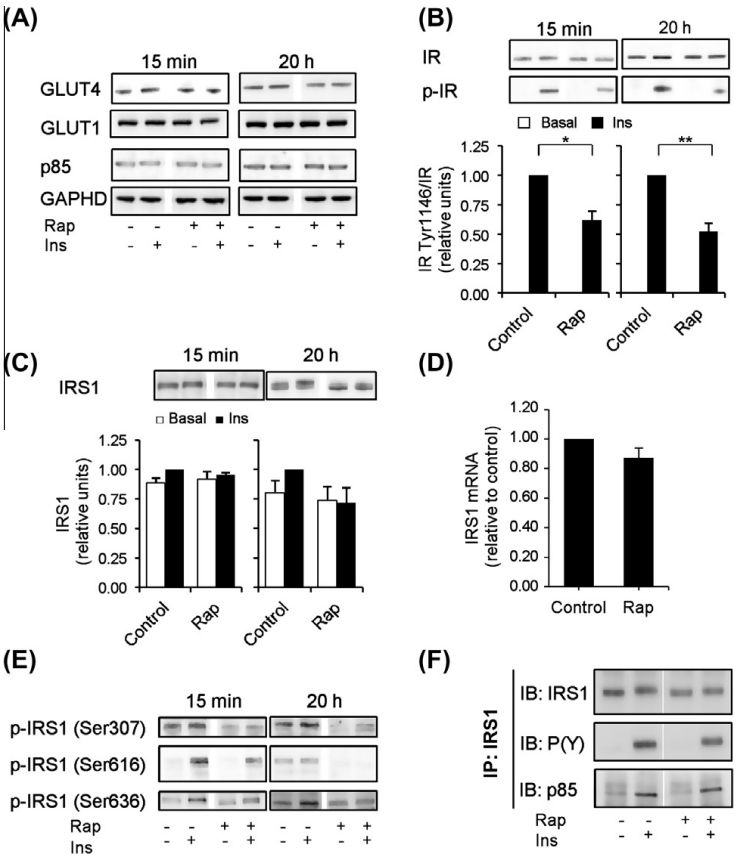

Fig. 4. Effect of the mTOR/p70S6K1 pathway down-regulation by rapamycin on the activation of IR/IRS1 in human sc adipocytes. (A–C) Isolated adipocytes were exposed for the indicated times, in absence or presence of 0.01 μM rapamycin and thereafter stimulated with insulin (1000 μU/ml) for additional 15 min for immunoblotting analysis of (A) GLUT4, GLUT1, p85 subunit of PI3K and GAPDH (n = 5–8), (B) IR and phosphospecific antibody against IR Tyr1146 (n = 4–6) and (C) IRS1 (n = 5–6). (D) IRS1 mRNA levels measured by real-time PCR in sc adipose tissue incubated in absence and presence of 0.01 μM rapamycin for 20 h (n = 21). (E) Isolated adipocytes were exposed for the indicated times, in absence or presence of 0.01 μM rapamycin and thereafter stimulated with insulin (1000 μU/ml) for additional 15 min for immunoblotting analysis of phosphospecific antibody against IRS1 Ser307, 616 and 636 (n = 4–5). (F) IRS1 immunoprecipitates prepared from isolated sc adipocytes incubated for 3 h in absence or presence of 0.01 μM rapamycin were analysed by immunoblotting for levels of IRS1, tyrosine phosphorylation and p85 subunit of PI3K (n = 3). Results (A–C, E, and F) show representative gels and mean ± SEM of densitometry analysis. Control and rapamycin treated conditions for each incubation time are different parts of the same gel. (B) Densitometry measures of phosphorylation were normalised for the respectively protein levels. Ins, insulin; Rap, rapamycin; p(Y), phospho-tyrosine. $^{*}p < 0.05$; $^{**}p < 0.01$.

could be a potential explanation of the previously described impairment in glucose metabolism in rapamycin/sirolimus-treated subjects (Johnston et al., 2008; Teutonico et al., 2005).

Since the effect of rapamycin on glucose uptake was similar in the sc and omental adipocytes, and due to limited amounts of omental adipose tissue, the effect of rapamycin on the content and phosphorylation of critical insulin signalling proteins was assessed only in sc adipocytes. As expected, incubation of human adipocytes with rapamycin resulted in almost complete prevention of p70S6K phosphorylation (mTORC1 substrate). These results demonstrate that rapamycin can, already after short-term incubation, effectively block the mTOR activity *ex vivo* in human sc adipocytes.

Rapamycin treatment inhibited insulin induced PKB Ser473 phosphorylation at all incubation time points, but did not change PKB Thr308 phosphorylation. A marked decrease in PKB Ser473 phosphorylation has previously been described in adipose tissue from rapamycin treated mice (Sarbassov et al., 2006) and in circulating mononuclear cells of rapamycin treated transplanted patients (Di Paolo et al., 2006). PKB activation requires the phosphorylation of Thr308 by PDK1 and of Ser473 by mTORC2 (Sarbassov et al., 2005; Stephens et al., 1998). Rapamycin is generally known to be an mTORC1 inhibitor (Guertin and Sabatini, 2007), however, it has been proposed, that prolonged rapamycin treatment of 3T3-L1 adipocytes and other cells can disrupt the mTORC2 and thereby decrease phosphorylation of PKB at Ser473 (Hagan et al., 2008; Sarbassov et al., 2006). Fat cell specific ablation of rictor in mice has previously been shown to impair insulin-regulated glucose transport in adipocytes and whole body (Kumar et al., 2010). In fact, in the present study we also demonstrated that rapamycin reduces the mTOR–rictor and mTOR–Sin1 interaction (mTORC2) in human sc adipocytes after 3 h incubation. The reduction in mTOR–rictor and mTOR–Sin1 interaction by rapamycin might indicate a reduction in mTORC2 assembly and cause impaired insulin stimulated PKB Ser473 phosphorylation and, conse-

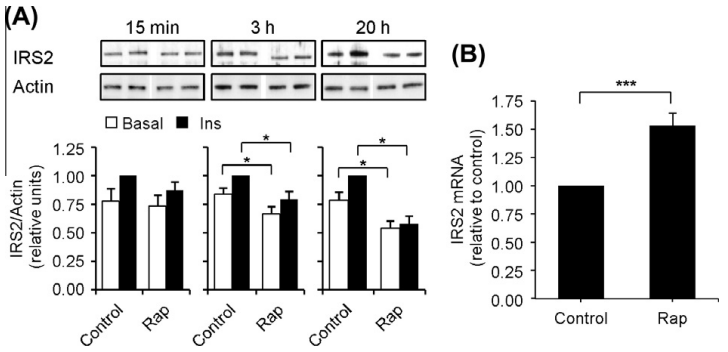

Fig. 5. Rapamycin regulates sc adipocyte IRS2 protein level and mRNA expression. (A) Isolated adipocytes were exposed for the indicated times, in absence or presence of 0.01 μM rapamycin and thereafter with or without insulin (1000 μU/ml) for additional 15 min for immunoblotting analysis of IRS2 protein levels in human sc adipocytes (n = 4–7). Control and rapamycin treated conditions for each incubation time are different parts of the same gel. Results show representative gels and mean ± SEM of densitometry analysis. (B) The IRS2 mRNA levels was measured by real-time PCR in sc adipose tissue incubated in absence or presence of 0.01 μM rapamycin for 20 h, n = 21. $^{*}p < 0.05$; $^{***}p < 0.001$.

quently, a reduction in glucose uptake. While the reduced phosphorylation of PKB at Ser473 was observed after 15 min pre-incubation with rapamycin, the mTOR–rictor and mTOR–Sin1 dissociation was observed at 3 h incubation with the compound. Thus, the time periods of incubation are different and we cannot firmly conclude by what mechanism rapamycin is affecting PKB phosphorylation at 15 min.

In addition, we demonstrated that phosphorylation of the PKB substrate AS160 on residue Thr642 was significantly reduced by rapamycin at both short- and long-term incubations. Phosphorylation of AS160 at Thr642 is required for insulin stimulated GLUT4 trafficking and glucose transport in adipocytes (Ramm et al., 2006). Due to limited amounts of tissue the translocation of GLUT4 to the cellular membrane was not assessed in the present study. However, impaired translocation of GLUT4 is a likely consequence of the attenuated insulin signalling following rapamycin treatment, and could explain the reduction in glucose uptake observed, since the GLUT4 protein levels were unchanged even after 20 h with rapamycin treatment.

Chronic activation of mTORC1 by nutrients, growth factors or insulin, increase IRS1 serine phosphorylation, which generally leads to reduced IRS1 function and impaired insulin signalling in several cell lines (Tremblay and Marette, 2001; Tzatsos and Kandror, 2006), but it can also positively modulate insulin action (Copps et al., 2010; Greene and Garofalo, 2002). IRS1 Ser307 in mice, is a positive regulatory site that moderates the severity of insulin resistance rather than inhibiting insulin sensitivity (Copps et al., 2010). mTOR regulates phosphorylation of IRS1 Ser307 in 3T3-L1 cells (Carlson et al., 2004), in adipocytes from type 2 diabetes patients (Danielsson et al., 2005), in adipocytes from healthy lean volunteers after overeating (Danielsson et al., 2009) and in adipocytes made insulin resistant by exposure to retinol-binding protein-4 (Ost et al., 2007). In agreement with these observations, the present study showed that the feedback signal to the phosphorylation on IRS1 Ser307 is prevented by rapamycin in human sc adipocytes. It has also previously been suggested that the mTOR pathway is involved in the phosphorylation of Ser616/Ser636 (corresponding to Ser612/Ser632 in mice) following prolonged insulin stimulation of 3T3-L1 adipocytes and in muscle and adipose tissue from insulin-treated mice (Gual et al., 2003). In the present study, the ability of rapamycin to prevent the insulin phosphorylation of Ser616 and 636 on IRS1 is consistent with this hypothesis. The IRS1 serine phosphorylation sites at 616 and 636, located in the PI3K binding domain of IRS1, are well established to be inhibitory of the tyrosine phosphorylation of IRS1 that leads to the termination of the insulin signalling (Tanti and Jager, 2009). However, the described prevention of insulin-stimulated phosphorylation of IRS1 Ser616/Ser636 and of IR Tyr1146 induced by rapamycin was not associated, as expected, with changes in the insulin-stimulated IRS1 tyrosine phosphorylation or in IRS1 associated p85 subunit of PI3K. A likely explanation is that the remaining IR phosphorylation has sufficient catalytic activity towards the IRS1 substrate. Moreover it is also possible that prevention of IRS1 serine phosphorylation by rapamycin reduces the pathways that normally act to attenuate the IRS1 signalling. This also suggests that PI3K-mediated PDK1 translocation to the membrane is intact and under these conditions rapamycin inhibitory effects in the IR tyrosine phosphorylation and IRS1 serine phosphorylation may be not physiologically relevant.

In the present study a time-dependent decrease in IRS2 protein levels was observed in rapamycin treated adipocytes. It has previously been shown that after prolonged insulin exposure (4–48 h), or in insulin resistant states, a reduction in IRS2 gene expression is observed in rat adipocytes (Renstrom et al., 2005). Therefore, a reduction or dysfunction on the IRS2 protein levels may contribute to the development of impaired insulin signalling. In addition, a polymorphism in the IRS2 gene is known to be a determinant of insulin sensitivity in both healthy, glucose tolerant individuals and those with type 2 diabetes (Mammarella et al., 2000). To determine if the reduction in protein content was related with the protein gene expression, the mRNA levels were determined and showed that IRS2 gene expression was increased after rapamycin treatment. Thus, the decrease IRS2 protein levels may instead be due to protein degradation or post-translational modifications and the increase in mRNA expression could therefore be a compensatory phenomenon.

Taken together (Fig. 6), this study demonstrated that therapeutic rapamycin concentrations impaired glucose uptake in human sc and omental adipocytes. This effect may be explained by decreased mTORC2 assembly and PKB Ser473 phosphorylation, with a consequent down-regulation of AS160 Thr642 phosphorylation, which is required for efficient glucose uptake. Rapamycin also promotes reduced IRS2 protein levels and pTyr–IR. These findings reinforce mTOR as being a central regulator of glucose metabolism in human adipocytes and its inhibition may potentially contribute to the development of new onset diabetes associated with rapamycin therapy in organ-transplanted patients. Future studies on

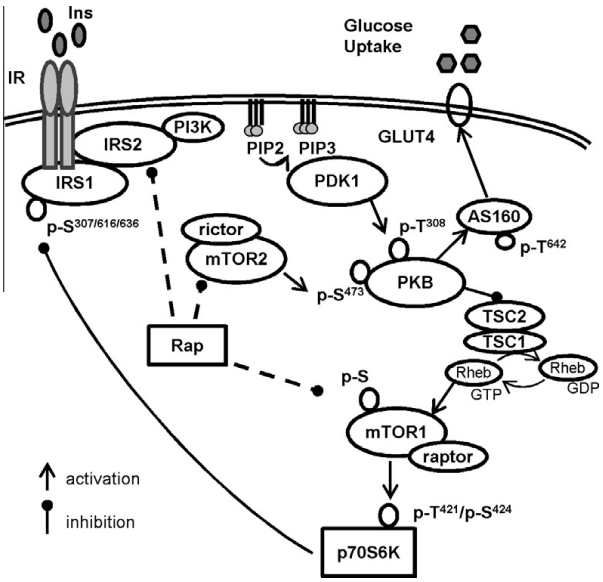

Fig. 6. Schematic diagram illustrating rapamycin effect on the activation of IRS1/2–PKB–mTORC1/2–p70S6K kinase cascade in response to insulin, in human adipocytes. Inhibition of the mTOR complex 1 by exposure to rapamycin, blocks p70S6K phosphorylation on Thr421/Ser424 and prevent IRS1 phosphorylation on Ser307/616/636. Rapamycin incubation also causes a reduction on IRS2 protein levels and disrupts mTOR complex 2 assembly, thereby decreasing phosphorylation of PKB at Ser473. Consequently phosphorylation of AS160 in Thr642 is reduced, with a consequent down-regulation of glucose uptake. Taken together, rapamycin treatment may contribute to the development of new onset diabetes associated with its therapy by reducing the insulin stimulated glucose uptake in human adipocytes. p-S, phospho-serine; p-Y, phospho-tyrosine; p-T, phospho-threonine; Rap, rapamycin; Ins, insulin.

adipocytes and adipose tissue from transplanted patients with and without rapamycin therapy are suggested to further assess these findings.

Disclosure statement

J.W. Eriksson is employed by AstraZeneca R&D. All other authors declare that there is no duality of interest associated with this manuscript.

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II

Cyclosporin A and tacrolimus reduce cell-surface amount of GLUT4 via increased endocytosis: a potential mechanism for the diabetogenic effects of immunosuppressive agents

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Abbreviated title: Cyclosporin A and tacrolimus reduce cell-surface GLUT4

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Abbreviations:

2-DG	2-Deoxy-glucose
AS160	Protein kinase B substrate of 160 kDa
CsA	Cyclosporin A
IR	Insulin receptor
mTOR	Mammalian target of rapamycin
NODAT	New onset diabetes after transplantation
p70S6K1	p70 ribosomal S6 kinase
PEST	Penicillin-streptomycin
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
WST-1	Water-soluble tetrazolium-1

ABSTRACT

Immunosuppressive agents are associated with metabolic side effects including new-onset diabetes after organ transplantation. We investigated the effects of therapeutic concentrations of the calcineurin inhibitors, cyclosporin A (CsA) or tacrolimus, on glucose uptake and insulin signalling in human isolated adipocytes and on the regulation of cellular trafficking of the GLUT4 in differentiated human pre-adipocytes and L6 cells.

CsA and tacrolimus had a concentration-dependent inhibitory effect on basal and insulin-stimulated ^{14}C-glucose uptake in adipocytes. Although phosphorylation at Tyr1146 of insulin receptor was inhibited by tacrolimus, the phosphorylation and/or protein levels of insulin signalling proteins: IRS1/2, p85-PI3K, PKB, AS160 and mTOR1C as well as GLUT4 and GLUT1 were unchanged by CsA and tacrolimus incubation. Furthermore, CsA and tacrolimus reduced the GLUT4 amount localized at the cell surface of differentiated human pre-adipocytes, and they reduced cell surface amount of myc-tagged GLUT4 in L6 cells in the presence of insulin. This occurred by increased GLUT4 rate constant for endocytosis, with no changes in the exocytosis trafficking.

These results suggest that therapeutic concentrations of CsA and tacrolimus can inhibit glucose uptake independent of insulin signalling by removing GLUT4 from the cell surface via increased rate constant for endocytosis. Such mechanisms can contribute to the development of insulin resistance associated with immunosuppressive therapy, and they may provide novel pharmacological approaches for treatment of diabetes.

Keywords: Cyclosporin A, Tacrolimus, Glucose uptake, Insulin signalling, Immunosuppressive agents, New onset diabetes after transplantation, Adipocytes, GLUT4 endocytosis, L6 cells

INTRODUCTION

The calcineurin inhibitors, cyclosporin A (CsA) and tacrolimus, are immunosuppressive agents frequently used to prevent rejection after solid organ transplantation, as well as for treatment of autoimmune diseases (1). Despite the expected action on the immune system, long-term treatment with either CsA or tacrolimus is associated with toxic effects, which have a negative impact in the patient's long-term outcome (2). Experimental and clinical studies suggest that CsA and tacrolimus are associated with increased risk for developing new-onset diabetes after transplantation (NODAT) (3, 4). NODAT has been reported to occur in 15-50% of the transplant recipients and is recognized as a risk factor for cardiovascular diseases, graft rejection and death (5, 6).

Both *in vitro* and *ex vivo* biopsy studies, from either animals or humans, indicate that calcineurin inhibitors have dose-dependent inhibitory effects on beta-cell function, resulting in reduced insulin synthesis and secretion (7, 8). Furthermore, recent studies in both mice and humans have indicated that treatment with calcineurin inhibitors is associated with reduced insulin sensitivity in peripheral tissues, as well as, impaired endothelial function (9-14). However, the mechanisms for glucose intolerance are not known, and the direct effects of CsA and tacrolimus on human adipocytes have never been studied.

CsA and tacrolimus mediate their immunosuppressive action by interrupting Ca^{2+}/calmodulin – calcineurin signalling pathways on T cells, thereby blocking antigen-stimulated gene expression of interleukin-2, a growth factor critical for T cell proliferation (15). In other cells types, calcineurin is also implicated in the control of transcription factors (16), ion homeostasis (17), intracellular inositol 1,4,5-triphosphate receptor expression (18) and actin cytoskeleton organization (19).

Glucose uptake into muscle and adipose tissue is the major mechanism by which normal whole-body glycaemia levels are maintained (20). This process is stimulated by insulin, and mediated by glucose transporters, mainly GLUT4 (20). In the absence of insulin, only 5-10% of the total cellular GLUT4 content is present at the cell surface in both adipocytes and muscle cells (21, 22). Insulin induces the translocation of GLUT4 from intracellular vesicles to the plasma membrane, mostly through signalling from the insulin receptor via the insulin-receptor substrates (IRS), to phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB) (23). Skeletal muscle is the main tissue responsible for insulin-dependent glucose uptake, whereas adipose tissue accounts for only a small fraction (24). Despite this, mice with adipose-selective depletion of GLUT4 develop

impaired glucose tolerance, apparently due to insulin resistance induced in muscle and liver (25). Thus, adipose tissue is crucial in regulating glucose metabolism beyond its intrinsic contribution to whole-body glucose handling and signalling, e.g. via adipokines and neuroendocrine pathways, it can contribute to insulin resistance and the development of type 2 diabetes (26).

The main objectives of this study were to investigate the direct effects of therapeutic concentrations of either CsA or tacrolimus on glucose uptake and effects on insulin signalling in human isolated adipocytes, and on the regulation of cellular trafficking of the glucose transporter GLUT4 in differentiated human pre-adipocytes and L6 cells.

MATERIAL AND METHODS

Adipose tissue donors

Human abdominal subcutaneous (n=44) and omental (n=11) adipose tissue biopsies were obtained from a total of 44 non-diabetic subjects (19 men/25 women; age 23-70 years; body mass index (BMI) 20-36 kg/m^2). Subjects fasted overnight (>10 h) and venous blood samples were collected for analysis of glucose, insulin and lipids by routine methods at the Department of Clinical Chemistry, Sahlgrenska University Hospital. Adipose tissue biopsies were performed by needle aspiration from the lower part of the abdomen after local dermal anaesthesia with lidocaine (Xylocain; AstraZeneca, Södertälje, Sweden), or by elective abdominal surgery after induction of general anaesthesia.

Clinical and biochemical characteristics of the adipose tissues donors are shown in Table 1. Some of the subjects donating adipose tissue in this study were also part of a previous published study elucidating metabolic effects of rapamycin (27). Subjects with diabetes, other endocrine disorders, systemic illnesses or malignancy, as well as ongoing medication with systemic glucocorticoids and immune modulating therapies were excluded from the study. The study protocol was approved by the Regional Ethics Review Board in Gothenburg. Written informed consent was received from all subjects.

Adipocyte isolation

Isolated adipocytes were obtained from both needle and surgical biopsies after collagenase type II digestion (from *Clostridium histolyticum*, Sigma Chemical Co., St. Louise, MO, USA), in Hank's medium (Invitrogen Corporation, Paisley, UK) containing 6 mM glucose, 4% BSA (Sigma), 0.15 µM adenosine (Sigma), pH 7.4 for 60 min at 37ºC in a shaking water-bath. Isolated adipocytes

were filtered through a 250 μm nylon mesh and were washed four times in Hank's medium without glucose (4% BSA, 0.15 μM adenosine and pH 7.4). The average cell diameter was measured in isolated subcutaneous and omental adipocytes (28).

Culture of adipocytes

To investigate the effects of short-term pre-incubations (15 min) with either CsA (Sigma) (1-1000 nM) or tacrolimus (Sigma) (1-1000 nM) on glucose uptake, freshly isolated subcutaneous (n=8-17) and omental adipocytes (n=8) were diluted to a lipocrit of 5% in Hank's medium (4% BSA, 0.15 μM adenosine and pH 7.4) without glucose.

To further investigate the effects of long-term pre-incubations (20h) of either CsA or tacrolimus on glucose uptake, the subcutaneous and omental adipocytes (n=8 and n=5, respectively) were placed in polystyrene flasks containing DMEM (Invitrogen) with 6 mM glucose, 10% FBS (Invitrogen), 1% PEST (Invitrogen) in the absence or presence of either CsA or Tacrolimus (100 nM). Adipocytes were pre-incubated at 37ºC under a gas phase of 5% CO_2 with gentle agitation (~30 rpm) in a culture chamber (29). After incubation the cells were washed and diluted in fresh Hank's medium without glucose and placed in a shaking water-bath in the absence or presence of CsA or tacrolimus (100 nM) before glucose uptake was assessed.

For immunoblotting analysis, freshly isolated subcutaneous adipocytes were diluted to a lipocrit of 5% in Hank's medium (4% BSA, 0.15 μM adenosine and pH 7.4) with 5.6 mM glucose, and pre-incubate with either CsA or tacrolimus (100 nM) for 15 min (n=5) or 20 h (n=4-6).

Assessment of cell viability

The effect of CsA and tacrolimus on adipocyte viability was assessed by using the water-soluble tetrazolium-1 colorimetric assay (WST-1, Roche, Mannheim, Germany). Adipocytes were placed in a 48 well plate at a lipocrit of 5% in Hank's medium (5.6 mM glucose, 4% BSA, 0.15 μM adenosine and pH 7.4) with or without CsA or tacrolimus (100 and 1000 nM). After 24 h, WST-1 was added to each well, and the cells were incubated for 2 h at 37ºC before absorbance was measured at 450 nm.

The viability of adipocytes was not significantly affected after 24 h incubation with CsA or tacrolimus (90 to 100%, p=ns), when compared to untreated cells.

Glucose uptake

Glucose uptake in subcutaneous and omental adipocytes was assessed as previously reported (27, 30). Briefly, after short-term (15 min) and long-term (20 h) pre-incubations with or without either CsA or tacrolimus, adipocytes were incubated at 37ºC for a further 15 min with or without human insulin (6 nM; Actrapid, Novo Nordisk A/S, Copenhagen, Denmark) and/or okadaic acid (1 μM; Alexis Biochemicals, Lausen, Switzerland) before the addition of D-[U-^{14}C] glucose (0.26 mCi/L, final conc. 0.86 μM; Amersham Biosciences GE HealthCare, Buckinghamshire, UK) for another 45 min (total incubation time was 75 min and 21 h for short-term and long-term incubation, respectively). Glucose uptake was stopped and the cell-associated radioactivity was determined by scintillation counting. Experiments were performed in triplicates.

Adipocyte lysates and immunoblotting

Immunoblotting was performed as previously described (27). Briefly, after the indicated pre-incubations, adipocytes were stimulated with or without a maximal insulin concentration (6 nM) for an additional 15 min, before the cells were lysed in ice-cold buffer containing 25 mM Tris-HCl pH 7.4, 0.5 mM EGTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine, 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochlorine and 0.1 μM okadaic acid. Aliquots of total lysate (15 μg/lane), a fat free extract, were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and were immunoblotted with primary antibodies: anti-IRS1, anti-IRS2, anti-insulin-receptor β-subunit (IR), anti-p85 subunit of phosphoinositide 3-kinase (p85-PI3K) and anti-GLUT1 (Upstate Biotechnology, Lake Placid, NY, USA); and anti-AS160, anti-phospho-AS160 Thr642, anti-PKB1, anti-phospho-PKB Thr308, anti-phospho-PKB Ser473, anti-mammalian target of rapamycin (anti-mTOR), anti-p70S6 Kinase, anti-phospho p70S6K (Thr421/Ser424), anti-insulin receptor β (Tyr1146), anti-GLUT4 (Cell Signaling Technologies, Beverly, MA, USA). Antibodies were diluted according to the manufacturer's instructions and incubated over-night at 4ºC. Thereafter membranes were incubated during 1 h with HRP-conjugated secondary antibody (anti-rabbit or anti-mouse secondary antibodies, Cell Signaling Technologies). Detection was made with chemiluminescence reagent (ECL, Amersham Biosciences) using a high performance chemiluminescence film (Amersham Biosciences).

Human preadipocytes differentiation and GLUT4 immunofluorescence analysis

Human primary preadipocytes (n=4) were cultured in DMEM supplemented with 10% FBS and 1% PEST in Lab Tek II chamber glass slides (Nalgene Nunc International, Naperville, IL, USA). Confluent pre-adipocytes were differentiated with DMEM containing 3% FBS, 850 nM insulin, 10 µM dexamethasone (Sigma), 0.50 mM 3-isobutyl 1-methylxanthine (Sigma), 10 µM pioglitazone (Sigma), 33 µM biotin (Sigma) and 17 µM pantothenate (Sigma). After 4 days the differentiation medium was replaced with growth medium (10% FBS, 142 mU/ml insulin, 1 µM dexamethasone and 1 µM pioglitazone), and incubated for additional 15 days. Differentiated adipocytes were serum starved over-night and were incubated with or without CsA or tacrolimus (100 nM) for 75 min, and 6 nM insulin was added in the last 20 min.

For GLUT4 cell-surface labelling the cells were washed with ice-cold PBS and then fixed in 4% paraformaldehyde/PBS (Histolab Products AB, Gothenburg, Sweden) for 20 min. This and all subsequent steps were performed at room-temperature. After fixation, non-specific staining was reduced by incubation cells in blocking buffer containing 20% FCS in PBS during 30 min. The cells were incubated with rabbit polyclonal anti-GLUT4 antibody (diluted 1:200, antibody recognizing several epitopes in various regions of GLUT4 potentially including extracellular parts, from Millipore Corporation, Darmstadt, Germany) in 5% FCS in PBS for 3 h. After washing, the cells were incubated with the secondary antibody conjugated with the fluorochrome Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) diluted 1:500 in 5% FCS in PBS during 1 h. After washing in PBS, the cover slips were mounted in vectashield H-1000 (Vector Laboratories, Burlingame, CA, USA) for confocal microscopy. Confocal microscopy was performed with a Leica SP5 confocal system, attached to a Leica DMI6000 inverted microscope and using a 63x oil immersion objective. Images were processed with NIH ImageJ software (Java-based public version).

Culture of L6-GLUT4myc cells and assessment of its 2-deoxy-D-[^{3}H] glucose uptake

L6 cells that stably expresses the GLUT4 protein containing a fourteen amino acid epitope human c-myc within its first exofacial loop, were kindly provided by Dr. A. Klip (The Hospital for Sick Children, Toronto, Ontario, Canada), and were cultured as previously described (31). By allowing highly reproducible quantitative measurements of the cell surface availability of the myc-epitope, L6 cells expressing GLUT4myc are an ideal model to study GLUT4 traffic and have been extensively characterized (31-33). The L6 cells were maintained in minimal essential medium-α (Invitrogen) supplemented with 10% FCS (at 37ºC, 5% CO_2), and used at confluence

2 days after seeding. The cells were serum-starved for 3-4 h and were incubated with or without CsA and tacrolimus (100 nM) for 75 min, and 100 nM insulin (Humulin R, Lilly, Solna, Sweden) was added, or not, in the last 20 min. 2-Deoxy-glucose (2-DG) uptake was measured as described previously (34). Briefly, 2-DG uptake was measured at room-temperature for 10 min in the respective ionic solution containing 10 μM nonradioactive 2-DG (Sigma) and 24.4 nM 2-deoxy-[^{3}H] glucose (0.5 μCi/ml; Amersham Biosciences). The reaction was stopped by washing twice with ice-cold 0.9% NaCl containing 25 mM β-D-glucose (Sigma). Specific uptake was normalized to total protein content.

Immunodetection of cell surface GLUT4myc in L6 cells

The amount of cell surface GLUT4myc was determined by an antibody-coupled colorimetric absorbance assay as previously described (32), with some modifications. Briefly, following stimulation, L6 cells were fixed with 4% paraformaldehyde/PBS for 10 min, and then neutralized with 1% glycine in PBS at 4ºC for 10 min. Cells were blocked with 10% goat serum (Invitrogen) in PBS at 4ºC for 30 min and then incubated with polyclonal anti-Myc antibody (diluted 1:100, Sigma) for 60 min. After washing with PBS, the L6 cells were incubated with peroxidase-conjugated goat anti-rabbit IgG (diluted 1:1000) for 1 h. Cells were washed 6 times with ice-cold PBS, before OPD reagent (Sigma) was added for 30 min at room-temperature. The reaction was stopped with HCl, and optical absorbance of the supernatant was measured at 492 nm.

GLUT4myc internalization (endocytosis) and externalization (exocytosis)

GLUT4myc internalization and externalization was measured in L6 myoblasts as previously reported (32). Briefly, cells were stimulated with 100 nM insulin, rinsed with ice-cold PBS and incubated with polyclonal anti-Myc antibody (1:100; Sigma) at 4ºC during 1 h.

For internalization measurements, the surface-labelled GLUT4myc was allowed to internalize by placing the cells at 37ºC in the presence or absence of insulin (100 nM) and either CsA or tacrolimus (100 nM). At indicated time points, cells were placed on ice, washed 3 times with ice-cold PBS, fixed with 4% paraformaldehyde/PBS for 10 min, and incubated with goat anti-rabbit IgG for 1 h at 4ºC. The amount of GLUT4myc remaining at the cell surface was measured as previously indicated. The data were fitted by nonlinear regression for a single exponential association, and the kinetics of GLUT4 traffic was measured with the equation $Y_{(t)}=Y_{min}+(Y_{max}-Y_{min})(1-e^{-kt})$, where $Y_{(t)}$ represents the cell surface GLUT4myc-antibody at time t; Y_{min} is the amount of GLUT4myc-antibody bound at the earliest time point; Y_{max} is the calculated maximum

amount of GLUT4myc-antibody bound at $t = \infty$; and k is the rate constant for endocytosis (k_{en}), as previously reported (35).

For measurement of externalization the surface-labelled GLUT4myc was allowed to internalize by warming up the cells at 37ºC for 30 min. The cells were then stimulated without or with insulin (100 nM) and either CsA or tacrolimus (100 nM) during 5, 10 and 20 min. Cells were placed on ice, washed 3 times with ice-cold PBS, fixed with 4% paraformaldehyde/PBS for 10 min before incubation with goat anti-rabbit IgG for 1 h at 4ºC. The amount of GLUT4myc remaining at the cell surface was measured as described above.

Statistical Analysis

Differences between control and treated conditions were performed pair-wise using Wilcoxon signed-rank test or paired Student's t-test, as appropriate. Thus, comparisons between treated and untreated cells were performed pair-wise within the same subject and experiment to minimize confounding variables. Results are given as mean ± standard error of the mean (SEM), or as indicated. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using the SPSS package version 18 (SPSS Inc., Chicago, IL).

RESULTS

Cyclosporin A (CsA) and tacrolimus inhibit glucose uptake in human adipocytes

Short-term incubation of subcutaneous isolated adipocytes with CsA (5-1000 nM) or tacrolimus (1-1000 nM) significantly decreased basal and insulin-stimulated (6 nM) glucose uptake in a dose depended manner by up to 40% (Fig. 1A). The inhibitory effect on insulin-stimulated glucose uptake was similar when a lower concentration of insulin (0.15 nM) was used (data not shown). In omental adipocytes, similar effects were observed after short-term incubation, both basal and insulin-stimulated (6 nM) glucose uptake were significantly decreased by up to 30% (Fig. 1B) upon treatment with either drug. After long-term incubation with CsA or tacrolimus, basal glucose uptake was inhibited by ~10%, while insulin stimulated glucose uptake was inhibited by ~20% in subcutaneous as well as omental adipocytes (Fig. 1C). Moreover, short-term pre-incubation with CsA or tacrolimus (100 nM) also inhibited glucose uptake stimulated by okadaic acid by ~20% (Fig. 1D).

CsA and tacrolimus inhibit insulin-stimulated insulin receptor (IR) phosphorylation

CsA and tacrolimus did not change total IR protein levels after 15 minutes or 20 h pre-incubations (Figure 2). Instead, tacrolimus reduced insulin-stimulated IR Tyr1146 phosphorylation at both incubation times by ~30% ($p<0.05$, Fig. 2A and B).

No effects of CsA and tacrolimus on insulin signalling proximal to AS160

Incubation with CsA or tacrolimus did not change the protein levels or the insulin-stimulated phosphorylation of IRS1, IRS2, p85-PI3K or PKB (Fig. 3A and B). Similar results were obtained for AS160 protein levels or phosphorylation, as well as GLUT4 and GLUT1 protein expression levels (Fig. 3C). Moreover, CsA and tacrolimus did not affect protein levels or phosphorylation of mTOR or its downstream target p70S6K (Fig. 3D).

CsA and tacrolimus reduce the amount of GLUT4 localized at the cell surface

Fluorescence labelling with anti-GLUT4 in the plasma membrane of adipocytes is shown in Fig. 4. Insulin increased by ~4-fold the amount of GLUT4 at cell surface, compared with basal (Fig. 4A and B). Short-term incubation with CsA or tacrolimus (100 nM) significantly reduced the amount of insulin-stimulated GLUT4 at the adipocyte surface by ~60% (Fig. 4A and B), and had no effect on basal amount of GLUT4 at cell surface (data not shown).

Effects of CsA and tacrolimus on glucose uptake and GLUT4myc trafficking in L6 cells

Short-term incubation of L6 cells with CsA or tacrolimus (100 nM), significantly reduced insulin-stimulated (100 nM) glucose uptake by ~15% (Fig. 5A) and cell surface GLUT4myc by ~20% (Fig. 5B).

GLUT4myc internalizes with similar rate constants for endocytosis in basal and insulin-stimulated conditions (k_{en}= 0.29 and 0.30 min^{-1}, respectively, Fig. 6A and B). CsA or tacrolimus increased the insulin-stimulated GLUT4myc rate constants for endocytosis ($k_{en\ insulin}$ = 0.30 min^{-1}, $k_{en\ insulin+CsA}$ = 0.39 min^{-1} and $k_{en\ insulin+tacrolimus}$ = 0.44 min^{-1}, Fig. 6B), but had modest effect on the basal endocytosis ($k_{en\ basal}$ = 0.29 min^{-1}, $k_{en\ CsA}$ = 0.31 min^{-1} and $k_{en\ tacrolimus}$ = 0.33 min^{-1}, Fig. 6A). In addition, CsA and tacrolimus, significantly decreased the fraction of cell surface GLUT4myc remaining at the cell surface by up to 30%, after 10 or 20 min incubations and in insulin-stimulated conditions ($Y_{insulin}$ ~ 0.30, $Y_{CsA+insulin}$ ~ 0.19 and $Y_{tacrolimus+insulin}$ ~ 0.21, Fig. 6B). To identify the effects of CsA and tacrolimus on exocytosis, the re-exocytosis of the internalized anti-Myc antibody labelled GLUT4myc was measured (Fig. 6C). The amount of GLUT4myc

recycled back to the membrane after 20 min incubation was significantly increased by ~ 2-fold by the presence of insulin. CsA and tacrolimus had no effect on the basal or insulin-stimulated re-exocytosis rate (Fig. 6C).

DISCUSSION

In this study we demonstrate that the calcineurin inhibitors, CsA and tacrolimus, at therapeutic concentrations commonly used in clinic, inhibited glucose uptake in human subcutaneous and omental adipocytes and in L6 muscle cells. This appears to be independent of the insulin signalling cascade, and removal of GLUT4 from the cell surface via increased rate constant for endocytosis may be a novel mechanism for the diabetogenic effects of the calcineurin inhibitors. These mechanism clearly differ from that of another class of immunosuppressive agents, namely mTOR inhibitors, and we recently reported major alterations in insulin signalling and action in human adipocytes exposed to rapamycin (27).

The majority of the studies that have examined the diabetogenic effects of CsA and tacrolimus, indicate that these calcineurin inhibitors inhibit insulin production and secretion from the beta-cells of the islets of Langerhans (7, 8), but a reduction in peripheral insulin sensitivity has also been suggested (13, 14). The reduction of glucose uptake in both subcutaneous and omental adipocytes after both short- and long-term incubation with either CsA or tacrolimus, as well as in L6 cells, suggests that these drugs may contribute to the development of insulin resistance by inhibiting glucose uptake in insulin-sensitive cells. Short-term incubation with CsA or tacrolimus (100 nM) seems to have higher inhibitory effect on glucose uptake in human adipocytes (~30%) than in L6 cells (~15%).

Results from several randomised clinical trials and meta-analyses, have suggested that tacrolimus is more diabetogenic than CsA (36-38), although this has not been confirmed by others (39, 40). In our study, however, the dose response relationships with respect to impairment of glucose uptake were similar for the two drugs. These effects of CsA and tacrolimus could be detected at low concentrations (1-5 nM) that were even bellow the recommended therapeutic ranges for both CsA (40-80 nM) and tacrolimus (6-12 nM) (41) and with similar dose-response effects. The proportion of inhibition of basal glucose uptake by CsA and tacrolimus after long-term incubation appear to be less severe than short-term inhibition. This effects may be due to a pre-

existing impairment of glucose uptake in these adipocytes caused by a more prolonged pre-incubation time (20 h), masking any further effects of the drugs.
In addition, we show that CsA and tacrolimus inhibit glucose uptake stimulated by okadaic acid. Okadaic acid, a protein phosphatases type 1 (PP1) and 2A (PP2A) inhibitor, stimulates adipocyte PKB kinase activity and glucose transport independent of PI3K activation (42, 43). These observations support that CsA and tacrolimus mediate their effects independent of PI3K activation.

A reduction in IR Tyr1146 activation by ~30% was found in adipocytes incubated with tacrolimus, when compared with untreated adipocytes. However, the concentration of insulin required to maximally activate glucose transport elicits only about 15% of the maximal receptor kinase activity (44), compatible with the spare receptor concept. Indeed, the observed inhibitory effect on the phosphorylation of the IR, was not associated with any change in expression or phosphorylation of the proximal insulin signalling cascade proteins (IRS1/2, p85-PI3K, PKB, mTOR, p70S6K), or GLUT4 and GLUT1 protein levels.

Taken together, these data indicate that the inhibitory effects of CsA and tacrolimus on glucose uptake are not mediated through effects on the early steps of the insulin signalling cascade. Therefore we propose that the reduced insulin-stimulated glucose uptake observed in subcutaneous and omental adipocytes treated with either CsA or tacrolimus could be due to effects on the intracellular trafficking of GLUT4, independent of the insulin signalling machinery. Our results support this hypothesis as they show that CsA and tacrolimus reduced GLUT4 amounts at the plasma membrane of differentiated human preadipocytes by up to ~60%, returning almost to basal levels. Effects on cell-surface GLUT4 are consistent with the inhibitory effects on insulin-stimulated glucose uptake in subcutaneous adipocytes which also almost return to basal levels. We also demonstrated that in L6 cells, CsA and tacrolimus inhibited insulin-stimulated glucose uptake and cell surface GLUT4myc to a similar extent (~15-20%). Moreover, CsA and tacrolimus enhanced the insulin-stimulated rate constant for endocytosis of the GLUT4myc, but they had no effect on the exocytosis trafficking. Chronic insulin treatment has been reported to reduce glucose transport, and to double the rate constants for glucose endocytosis in rat adipocytes (45). These effects are ameliorated by inclusion of metformin in the culture medium, but the mechanism is not known. It thus could be hypothesised that targeting this endocytic pathway by novel pharmacological approaches may be a way to increase cell-surface

GLUT4. Since transmembrane glucose transport is considered to be rate-limiting for glucose metabolism, such a treatment, could mitigate impaired glucose metabolism during calcineurin and moreover, it could potentially provide a novel principle to improve glycaemic control in type 2 diabetes.

Effects of CsA and tacrolimus on basal glucose uptake seem to differ between human adipocytes and L6 cells. Furthermore, neither drug affected basal GLUT4 translocation in human adipocytes and L6 cells. These effects seem to suggest that differences in the sensitivity of internalization of GLUT4 to CsA and tacrolimus may involve a divergent signalling pathway with different insulin dependence. The question remains, about how CsA and tacrolimus increase the rate constant for GLUT4 endocytosis. GLUT4 is internalized mainly via clathrin-coated vesicles and a dynamin-dependent route in adipocytes and muscle cells (46).
The effect of CsA and tacrolimus on clathrin, dynamin and other adaptors proteins involved in docking, fusion and endocytosis of GLUT4 vesicles (e.g. adaptor protein 2, transferrin receptor, vesicle-associated membrane protein 2 and insulin-responsive aminopeptidase) should be further studied, since changes in the protein composition of GLUT4 vesicles may alter their subcellular distribution (47). Another intracellular mechanism required for insulin-mediated GLUT4 trafficking is dynamic remodelling of actin filaments (48). Calcineurin regulates actin dynamics in renal podocytes by dephosphorylation of synaptopodin, thereby blocking the phosphorylation-dependent synaptopodin-14-3-3β interaction (19). CsA also was reported to affect the actin dynamics in podocytes (19), thereby suggesting a possible mechanism affecting cytoskeleton function that can lead to altered vesicle trafficking.

The present results indicate that the reduction in glucose uptake on insulin-sensitive adipocytes by CsA and tacrolimus could be involved in the pathogenesis of new-onset diabetes in patients treated with calcineurin inhibitors, but this *in vitro* study has several limitations. The number of subjects per experiment is limited and the same subjects were not used for all analysis. In addition, it should be noted that other cell types (differentiated humans preadipocytes and L6 cells) were used for mechanistic experiments on GLUT4 localization and trafficking. In addition, the *in vitro* models do not take into account the complex cross-talk between tissues occurring *in vivo* in the regulation of glucose metabolism (26). Thus, the findings in this study need to be confirmed *in vivo* experiments in order to better understand the adverse mechanisms of calcineurin inhibitors and to develop strategies to overcome them.

In conclusion, we have demonstrated that the calcineurin inhibitors CsA and tacrolimus, impaired glucose uptake in subcutaneous and omental adipocytes and in L6 muscle cells. The effects appear to be independent of the insulin signalling cascade. Our data also suggest that CsA and tacrolimus enhanced rate constant for GLUT4 endocytosis in L6 cells. The described effect of CsA and tacrolimus on adipocytes and other insulin-sensitive cells, may contribute to impaired glucose handling in peripheral tissues, as reported with calcineurin inhibitor therapy in organ-transplanted patients. In addition, these findings could potentially point to novel pharmacological mechanisms to inhibit endocytosis and increase cell surface availability of GLUT4. Such a treatment, would enhanced cellular glucose uptake and potentially improve glycaemic control in patients with impaired cellular glucose handling due to calcineurin inhibitor therapy.

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Table 1 – Characteristics of the adipose tissue donors (n = 44).

Variable	Value
Sex (male/female; n)	19/25
Age (years)	50 ± 13
Body mass index (kg/m^2)	27.7 ± 4.0
Waist-hip ratio (WHR)	0.92 ± 0.09
Systolic blood pressure (mmHg)	132 ± 18
Diastolic blood pressure (mmHg)	83 ± 11
Subcutaneous adipocyte diameter (μm) [a]	99.8 ± 11.7
Omental adipocyte diameter (μm) [b]	88.4 ± 19.7
HbA_{1c} (mmol/mol, IFCC) [c]	34 ± 4
Glucose (mmol/L)	5.1 ± 0.6
Insulin (mU/L)	8.6 ± 6.0
HOMA-IR [d]	1.7 ± 1.1
Body fat mass (%)	32.1 ± 7.1
Triglycerides (mmol/L)	1.2 ± 0.4
Cholesterol (mmol/L)	6.0 ± 1.3
LDL-cholesterol (mmol/L)	3.9 ± 1.1
HDL-cholesterol (mmol/L)	1.7 ± 0.6

Data are means ± SD.
[a] n=44; [b] n=11; [c] Normal range 27-46 mmol/mol (IFCC standard); [d] Homeostasis model assessment-estimated insulin resistance, calculated as: fasting insulin (mU/L) x fasting glucose (mM)/22.5 (49); HbA_{1c}, glycosylated haemoglobin; LDL, low-density lipoprotein; HDL, high density lipoprotein

FIGURES

Figure 1.

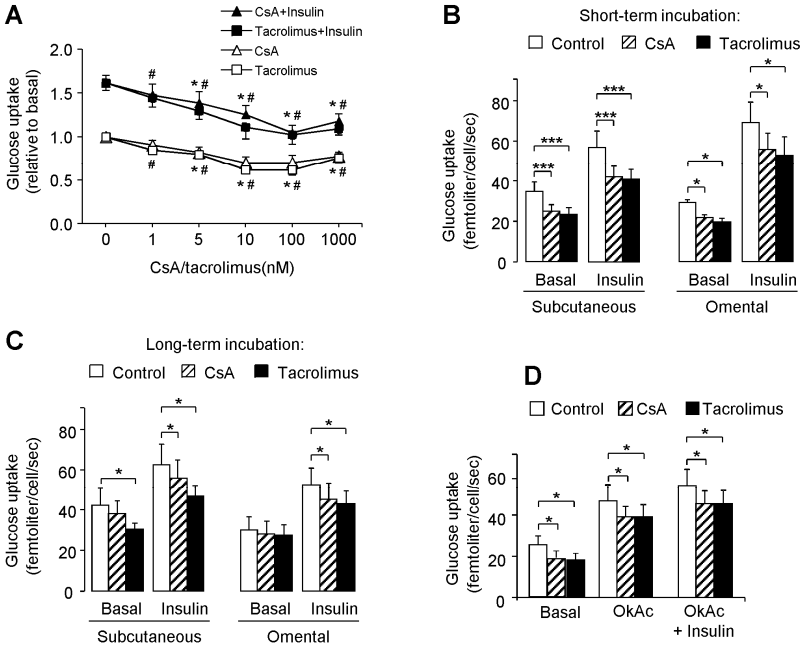

Figure 1. Cyclosporin A (CsA) and tacrolimus reduce glucose uptake in subcutaneous and omental adipocytes.

A: Freshly isolated human subcutaneous adipocytes (n=5-9) were pre-incubated during 15 min with or without different concentrations of CsA or tacrolimus (1 –1000 nM) and the incubation continued with or without insulin stimulation (6 nM) for an additional 15 minutes, before D-[U-^{14}C]-glucose was added, and glucose uptake was then assessed during the following 45 min. **p*<0.05 and #*p*<0.05 CsA and tacrolimus treated vs. untreated. *B* and *C*: Freshly isolated human subcutaneous and omental adipocytes were pre-incubated short-term (15 min) (*B*) and long-term (20 h) (*C*) with CsA or tacrolimus (100 nM) and thereafter glucose uptake was measured as previously explained. (*B*, n=17 and n=8; *C*, n=8 and n=5 for subcutaneous and omental adipocytes, respectively). Total incubation times were 75 min (short-term) and 21 h (long-term). **p*<0.05 and ****p*<0.001 CsA and tacrolimus treated vs. untreated. *D*: Freshly isolated human subcutaneous adipocytes were pre-incubated during 15 min with CsA or tacrolimus (100 nM) and the incubation continued with okadaic acid (1 μM) as well as with or without insulin (6 nM) for an additional 15 minutes, and thereafter as previously reported (n=5). **p*<0.05 CsA and tacrolimus treated vs. untreated. OkAc, okadaic acid.

Figure 2.

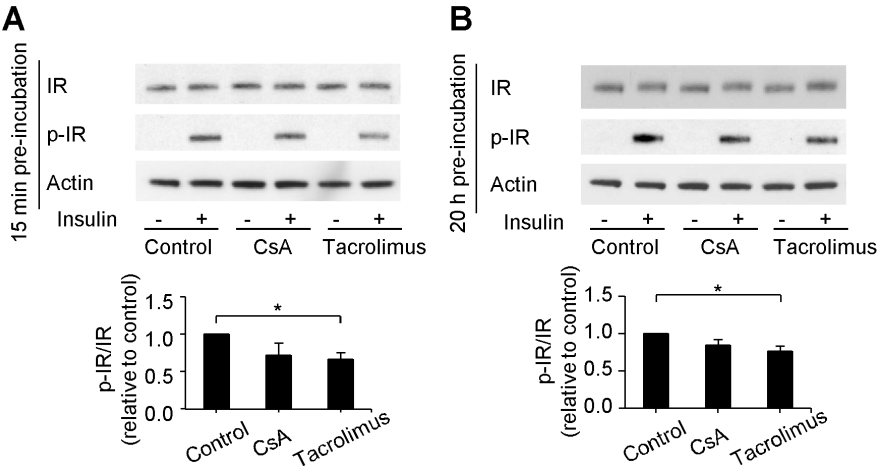

Figure 2. Effects of Cyclosporin A (CsA) and tacrolimus on insulin-stimulated insulin receptor (IR) Tyr1146 phosphorylation.

Insulin stimulated IR Tyr1146 phosphorylation is inhibited by 15 min and 20 h pre-incubation of adipocytes with tacrolimus (100 nM). Freshly isolated human subcutaneous adipocytes were incubated without (control) or with CsA and tacrolimus (100 nM) for 15 min *(A)* or 20 h *(B)*, prior to insulin stimulation (6 nM) for additional 15 min. Lysates were analysed by immunoblotting analysis of IR protein levels and phosphospecific antibody against p-IR (Tyr1146), n=4-5. Results show representative blots. Data are mean ± SEM of densitometry analysis of insulin-stimulated IR Tyr1146 phosphorylation after CsA and tacrolimus treatment compared to untreated adipocytes (set to 1) and normalised to the respective protein levels (p-IR/IR). **p*<0.05 and ***p*<0.01 CsA and tacrolimus treated vs. untreated.

Figure 3.

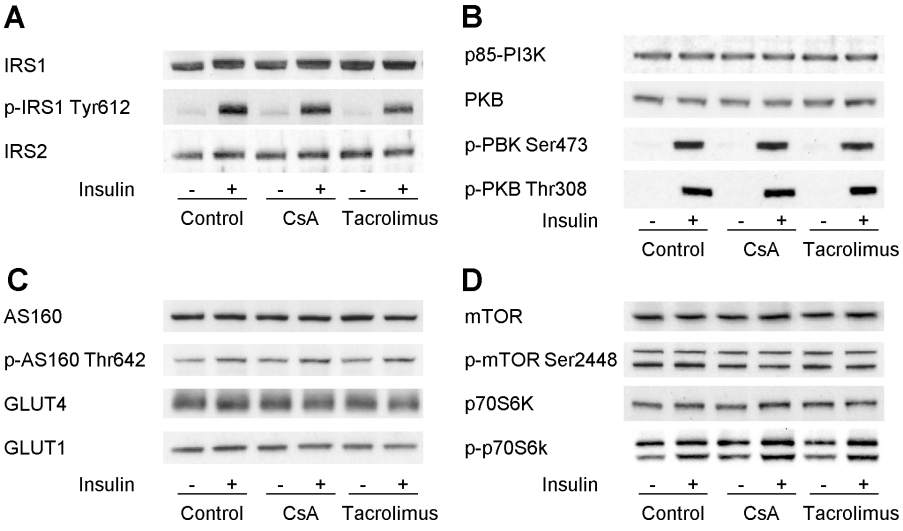

Figure 3. Cyclosporin A (CsA) and tacrolimus do not alter insulin signalling or total GLUT1 or 4 levels.

Freshly isolated human subcutaneous adipocytes were treated without (control) or with CsA or tacrolimus (100 nM) during 20 h and thereafter stimulated with or without insulin (6 nM) for additional 15 min. Immunoblotting analysis showed no effects of CsA or tacrolimus on protein levels or phosphorylation of IRS1/2 and phosphospecific antibody against p-IRS1 Tyr612, n=4-5 (*A*); p85 subunit of PI3K, PKB and phosphospecific antibodies against p-PKB Ser473 and Thr308, n=4-5 (*B*); AS160, phosphospecific antibody against p-AS160 Thr642 and GLUT4/GLUT1, n=4-6 (*C*); and mTOR, p70S6K and phosphospecific antibodies against p-mTOR Ser2448 and p-p70S6K Thr421/Ser424, n=3-4 (*D*).

Figure 4.

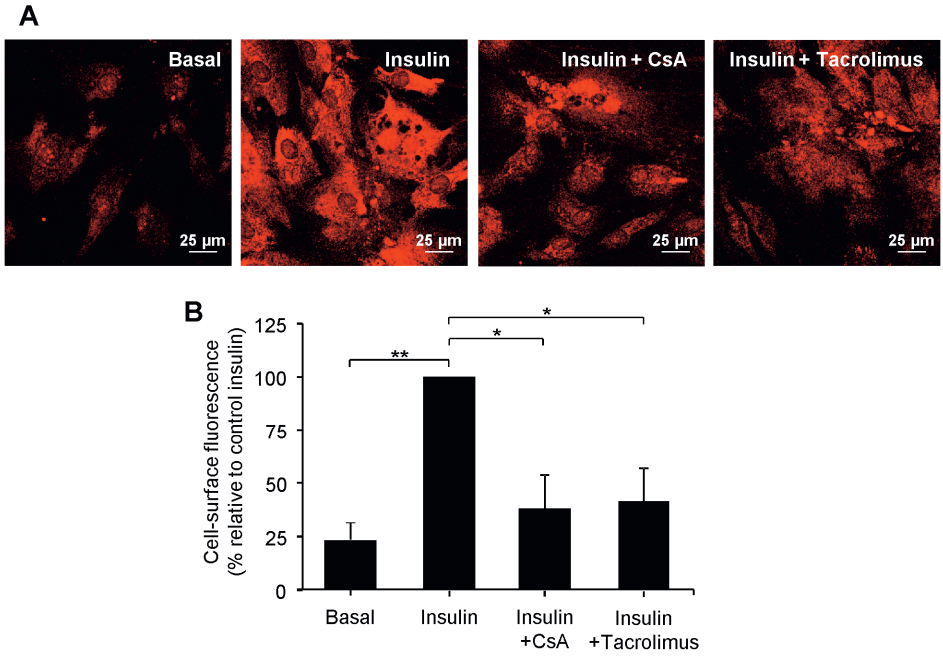

Figure 4. Cyclosporin A (CsA) and tacrolimus inhibit the amount of GLUT4 localized at the adipocyte surface.

Human pre-adipocytes differentiated into adipocytes were serum starved and incubated without or with CsA or tacrolimus (100 nM) during 75 min, and insulin (6 nM) was added in the last 20 min. Cells were fixed and probed with anti-GLUT4 and with Alexa Fluor 594 goat anti-rabbit IgG. *A*: Immunofluorescence was detected using laser confocal microscopy. A representative image of 4 separate experiments is presented. Bars represent 25 µm. *B*: The fluorescence intensity (mean pixel density per are unit) was quantified and is represented as % relative to insulin. **p*<0.05 and ***p*<0.01.

Figure 5.

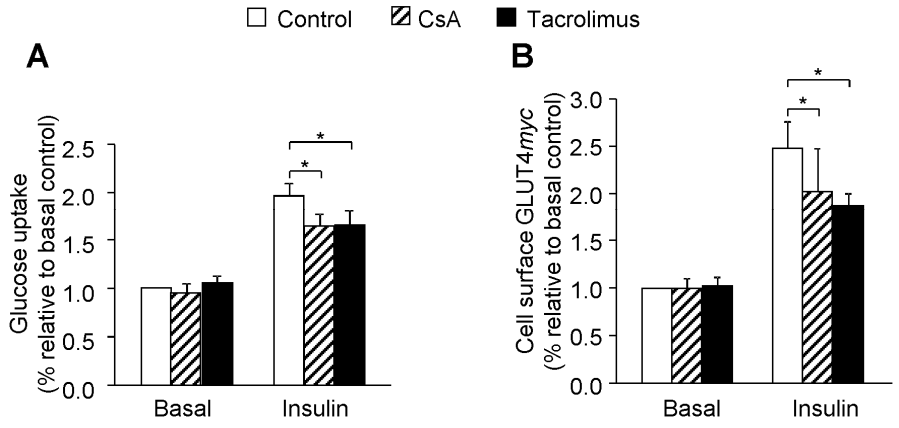

Figure 5. Cyclosporin A (CsA) and tacrolimus reduce glucose uptake and the amount of GLUT4myc at the L6 cell surface.

Confluent L6-GLUT4myc cells were incubated in the presence or absence of CsA or tacrolimus (100 nM) for 75 min, and 100 nM insulin was added in the last 30 min. 2-deoxyglucose uptake (*A*), or cell surface density of GLUT4myc (*B*) were measured in culture plates. Glucose uptake and cell surface GLUT4myc detection are expressed relative to the respective basal control values. Data are presented as mean ± SEM of 8 separate experiments performed in duplicates. **p*<0.05 CsA and tacrolimus treated vs. untreated.

Figure 6.

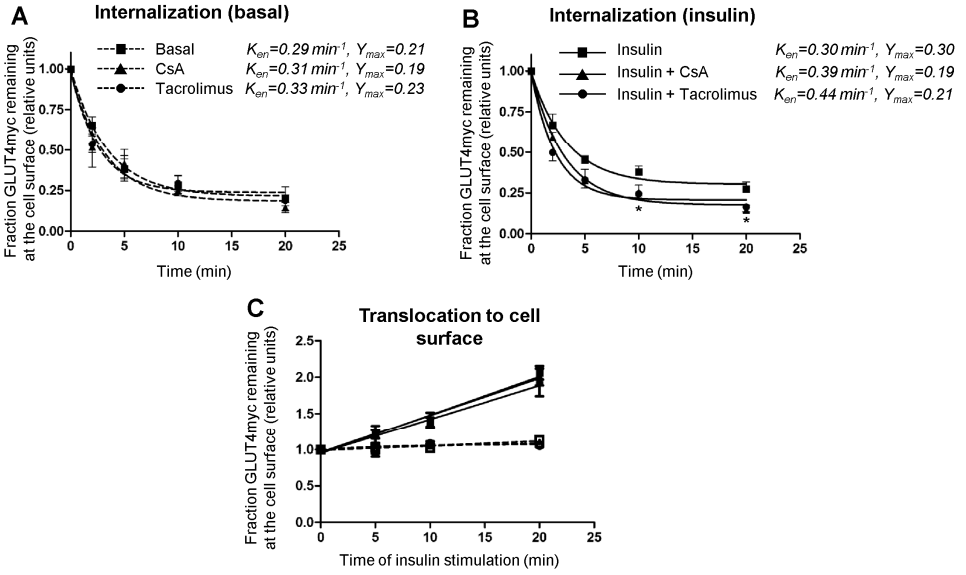

Figure 6. Cyclosporin A (CsA) and tacrolimus increase insulin-stimulated endocytosis of GLUT4myc in L6 cells, and have little effect on the exocytosis.

Confluent L6-GLUT4myc cells were incubated in the absence or presence of 100 nM insulin at 37ºC for 30 min and thereafter with anti-Myc antibody for 1 h to label cell surface GLUT4myc. To measure the time course of internalization, cells were then re-warmed to allow endocytosis in the absence (A) or presence of 100 nM insulin (B) and CsA or tacrolimus (100 nM) for 2-20 min. At the indicated times cells were put on ice and the Myc antibody-labelled GLUT4myc remaining on the surface was measured with the optical densitometry detection assay. *C*: To measure the time course of stimulation, the cells were re-warmed to allow endocytosis in absence of insulin during 30 minutes. Cells were then incubated in absence or presence of insulin (100 nM) and CsA or tacrolimus (100 nM) for 5, 10 or 20 minutes. Cells were placed on ice again and the Myc antibody-labelled GLUT4myc remaining on the surface was analysed by the OPD optical densitometry detection assay. The amount of GLUT4myc remaining at the cell surface at any time point is expressed as a percentage of the cell surface GLUT4myc level at 0 min of incubation. Data points are mean ± SEM of 4-5 separate experiments each performed in duplicates. *Line*, single exponential fits. Rate constants for endocytosis (k_{en}) were calculated from single exponential fits. **p*<0.05 CsA or tacrolimus and insulin-treated vs. insulin.

III

The immunosuppressive agents rapamycin, cyclosporin A and tacrolimus increase lipolysis, inhibit lipid storage and alter expression of genes involved in lipid metabolism in human adipocytes

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Abbreviations:

AMPK	5'-AMP-activated protein kinase
aP2	Fatty acid binding protein 4
ATGL	Adipose triacylglycerol lipase
CGI-58	α/β-hydrolase domain containing 5
CNI	Calcineurin inhibitor
CsA	Cyclosporin A
DGAT1	Diglyceride acyltransferase 1
FFA	Free fatty acids
HSL	Hormone-sensitive lipase
IA	Immunosuppressive agents
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MGL	Monoacyl lipase
mTOR	Mammalian target of rapamycin
NODAT	New-onset diabetes after transplantation
P70S6K	p70 ribosomal S6 kinase
PPARγ	Peroxisome proliferator-activated receptor γ
PKA, B	Protein kinase A, B

Abstract

Cyclosporin A (CsA), tacrolimus and rapamycin are immunosuppressive agents (IA) associated with insulin resistance and dyslipidemia, although their molecular effects on lipid metabolism in adipose tissue are unknown.

We explored IAs effects on lipolysis, lipid storage and expression of genes involved on lipid metabolism in human isolated adipocytes and/or adipose tissue obtained via subcutaneous and omental fat biopsies.

CsA, tacrolimus and rapamycin increased isoproterenol-stimulated lipolysis and inhibited lipid storage by 20-35% and enhanced isoproterenol-stimulated hormone-sensitive lipase Ser552 phosphorylation. Rapamycin also increased basal lipolysis (~20%) and impaired insulin's antilipolytic effect. Rapamycin, down-regulated the gene expression of perilipin, sterol regulatory element-binding protein 1 and lipin 1, while tacrolimus down-regulated CD36 and aP2 gene expression. All three IAs increased IL-6 gene expression and secretion, but not TNF-α or adiponectin.

These findings suggest that CsA, tacrolimus and rapamycin enhance lipolysis, inhibit lipid storage and expression of lipogenic genes in adipose tissue, which may contribute to the development of dyslipidemia and insulin resistance associated with immunosuppressive therapy.

Keywords: Cyclosporin A, Tacrolimus, Rapamycin, Lipolysis, Adipocytes, Lipid storage

1. Introduction

The calcineurin inhibitors (CNIs), cyclosporin A (CsA) and tacrolimus, as well as the mTOR (mammalian target of rapamycin) inhibitor, rapamycin, are frequently used immunosuppressive agents (IA) to prevent rejection after solid organ transplantation and treat autoimmune diseases (Scherer et al., 2007). However, standard recommended doses are associated with the development of metabolic complications, including hyperlipidemia, and new-onset diabetes after transplantation (NODAT) (Parekh et al., 2012). The reported prevalence of these conditions has varied greatly, with estimates up to 50% for diabetes (Montori et al., 2002) and 20% to 80% for dyslipidemia (Kesten et al., 1997; Parekh et al., 2012), depending on the population, diagnostic criteria, mean follow-up time and type of IA therapy. These complications are well known risk factors for cardiovascular diseases and are associated with reduced graft and patient survival in transplant recipients (Massy, 2001). Clinical studies have shown that the IA increase serum levels of cholesterol, triglycerides and low-density lipoprotein (LDL), usually in a dose-dependent manner (Ichimaru et al., 2001; Li et al., 2012; Morrisett et al., 2002; Spinelli et al., 2011). In addition, rapamycin increases plasma free fatty acids (FFA) levels (Morrisett et al., 2002). Although CsA and tacrolimus are similar in terms of their mechanism of action, tacrolimus is associated with less adverse effects on the patient's lipid profile (Bakar et al., 2009; Ichimaru et al., 2001; Vincenti et al., 2007), and one study even suggested beneficial effects (Perrea et al., 2008).

The underlying causes for this dyslipidemia are not clear, but CNIs therapy have been associated with reduced lipoprotein lipase (LPL) activity in plasma samples (Derfler et al., 1991; Tory et al., 2009), thereby limiting triglycerides-rich lipoprotein clearance, while rapamycin has been shown to increase lipolysis rates in 3T3-L1 cells (Chakrabarti et al., 2010; Soliman et al., 2010). However, effects of immunosuppressive agents on lipid metabolism and on gene and protein expression of factors involved in regulation of lipid metabolism in human adipose tissue have never been studied.

Adipose tissue is an important metabolic and endocrine organ involved in the regulation of energy intake and expenditure (Ahima, 2006; Ost et al., 2010). Postprandially, adipose tissue takes up glucose and FFA from the circulation and stores it as energy in the form of triglycerides (Large et al., 2004). During starvation, adipose tissue provides energy via breakdown of triglycerides into FFA and glycerol, a process known as lipolysis. Lipolysis is regulated by

various lipolytic and anti-lipolytic hormones. Adipocyte lipolysis is activated by catecholamines (e.g., epinephrine) binding to the β-adrenergic-receptors (β1, β2 and β3), which results in increased adenylate cyclase activity and cAMP formation (Jaworski et al., 2007). This leads to subsequent activation of protein kinase A (PKA) (Kraemer and Shen, 2002) and downstream targets, including adipose triacylglycerol lipase (ATGL), hormone-sensitive lipase (HSL) and perilipin (Kraemer and Shen, 2002; Schweiger et al., 2006; Tansey et al., 2004). Insulin is a powerful anti-lipolytic hormone in adipose tissue that inhibits lipolysis by stimulating phosphodiesterase 3B resulting in decreased cAMP levels and suppressed activation of PKA and, subsequently, HSL (Degerman et al., 1990).

Dysregulation of fatty acid metabolism in adipose tissue may contribute to elevation of FFA in plasma and contribute to ectopic fat deposition in liver and skeletal muscle (Roden et al., 1996). These perturbations can promote systemic insulin resistance through several mechanisms, such as increased hepatic gluconeogenesis and decreased glycogen synthesis and glucose oxidation in skeletal muscle (Jensen, 2006).
In addition to fatty acids, cytokines and adipokines are produced by human adipocytes and play a major role in glucose and lipid metabolism (Bastard et al., 2006). Increased levels of IL-6 and TNF-α contribute to insulin resistance and dyslipidemia, while adiponectin is an insulin sensitizing adipokine (Bastard et al., 2006).

The main objective of this study was to investigate the direct effects of the IA CsA, tacrolimus and rapamycin, at therapeutic concentrations, on lipolysis including interactions with catecholamine (isoproterenol) and insulin, and effects on lipid storage in human adipocytes, and expression of genes involved in regulation of lipid metabolism in human adipose tissue.

2. Methods

2.1 Subjects- Adipose tissue donors

Human abdominal subcutaneous (n=57) and omental (n=18) adipose tissue biopsies were obtained from 60 non-diabetic subjects (26 men/34 women; age 18-72 years; body mass index (BMI) 20.9-36.4 kg/m^2). Paired subcutaneous and omental adipose samples were obtained from 15 of these subjects. Due to limited amount of adipose tissue obtained, not all experiments were performed in all biopsies and the number of experiments is indicated in each section bellow.

Subjects fasted overnight (>10 h) and fasting venous blood samples were collected in the morning for analysis of glucose, insulin and lipids by routine methods at the Department of Clinical Chemistry, Sahlgrenska University Hospital. Thereafter, adipose tissue biopsies were performed by needle aspiration of subcutaneous fat from the lower part of the abdomen after dermal local anaesthesia with lidocaine (Xylocain; AstraZeneca, Södertälje, Sweden) (n=36), or during elective abdominal surgery after induction of general anaesthesia (subcutaneous and/or omental, n=24).

The clinical and biochemical characteristics of the adipose tissue donors are shown in Table 1. Anthropometric measurements including body composition assessed by bioimpedance were measured in all subjects (Lukaski et al., 1986). Subjects with diabetes, other endocrine disorders, systemic illnesses or malignancy, as well as ongoing medication with systemic glucocorticoids and immune modulating therapies were excluded from the study. One subject used local inhalation treatment with β2-agonist and glucocorticoids for asthma, two subjects had ongoing treatment due to depressive disorder (low-dose venlafaxine and lamotrigine plus aripiprazol, respectively) and one subject used oral contraception. The study protocol was approved by the Regional Ethics Review Board in Gothenburg. Written informed consent was obtained from all subjects.

2.2 Isolation of subcutaneous adipocytes

Adipocytes were isolated from subcutaneous fat obtained from both needle and surgical biopsies after collagenase type II digestion (from *Clostridium histolyticum,* Sigma Chemical Co., St. Louise, MO, USA) in Hank's medium 199 (Invitrogen Corporation, Paisley, UK) that contained 6 mM glucose, 4% BSA (Sigma), 0.15 μM adenosine (Sigma) (pH 7.4) for 60 min at 37ºC in a shaking water-bath. Isolated adipocytes were filtered through a 250-μm nylon mesh and washed four times in Hank's medium. The average cell diameter was measured in isolated subcutaneous and omental adipocytes from all subjects (Lundgren et al., 2007).

2.3 Lipolysis

Effects of the IA on lipolysis in isolated subcutaneous adipocytes were essentially performed as previously reported (Lundgren and Eriksson, 2004; Palming et al., 2006). The adipocyte suspension (lipocrit 3-5%) were incubated in the presence or absence of CsA (0.1 μM) (Sigma), tacrolimus (0.1 μM) (Sigma) or rapamycin (0.01 μM) (Sigma) in Hank's medium containing 5.6 mM glucose, 4% BSA, 0.15 μM adenosine, pH 7.4 in a gently shaking water bath at 37ºC for 2 h.

To test the effect of the IA on lipolysis (n=13 with all conditions and additional 3 with all but 0.01 μM isoproterenol-stimulated conditions; duplicates) and on the antilipolytic effect of insulin (n=6; duplicates), the medium was supplemented or not with isoproterenol (0.01 or 1 μM) and insulin (0-100 μU/ml) and glycerol released into the medium was measured by colorimetric absorbance in a kinetic enzymatic analyser (Microdialysis Analyser CMA600; CMA Microdialysis AB, Sweden), and used as an index of lipolysis.

2.4 Lipid storage

Lipid storage in subcutaneous adipocytes was measured using glucose (lipogenesis) and palmitate, as substrates, and essentially performed as previously reported (Gathercole et al., 2011; Palming et al., 2006). For the ^{14}C-glucose experiments (n=8; duplicates), the adipocyte suspension (lipocrit 3-5%) were incubated without or with CsA (100 nM), tacrolimus (100 nM) (Sigma) or rapamycin (10 nM) in Hank's medium (5.6 mM glucose, 4% BSA, 0.15 μM adenosine, pH 7.4) supplemented or not with insulin (1000 μU/ml) and with D-[U-^{14}C] glucose (0.26 mCi/L, 0.86 μM, Amersham Biosciences GE Healthcare, Buckinghamshire, UK) in a gently shaking water bath at 37ºC for 2 h. In the ^{14}C-palmitate assay (n=5; duplicates), the adipocyte suspension (lipocrit 3-5%) was treated without or with CsA (100 nM), tacrolimus (100 nM) (Sigma) or rapamycin (10 nM) in DMEM (5.6 mM glucose, 10% FCS, 1% penicillin-streptomycin) (Invitrogen) and sodium-palmitate/BSA-mix (Sigma) (0.12 mM sodium palmitate, 40% BSA) supplemented with [1-^{14}C] palmitic acid (1.0 μCi/ml, Perkin Elmer, Boston, MA, USA), in a gently shaking water bath at 37ºC for 2 h. Then, the adipocytes were separated by centrifugation through silicone oil and the cellular lipids, which mainly consist of triglycerides (Large et al., 2004), were immediately extracted according to the two-phase method described by Dole and Meinertz (Dole and Meinertz, 1960). The upper *n*-heptane phase (1 ml) was evaporated to dryness and the 14C-glucose incorporation in the glyceride-glycerol moiety (lipogenesis) and the 14C-palmitate esterification in cellular triglycerides, was determined by scintillation counting as measures of lipid storage.

2.5 Adipocyte lysates and immunoblotting

Isolated subcutaneous adipocytes (n=6, single) were diluted (lipocrit 3-5%) in Hank's medium [6 mM glucose, 4% BSA, 0.15 μM adenosine, (pH 7.4)] and single samples were pre-incubated for 15 minutes in the absence or presence of CsA (0.1 μM), tacrolimus (0.1 μM) or rapamycin (0.01 μM) at 37ºC in a shaking water-bath, before isoproterenol (1 μM) was added for additional 60 min. In addition, to test effects of CsA (0.1 μM) and tacrolimus (0.1 μM) on mTOR activation,

isolated adipocytes were pre-incubated for 15 minutes or 20 h in the absence or presence of the agents, before insulin (6 nM) was added for additional 15 min.

Immunoblotting was performed as previously described (Pereira et al., 2012). In brief, adipocytes were separated from medium and total protein was extracted in lysis buffer [25 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine, 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochlorine and 0.1 μM okadaic acid] and insoluble substances were sedimented through centrifugation. Aliquots of total lysate (15 μg/lane) were loaded into 4–12% gels (Invitrogen Corporation, Paisley, UK), subjected to SDS-PAGE and transferred to a nitrocellulose membrane and immunoblotted with primary antibody: anti-HSL, anti-phospho HSL Ser552 (equivalent to Ser563 of rat HSL), anti-perilipin, anti-mammalian target of rapamycin (anti-mTOR), anti-phospho mTOR (Ser2448), anti-p70 ribosomal S6 kinase (p70S6K) and anti-phospho p70S6K (Thr421/Ser424) (Cell Signaling Technologies, Beverly, MA, USA); and anti-α/β-hydrolase domain containing 5 (CGI-58) and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies were diluted according to the manufacturer's instructions, and thereafter with the appropriate secondary antibody. Detection was made by Immuno-star HRP luminol/enhancer (Biorad Laboratories, Hercules, CA, USA), with Chemidoc XRS (CCD camera). Intensity of the bands was quantified by densitometry and phosphospecific protein quantifications were adjusted for the corresponding protein level.

2.6 Subcutaneous and omental adipose tissue incubation

Effects of long-term incubation (20h) with the immunosuppressive agents on gene and protein expression of lipogenic factors was performed in single samples of subcutaneous and omental adipose tissue instead of adipocytes. Preservation of the interaction between the different cell types in the adipose tissue may be critical for its function during long-term incubation. For this, adipose tissue pieces (300 mg) were incubated for 20 h without or with CsA (0.1 μM), tacrolimus (0.1 μM) or rapamycin (0.01 μM), in 6 well polystyrene plates containing 5 ml of DMEM (6 mM glucose, 10% FBS, 1% penicillin-streptomycin) (Invitrogen) at 37ºC under a gas phase of 5% CO_2 with gentle agitation (~30 rpm) in a culture cupboard. Adipose tissue was thereafter snap-frozen for gene and protein analysis, while incubation media was used to measure IL-6 and adiponectin secretion.

2.7 Immunoblotting analysis of adipose tissue

Adipose tissue was homogenized, and total protein was extracted in lysis buffer as previously described for the adipocytes. Subcutaneous and omental adipose tissue lysates (n=5, single) were used for western-blot analysis of anti-ATGL (Santa Cruz Biotechnology) anti-HSL, anti-perilipin, anti-CGI-58 and anti-actin, as previously described.

2.8 Adipose tissue gene expression

Total RNA from adipose tissue was isolated with RNeasy Lipid Tissue Mini Kit (Quiagen, Hilden, Germany), and used for cDNA synthesis using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA). The gene expression was analyzed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) applying gene-specific custom-designed primers and probes from Applied Biosystems (sequences used are available on request). Relative quantification of mRNA levels was plotted as the fold change compared with basal and were normalized to the housekeeping gene 18S rRNA (Applied Biosystems). The gene expression of the lipolytic genes ATGL, HSL and perilipin were investigated in subcutaneous and omental adipose tissue (n=21 and n=17, respectively, single). In addition, expression of genes involved in the uptake (LPL and CD36), transport (ap2/FABP4, fatty acid binding protein 4) and storage (SREBP1, sterol regulatory element-binding protein 1 (both SREBP1-a and 1c isoforms); DGAT1, diglyceride acyltransferase 1; lipin 1; FAS, and fatty acid synthase) of fatty acids and the adipokines IL-6, TNF-α and adiponectin were also investigated.

2.9 Determination of IL-6 and adiponectin in the culture medium

IL-6 and adiponectin released in the medium from the subcutaneous and omental adipose tissue incubations in the presence or absence of CsA, tacrolimus or rapamycin were measured after 20 h. Commercial sandwich ELISA kits were used (PeliKine Compact™ Human IL-6 ELISA kit, Sanquin Reagents, Amsterdam, The Netherlands and Human Adiponectin ELISA kit, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Samples were diluted 1:100 for IL-6 measurement and 1:30 for adiponectin measurement in the kit dilution buffer to be within the linear range of the assay (0.6-450 pg/ml for IL-6 and 1.56-100 ng/ml for adiponectin).

2.10 Assessment of cell viability

The viability of adipocytes was not significantly affected after 24 h incubation with either CsA (0.1 μM), tacrolimus (0.1 μM) or rapamycin (0.01 μM) (93 to 100%, *p*=ns), when compared to untreated cells. See supplementary Fig. 1 for details.

2.11 Statistical analysis

Differences between control (no-treatment) and treated conditions were performed pair wise using Wilcoxon signed-rank test. Comparisons between treated and untreated cells were performed within the same individual to minimize confounding variables. Results are given as mean ± standard error of the mean (SEM), or as indicated. A *p*-value <0.05 was considered statistically significant. Statistical analyses were performed using the SPSS package version 18 (SPSS Inc., Chicago, IL).

3. Results

3.1 Effects of CsA, tacrolimus and rapamycin on lipolysis

Treatment of subcutaneous adipocytes with CsA, tacrolimus or rapamycin significantly enhanced lipolysis rate during concomitant stimulation with isoproterenol (0.01 or 1 μM) by 20-35% ($p<0.05$), compared to control (Fig. 1A). In addition, the rate of basal lipolysis was significantly increased by ~20% ($p<0.05$), in adipocytes incubated with rapamycin, but not with CsA or tacrolimus. Rapamycin significantly reduced the antilipolytic effect of insulin (10-100 μU/ml, $p<0.05$) (Fig. 1B), while CsA and tacrolimus did not (Fig. 1C).

3.2 Effects of CsA, tacrolimus and rapamycin on lipid storage

CsA, tacrolimus and rapamycin decreased insulin-stimulated lipogenesis rate as measured by ^{14}C-glucose incorporation into lipids (~10%, $p<0.05$). However, after subtracting the basal from the insulin-stimulated lipogenesis, the changes induced by the immunosuppressive agents are not significant (Fig. 2A). In addition, CsA, tacrolimus and rapamycin reduced the ^{14}C-palmitate storage by 20-35%, compared to control (Fig. 2B).

Correlations between the effects of the IA on lipolysis/lipid storage and several metabolic variables (BMI, WHR, % body fat mass, adipocyte diameter, HbA1c, HOMA and serum glucose, insulin and lipids) were performed, but no significant correlations were identified. However, the number of subjects is limited (13-16 and 8 for lipolysis and lipid storage, respectively) and we

can not rule out the possibility that adipocytes from individuals with different insulin sensitivities may respond different to the effects of the immunosuppressive agents.

3.3 HSL and perilipin protein amount in incubated adipocytes

Although there were no differences in cellular HSL protein levels, CsA, tacrolimus and rapamycin significantly increased isoproterenol-stimulated HSL Ser552 phosphorylation in subcutaneous adipocytes (CsA ~100% increase; tacrolimus and rapamycin ~230% increase, $p<0.05$) (Fig. 3A and B). Perilipin and CGI-58 protein levels were not changed by any of the IA (Fig. 3C). There is a tendency to decrease HSL and increase perilipin protein levels in adipocytes treated with isoproterenol, when compared to non-treated (Fig. 3A and C). This may be explained by a loss of the proteins in the lipid fraction during preparation of the lysates, due to HSL and perilipin translocation between the cytosol and the lipid droplet upon lipolytic stimulation (Brasaemle et al., 2000; Tansey et al., 2001). Although we have not used optimized mobility shift assay to determine the phosphorylation state of perilipin, the 4-12% polyacrylamide gel used for the SDS-PAGE allows some degree of electrophoretic separation, as observed by the mobility shift in the isoproterenol-stimulated conditions (Fig. 3C). None of the IA seems to change the phosphorylation state of perilipin. However, we can not exclude the possibility that the immunosuppressive agents may change phosphorylation of individual sites of perilipin that are not detected by mobility shift.

3.4 Effects of CsA and tacrolimus on mTOR and p70S6K protein levels and phosphorylation

To study the effects of CsA and tacrolimus on the mTOR pathway, the phosphorylation of mTOR and its downstream substrate p70S6K was assessed (supplementary Fig. 2, n=4). CsA and tacrolimus did not affect phosphorylation of mTOR or p70S6K, in the absence or presence of insulin, and the cellular content of both these proteins was left intact.

3.5 Expression of genes and protein involved in the regulation of lipolysis and lipid storage in subcutaneous and omental adipose tissue

The gene expression of the lipolytic mediators ATGL, HSL and perilipin were investigated in human subcutaneous and omental adipose tissue after 20 h incubation in the absence or presence of CsA (0.1μM), tacrolimus (0.1 μM) or rapamycin (0.01 μM). Using real-time PCR we found that rapamycin significantly decreased perilipin gene expression in both subcutaneous and omental adipose tissue by ~20%, $p<0.05$, and had no effect on ATGL or HSL gene expression (Fig. 4A). The perilipin protein levels also decreased significantly in both subcutaneous and

omental adipose tissue by ~50%, $p<0.05$ (Fig. 4B and C), compared to control. CsA and tacrolimus did not change ATGL, HSL and perilipin gene expression or protein amount, and CGI-58 protein amount was not changed by any of the immunosuppressive agents (Fig. 4A-C).

In addition, the expression of several key lipogenic genes SREBP1, FAS, lipin 1, DGAT1, LPL, CD36 and aP2 involved in the clearance and storage of circulating lipids was assessed. Rapamycin reduced the gene expression levels for SREBP1 and aP2 by ~20%, in subcutaneous adipose tissue, $p<0.05$, (Fig. 5A and B) and lipin 1 by ~20%, in both subcutaneous and omental adipose tissue, $p<0.05$ (Fig. 5A). In addition, rapamycin increased the LPL gene expression by ~15% and 25%, in subcutaneous and omental adipose tissue, respectively, $p<0.05$, (Fig. 5B), compared to control. The CD36 and the aP2 gene expression was reduced by tacrolimus in both the subcutaneous and omental adipose tissue (20-30%, $p<0.05$, Fig. 5B), compared to control. CsA and tacrolimus had no effect on the gene expression levels for SREBP1, FAS, lipin 1, DGAT1 and LPL in subcutaneous and omental adipose tissue (Fig. 5A and B).

3.6 IL-6, TNF-α and adiponectin gene expression and secretion

CsA, tacrolimus and rapamycin increased IL-6 gene expression in subcutaneous and omental adipose tissue, although this effect was only significant in subcutaneous adipose tissue (subcutaneous: CsA ~35%; tacrolimus ~240%; rapamycin ~82%, $p<0.05$; omental: CsA ~85%, $p=0.55$; tacrolimus ~440%, $p=0.17$; rapamycin ~230%, $p=0.80$, Fig.6 A). The IL-6 release into the incubation media was also increased in both subcutaneous and omental adipose tissue incubated with IA, compared to control (Fig. 6B). However, this increase was only significant for the subcutaneous adipose tissue incubated with rapamycin ($p<0.05$). TNF-α and adiponectin gene expression and adiponectin release into the medium was not changed after incubation with any of the IA (Fig. 6C and D).

4. Discussion

In the present study, we show that CsA, tacrolimus and rapamycin increase isoproterenol-stimulated lipolysis in human subcutaneous adipocytes by 20-35%. Rapamycin also increased basal lipolysis and impaired insulin's antilipolytic effect. In addition, the IA, reduced lipid storage by 20-35%, which may potentially contribute to dyslipidemia. Rapamycin effects on

lipolysis and on the insulin antilipolytic effect, together with impaired lipid storage, suggest that rapamycin modulates FFA mobilization, which could contribute to insulin resistance.

In addition, we demonstrate that CsA, tacrolimus and rapamycin, in mature human adipocytes, significantly increase isoproterenol-stimulated phosphorylation of HSL on Ser552, one of the major sites controlling HSL activity (Anthonsen et al., 1998). These results are in agreement with previous studies that show that rapamycin increases basal and isoproterenol-stimulated lipolysis and enhanced isoproterenol-stimulated phosphorylation of HSL on Ser563 (equivalent to Ser552 of human HSL) in 3T3-L1 adipocytes (Chakrabarti et al., 2010; Soliman et al., 2010). In contrast, treatment of 3T3-L1 cells with CsA has been shown to inhibit basal and TNF-α-induced lipolysis (Holowachuk, 2007). The apparent discrepancy between the present and the previous study regarding the effects of CsA on lipolysis could be due to different effects of CsA in the different activated lipolysis pathways in these studies. The catecholamine isoproterenol is a synthetic β-adrenergic agonist that activates PKA activity by stimulating production of cAMP (Anthonsen et al., 1998), while the TNF-α stimulates lipolysis through the MAPKs (Ryden et al., 2004).

Lipogenesis was only modestly affected by the IA (~10% reduction) and this effect does not seem to be mediated through direct effects on insulin signalling. Notably, a reduced adipocyte glucose uptake caused by rapamycin (Pereira et al., 2012) and by CsA and tacrolimus (Pereira MJ *et al,* unpublished data) could also contribute to that finding. Hence the IA may in parallel inhibit glucose uptake, enhance lipolysis and attenuate lipid storage. These findings may contribute to higher circulating levels of glucose, glycerol and FFA and might lead to fatty acid deposition as ectopic triglycerides in insulin target tissues such as liver and skeletal muscle (Roden et al., 1996). High levels of FFA may also promote insulin resistance via several mechanisms and also directly compete for substrate utilization in skeletal muscle, thus reducing glucose utilization (Boden, 1997; Roden et al., 1996). In fact, recent studies in animal models have shown that treatment with either rapamycin (Deblon et al., 2012; Houde et al., 2010) or CsA (Delgado et al., 2012; Ikeuchi et al., 1992) cause both muscle and liver insulin resistance resulting in severe glucose intolerance. In addition, glycerol is as important substrate for hepatic gluconeogenesis directly contributing to glucose production (Baba et al., 1995). Therefore, increased lipolysis and reduced lipid storage could be a cellular basis for elevation of circulating FFA and VLDL-particles (Ichimaru et al., 2001; Morrisett et al., 2002) and also provide one explanation for insulin resistance and development of NODAT during immunosuppressive therapy.

Perilipin coats the surface of intracellular lipid droplets, and its down-regulation facilitates lipolysis (Brasaemle et al., 2000; Tansey et al., 2001). Thus, the reduced perilipin gene and protein expression during rapamycin treatment may contribute to a non-stimulated (basal) lipolysis. One must, however, bear in mind that perilipin gene and protein expression were reduced after 20 h of incubation, while lipolysis was measured after 2 h of incubation. So, the mechanism by which rapamycin is affecting basal lipolysis, remains unexplained. Our results do not suggest that ATGL, HSL and CGI-58 protein levels can account for the effects of the IA on the lipolysis, since levels of these proteins were similar in the subcutaneous and omental adipose tissue after 20 h incubation.

We demonstrate that the IA can enhance IL-6 adipose tissue gene expression and rapamycin also increased IL-6 secretion *in vitro.* This indicates that rapamycin can contribute to increased levels of IL-6 in circulation. Thus, an increase in IL-6 production and secretion in adipose tissue during rapamycin incubation may be an autocrine and paracrine mediator stimulating lipolysis (van Hall et al., 2003). On the other hand, we cannot exclude an indirect effect of FFA influencing IL-6 gene expression. Thus, effects of IA on IL-6 gene expression should be further evaluated *in vivo*.

Nonetheless it should be noted that the clinical significance of the modest changes we observed after short-term incubation with the immunosuppressive agents is uncertain. However, it is likely that the 20-35% increase in lipolysis and decrease in lipid storage may be clinically significant. This is supported by the fact that other well know therapies that induce dyslipidemia and insulin resistance, such as glucocorticoids (Lundgren et al., 2008) and anti-HIV protease inhibitors (Hadigan et al., 2002; Leroyer et al., 2011), cause 20-40% increase in lipolysis rate in human subcutaneous adipocytes/adipose tissue during long-term incubation at high exposures *in vitro*. Conversely, nicotinic acid derivatives that are used as treatment for dyslipidemia act mainly by inhibiting adipose tissue lipolysis, and *in vitro* this effect has been reported to be around 25% (Stirling et al., 1985). Importantly, our findings occurred at IA concentrations that are commonly present in the circulation of treated patients (Schiff et al., 2007; Wyeth, 1999).

It must be emphasized that culture of adipocytes *in vitro* may not mimic the biological processes in adipocytes in their native environment and does not take into account the neuroendocrine, e.g. sympathetic and parasympathetic nervous control of adipose tissue metabolism (Kreier et al., 2002; Sjostrand and Eriksson, 2009). Thus, the findings in this study should be confirmed with *in vivo* experiments. In addition, visceral adiposity is more strongly associated with insulin

resistance and cardiovascular diseases. Thus, future studies should also address the effects of the immunosuppressive agents on lipid metabolism in omental adipocytes.

The gene expression of LPL was up-regulated in both subcutaneous and omental adipose tissue after 20 h incubation with rapamycin. In contrast, others have shown that rapamycin reduces LPL gene expression and activity in the retroperitoneal adipose tissue of rats treated with the drug (Blanchard et al., 2012; Houde et al., 2010). Important differences between the present study and the previous ones include duration of treatment and the species studied. The increased expression of the LPL gene might be expected to promote FFA flux from the circulation into the adipose tissue. However, the use of only mRNA expression imposes limitations, as LPL activity displays a strong post-translational regulation and thus its activity does not necessarily correlate with LPL gene expression and protein amount (Ruge et al., 2012; Ruge et al., 2006).

It was previously demonstrated by our group (Pereira et al., 2012) that rapamycin inhibits formation of mTOR complex 1 and 2 and insulin-stimulated phosphorylation of p70S6K and protein kinase B (PKB) at Ser473 in human adipocytes. PKB activity is required for the insulin-induced activation of phosphodiesterase 3B and thus for the antilipolytic action of insulin (Berggreen et al., 2009). Thus, the interference with the antilipolytic effect of insulin could be attributed to rapamycin inhibitory effect on PKB activation. In addition, mTORC1 mediates the effects of insulin on lipid storage by regulating the expression and activation of PPARγ and SREBP1 (Porstmann et al., 2008), two master transcription factors that stimulate genes involved in fatty acid biosynthesis. This very well matches the observed reduction mediated by rapamycin on expression of the SREBP1 gene (both 1a and 1c isoforms) as well as downstream target genes involved in lipid uptake (aP2/FATP4) and triglyceride synthesis (lipin 1) in subcutaneous adipose tissue. By disrupting mTORC1 regulation of expression of genes involved in fatty acid biosynthesis, rapamycin may impair the capacity of adipose tissue for plasma lipid clearance, which likely contributes to hyperlipidemia. Additional support for the involvement of rapamycin in the reduced adipose storage of lipids is provided by the reduction in palmitate incorporation into the triglycerides. In contrast, FAS and DGAT1 gene expression were not affected by rapamycin, suggesting that SREBP1c activity was not affected by this drug. However we cannot exclude that SREBP1a and/or 1c activity will behave differently as compared to gene expression.
In contrast, neither CsA nor tacrolimus affected the amount or phosphorylation of mTOR or its downstream target p70S6K, indicating that these agents do not affect the mTOR pathway. Instead, tacrolimus reduced expression of both the CD36 and aP2 genes in subcutaneous and

omental adipose tissue, two target genes of proliferator-activated receptor γ (PPARγ) involved in cellular transport and metabolism of fatty acids. This suggests that impaired PPARγ activity may be a mechanism in the observed down-regulation of lipid storage.

Results are summarised on Table 2. In conclusion, therapeutic concentrations of calcineurin inhibitors CsA and tacrolimus and the mTOR inhibitor rapamycin enhance lipolysis, increase the phosphorylation of hormone-sensitive lipase (HSL) and inhibit lipid storage and expression of lipogenic genes in human adipose tissue. This may contribute to the development of dyslipidemia, insulin resistance and new-onset diabetes (NODAT) in patients treated with these immunosuppressive agents.

Disclosure statement:

JWE is employed by AstraZeneca R&D. All other authors have nothing to disclose.

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Table 1 – Clinical and biochemical characteristics of the adipose tissue donors (n = 60).

Variables			
Sex (male/female; n)	26/34		
Age (years)	48	±	14
Body mass index (kg/m^2)	27.2	±	4.1
Waist-hip ratio (WHR)	0.91	±	0.1
Systolic blood pressure (mmHg)	133	±	17
Diastolic blood pressure (mmHg)	83	±	11
Subcutaneous adipocyte diameter (µm) [a]	99.5	±	12.6
Omental adipocyte diameter (µm)[b]	86.0	±	16.3
HbA_{1c} (mmol/mol, IFCC) [c]	30	±	10
Glucose (mmol/L)	5.2	±	0.6
Insulin (mU/L)	9.2	±	5.7
HOMA-IR [d]	1.9	±	1.1
Body fat mass (%)	30.3	±	7.6
Triglycerides (mmol/L)	1.2	±	0.6
Cholesterol (mmol/L)	5.4	±	1.2
LDL-cholesterol (mmol/L)	3.4	±	1.0
HDL-cholesterol (mmol/L)	1.7	±	0.6

Data are means ± SD; [a] n=47; [b] n=18; [c] Normal range 27-46 mmol/mol (IFCC standard); [d] Calculated as: fasting insulin (mU/L) x fasting glucose (mM)/22.5 (Matthews et al., 1985); HbA_{1c}, glycosylated haemoglobin; LDL, low-density lipoprotein; HDL, high density lipoprotein

Table 2 – Summary of results

	CsA	*FK*	*Rap*
Adipocytes (sc)			
Lipolysis			
Basal	-	-	↑
Isoproterenol	↑	↑	↑
Lipid storage	↓	↓	↓
Lipolytic proteins			
p-HSL	↑	↑	↑
Adipose tissue (sc/om)			
Lipolytic protein			
Perilipin	-	-	↓
Lipolytic gene			
Perilipin	-	-	↓
Lipogenic genes			
SREBP1	-	-	↓
Lipin 1	-	-	↓
LPL	-	-	↑
CD36	-	↓	-
aP2	-	↓	↓
Adipokine			
IL-6	↑	↑	↑

CsA, cyclosporin A; FK, tacrolimus; Rap, rapamycin; sc, subcutaneous; om, omental. Increase (↑) and decrease (↓).

Figures

Figure 1

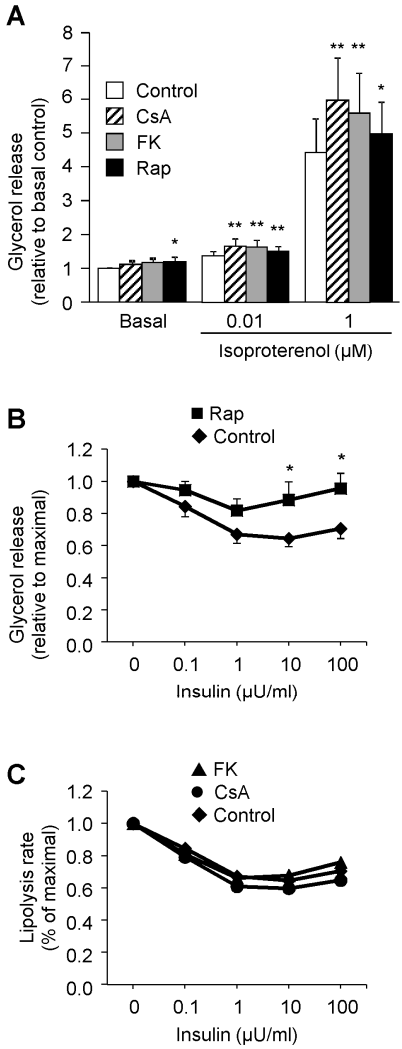


Figure 1. Effects of CsA, tacrolimus (FK) and rapamycin (Rap) on lipolysis and on the antilipolytic effect of insulin in human subcutaneous adipocytes. Adipocytes were incubated in absence (Control) or presence of CsA (0.1 μM), tacrolimus (0.1 μM) or rapamycin (0.01 μM). (A) For lipolysis experiments the medium was supplemented or not with isoproterenol (0.01 or 1 μM) and the amount of glycerol released into the medium, after 2 h incubation, was taken as an index of lipolysis. Lipolysis rate for each condition (n=13-16) was calculated relative to basal control in each experiment (overall mean glycerol release in basal condition: 2.0 ± 0.4 nmol/10^5

cells/h) and are presented as mean ± SEM. For the effects of rapamycin (B) and CsA and tacrolimus (B) on the antilipolytic effect of insulin (n=5-6), the medium was supplemented with isoproterenol (1 μM) and with the indicated insulin concentrations (0-100 μU/ml insulin) and the amount of glycerol released into the medium, after 2 hours of incubation, was taken as an index of lipolysis. Results are expressed as relative to maximal glycerol release (isoproterenol alone: 7.5 ± 0.4 nmol/10^5 cells/h), set to 1, and presented as mean ± SEM. ∗p<0.05 and ⁕ p<0.01 control vs. treated (CsA, tacrolimus or rapamycin) with otherwise identical conditions. Reducing the basal from isoproterenol-stimulated lipolysis, the changes are only significant (p<0.05) for isoproterenol (1 μM) stimulated conditions (A).

Figure 2

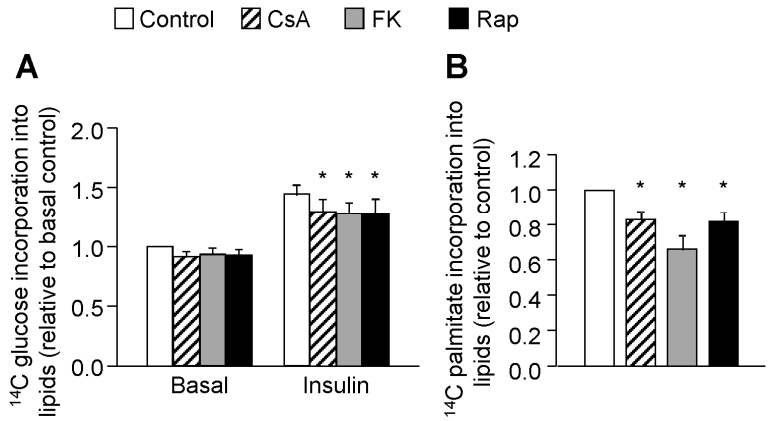


Figure 2. CsA, tacrolimus (FK) and rapamycin (Rap) inhibits lipid storage in human subcutaneous adipocytes. Adipocytes were incubated in absence (Control) or presence of CsA (0.1 μM), tacrolimus (0.1 μM) or rapamycin (0.01 μM) and the medium was supplemented with D-[U-^{14}C] glucose and with or without insulin (1000 μU/ml) (A) or with ^{14}C-palmitate (B) during 2 h. ^{14}C-glucose (lipogenesis) or ^{14}C-palmitate incorporation into lipids were taken as a measure for lipid storage, and calculated relative to basal control in each condition (^{14}C-glucose uptake overall basal: 0.70 ± 0.14 fl/cell/s, n=8; and ^{14}C-palmitate uptake overall basal: 92.0 ± 17,3 fl/cell/s, n=5) and are presented as mean ± SEM. ∗p<0.05 control vs. treated (CsA, tacrolimus or rapamycin) with otherwise identical conditions. Reducing the basal from the insulin-stimulated lipogenesis, the changes are not significant.

Figure 3

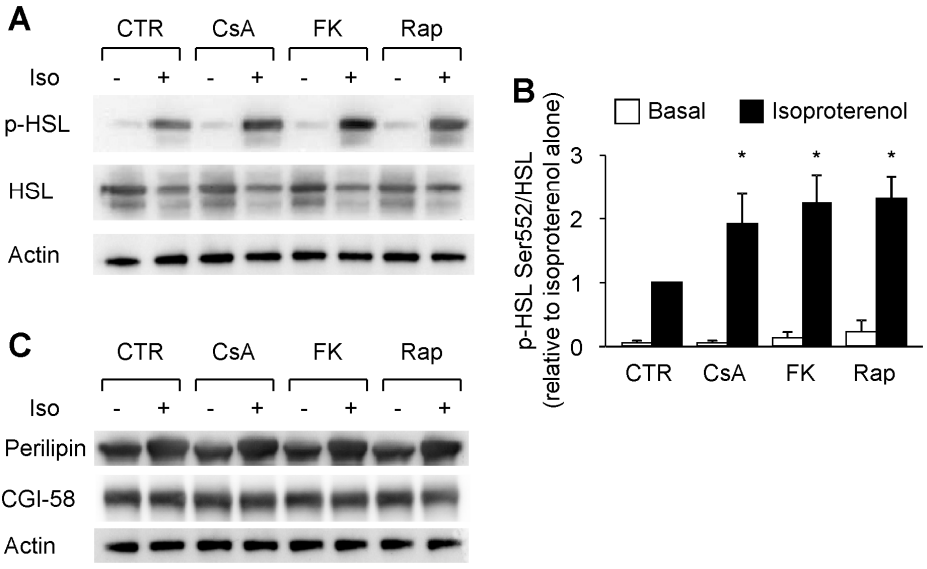


Figure 3. Cyclosporine (CsA), tacrolimus (FK) and rapamycin (Rap) increase HSL phosphorylation in human subcutaneous adipocytes. Isolated human subcutaneous adipocytes were incubated without (CTR) or with CsA (0.1μM), tacrolimus (0.1 μM) or with rapamycin (0.01 μM) for 15 min and thereafter stimulated with or without isoproterenol (1 μM) for an additional 60 min. Fat cell lysates were analysed by immunoblotting for the HSL protein and phospho-HSL (Ser552) (A, B), and perilipin and CGI-58 protein levels (C). CsA, tacrolimus and rapamycin increased isoproterenol-stimulated phosphorylation of Ser552 of HSL (A). The intensity of the phosphorylation of Ser552 of HSL after CsA, tacrolimus and rapamycin treatment was quantified, and the results are represented as mean ± SEM compared to isoproterenol alone (set to 1) and normalised to the respective protein levels (p-HSL/HSL) (B). Actin was used as the loading control protein. n = 5-6, *p<0.05, compared to isoproterenol alone.

Figure 4

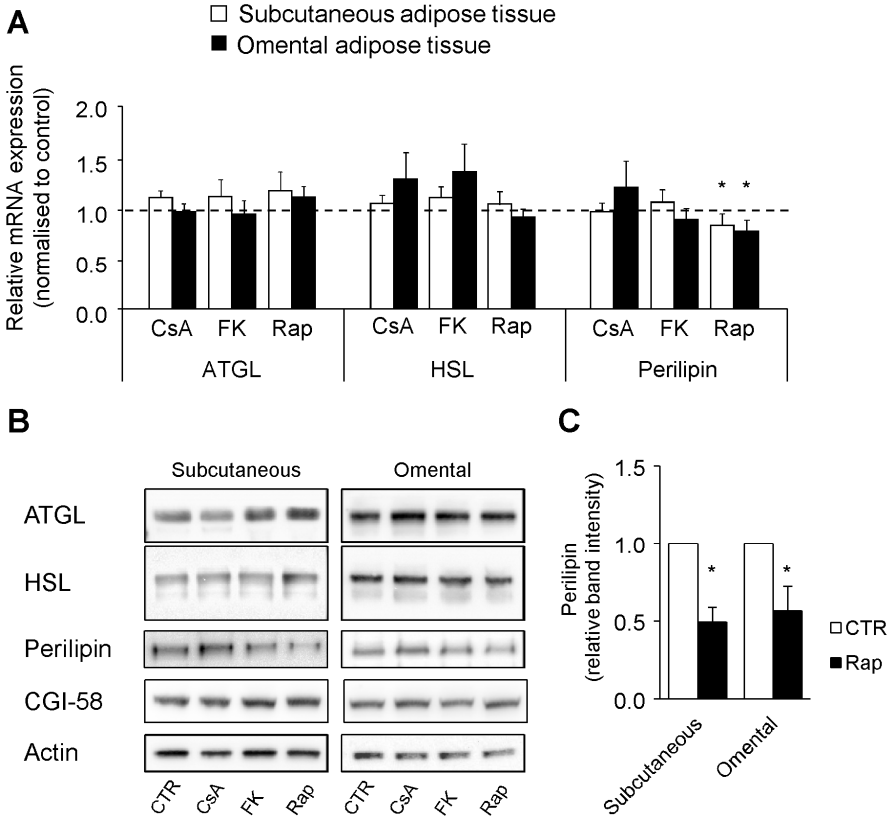


Figure 4. Regulation of gene expression and/or protein amount of adipose triacylglycerol lipase (ATGL), hormone-sensitive lipase (HSL), perilipin and CGI-58 in human subcutaneous and omental adipose tissue by CsA, tacrolimus (FK) and rapamycin (Rap). Subcutaneous and omental adipose tissue, were incubated for 20 h without (CTR) or with CsA (0.1 μM), tacrolimus (0.1 μM) or rapamycin (0.01 μM), and mRNA (n = 21 and 17, respectively) was extracted to measure gene expression for ATGL, HSL and perilipin (A); and total lysates (n = 4-5) were analysed by Western blotting for ATGL, HSL, perilipin, CGI-58 and actin (B). The relative band intensity for perilipin was quantified in subcutaneous and omental adipose tissue incubated without (control; CTR) or with rapamycin and represented as mean ± SEM compared to control (set to 1) (C). mRNA expression data are presented as mean ± SEM, and expressed as mRNA relative expression normalised to control (no-treatment). Rapamycin decreased perilipin gene expression and protein amount (A-C) in both subcutaneous and omental adipose tissue, compared to control. $*p<0.05$ compared with control.

Figure 5

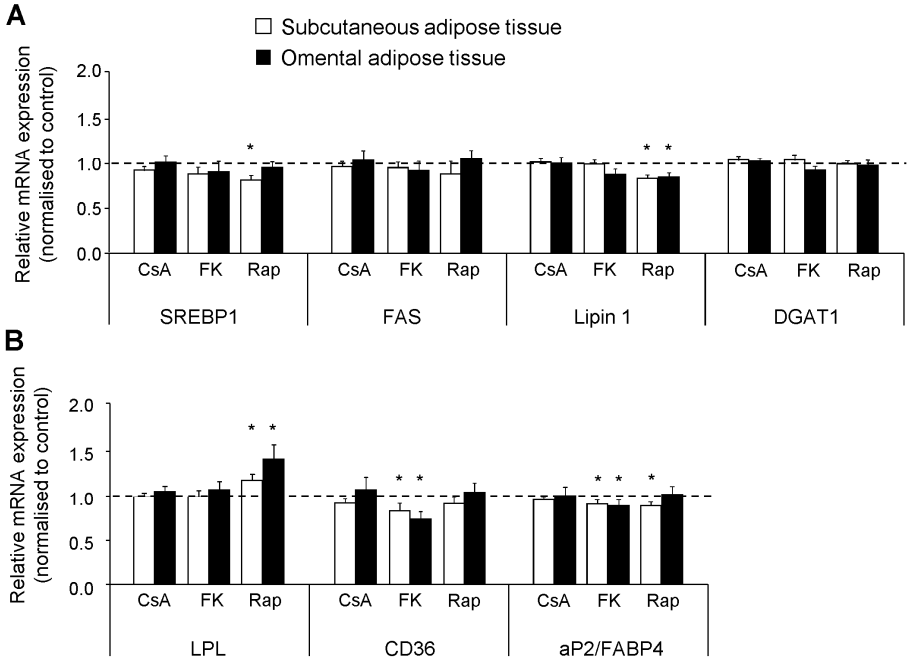


Figure 5. Regulation of expression of lipogenic genes in human subcutaneous and omental adipose tissue by cyclosporine A (CsA), tacrolimus (FK) and rapamycin (Rap). Subcutaneous and omental adipose tissue (n = 21 and n = 17, respectively), were incubated for 20 h without or with CsA (0.1 μM), tacrolimus (0.1 μM) or rapamycin (0.01 μM), and mRNA was extracted to measure mRNA expression for sterol regulatory element-binding protein 1 (SREBP1), fatty acid synthase (FAS), lipin 1 and diglyceride acyltransferase 1 (DGAT1) (A) and lipoprotein lipase (LPL), CD36 and fatty acid binding protein 4 (aP2/FABP4) (B). Data are presented as mean ± SEM, and expressed as mRNA relative expression normalised to control (no-treatment). $*p<0.05$ compared with control.

Figure 6.

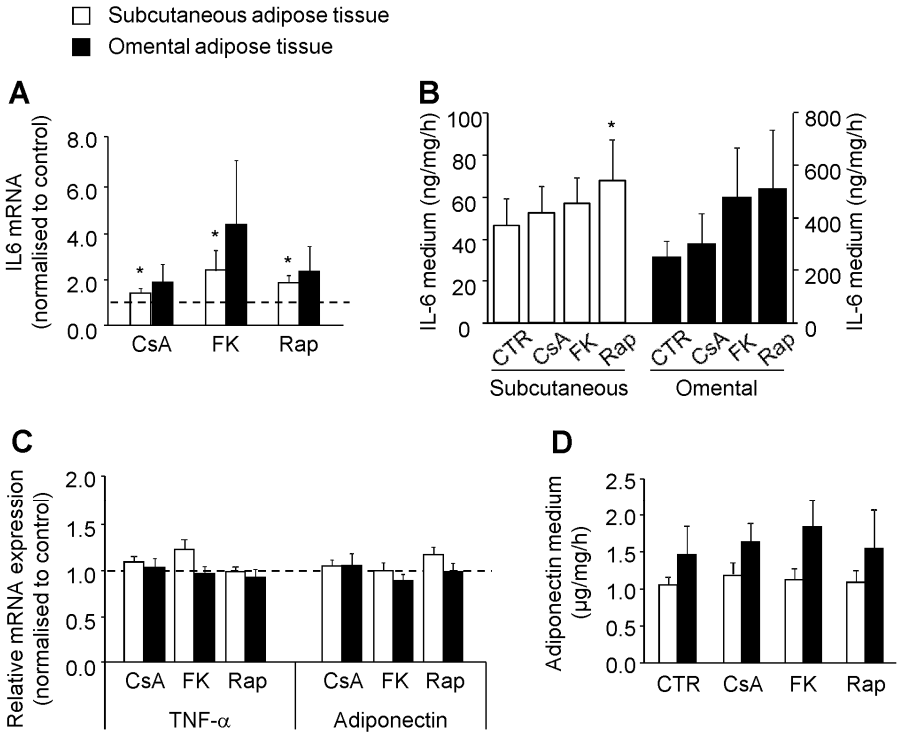


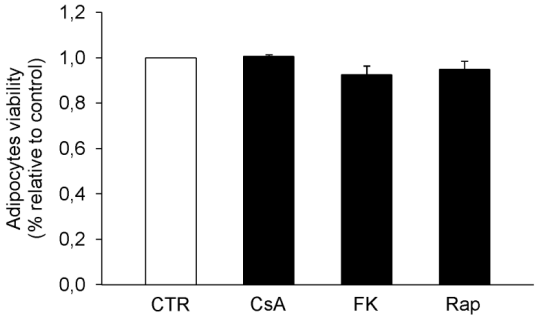
Figure 6. Regulation of IL-6, TNF-α and adiponectin gene expression and IL-6 and adiponectin secretion in human subcutaneous and omental adipose tissue by cyclosporine A (CsA), tacrolimus (FK) and rapamycin (Rap).

Subcutaneous and omental adipose tissue, were incubated for 20 h without or with CsA (0.1 µM), tacrolimus (0.1 µM) or rapamycin (0.01 µM), and mRNA was extracted to measure mRNA expression for IL-6 (A), TNF-α (C) and adiponectin (C). The IL-6 (B) and adiponectin (D) concentration in the culture medium was determined by enzyme-linked immunoassay.

Data for gene expression (n = 21 and n = 17, for subcutaneous and omental adipose tissue, respectively) are presented as mean ± SEM, and expressed as mRNA relative expression normalised to control (CTR; no treatment). Data for IL-6 (n = 17 and n = 6, for subcutaneous and omental adipose tissue, respectively) and adiponectin (n = 16 and n = 4, for subcutaneous and omental adipose tissue, respectively) concentrations in the medium are mean ± SEM and calculated as ng/mg/h and µg/mg/h, respectively. Data for IL-6 concentration in the media from subcutaneous and omental adipose tissue have different scales (y-axis). $*p<0.05$ compared with control.

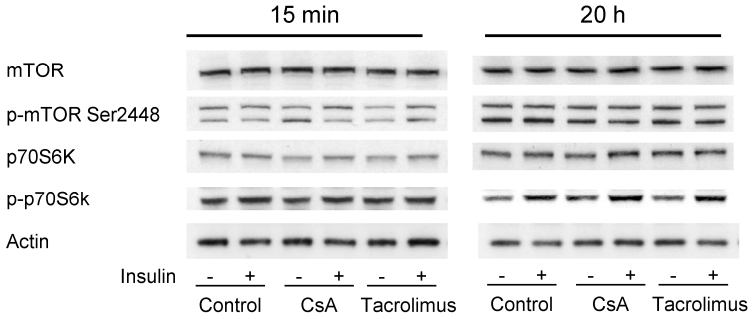
Supplement

Supplementary Figure 1



Legend - Incubation of adipocytes with CsA, tacrolimus and rapamycin during 24 h did not change cell viability. The effect of CsA, tacrolimus or rapamycin on adipocyte viability was assessed by using the water-soluble tetrazolium-1 colorimetric assay (WST-1, Roche, Mannheim, Germany). Adipocytes were placed in a 48 well plate at a lipocrit of 5% in Hank's medium (5.6 mM glucose, 4% BSA, 0.15 µM adenosine and pH 7.4) with or without CsA (0.1 µM), tacrolimus (0.1 µM) and rapamycin (0.01 µM). After 24 h, WST-1 was added to each well, and the cells were incubated for 2 h at 37ºC before absorbance was measured at 450 nm.

Supplementary Figure 2



Legend - CsA and tacrolimus do not alter protein levels or phosphorylation of mTOR and p70S6K.

Freshly isolated human subcutaneous adipocytes were treated for 15 min or 20 h without (control) or with CsA (0.1 µM) or tacrolimus (0.1 µM) and thereafter incubated with or without insulin (6 nM) for an additional 15 min. Immunoblotting analyses showed no effects of CsA or tacrolimus on protein levels or phosphorylation of mTOR and p70S6K. Actin was used as loading control. Representative blot is shown from one subject out of four. The average insulin effect on p70S6K phosphorylation was ~40 ± 20% in control adipocytes.

IV

Effects of dexamethasone on gene expression in human subcutaneous and omental adipose tissue – is FKBP5 a novel link between insulin resistance and immune modulation?

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Abbreviated title: Dexamethasone regulates FKBP5 in fat tissue

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ABBREVIATIONS:

CNR1	Cannabinoid receptor 1
FKBP5	FK506-binding protein 5
FKBP51	51 kDa FK506-binding protein 5
Hsp90	Heat shock protein 90
PEST	Penicillin-streptomycin
SERTM1	Serine-rich and transmembrane domain containing 1
TIMP4	Metallopeptidase inhibitor 4

ABSTRACT

Context: Glucocorticoid excess is linked to central obesity, adipose tissue insulin resistance and type 2 diabetes mellitus.

Objective: To study the effects of dexamethasone on gene expression in human subcutaneous and omental adipose tissue, in order to identify potential novel mechanisms and biomarkers for adipose tissue insulin resistance caused by glucocorticoids.

Design and Setting: Human subcutaneous and omental adipose tissue was incubated with dexamethasone (0.003-3 μM) for 24 h and glucose uptake, gene expression (microarray and real time-PCR) and levels of key proteins were assessed.

Participants: 25 non-diabetic adipose tissue donors (10 M/15 F; age 28-60 yrs; BMI 20.7-30.6 kg/m^2).

Results: Dexamethasone changed the expression of 527 genes in both subcutaneous and omental adipose tissue. *FKBP5* and *CNR1* were the most responsive genes in both depots (~7-fold increase). Dexamethasone increased *FKBP5* gene and protein expression in a dose-dependent manner in both depots, but *FKBP5* protein levels were 10-fold higher in omental that in subcutaneous adipose tissue. *FKBP5* gene expression in subcutaneous adipose tissue was positively correlated with serum insulin, HOMA-IR and subcutaneous adipocyte diameter, while fold change in gene expression exerted by dexamethasone was negatively correlated with HbA1c, BMI, HOMA-IR and serum insulin. Only one gene was identified, namely *SERTM1*, that clearly differed in response to dexamethasone between the two depots.

Conclusions: Dexamethasone at high concentrations, influences gene expression similarly in subcutaneous and omental adipose tissue and promotes gene expression of *FKBP5,* a gene that may be implicated in glucocorticoid-induced insulin resistance.

Key terms: Glucocorticoids; Adipose tissue; FKBP5; Gene expression; Insulin resistance, Human

INTRODUCTION

Cortisol, the active endogenous glucocorticoid secreted by the adrenal cortex, is a powerful insulin-antagonistic hormone (1). Excess cortisol promotes the development of insulin resistance, hyperglycaemia and features of the metabolic syndrome (2, 3). Cortisol opposes insulin's effects to stimulate peripheral glucose utilization and to suppress hepatic glucose output and can also impair insulin secretion from pancreatic β-cells. This is mediated mainly via effects on gene transcription (4, 5). Furthermore, elevated plasma cortisol, as in Cushing's syndrome or during prolonged corticosteroid therapy, is associated with redistribution of fat from peripheral to central depots and other components of the metabolic syndrome, such as hypertension and dyslipidemia (6, 7). Central obesity, in particular visceral adiposity, is associated with insulin resistance, cardiovascular disease and the metabolic syndrome (8, 9). Consequently, it is of interest to further explore the response of adipose tissue to cortisol.

Some adipose-secreted molecules are differentially regulated by glucocorticoids in different fat depots. For example, adipose tissue lipoprotein lipase production is stimulated to a higher extent in visceral fat, thus partitioning free fatty acids delivery to that depot (10). In contrast, in subcutaneous adipose tissue glucocorticoids induce lipolysis (11), possibly resulting in increase release of free-fatty acids and reduced lipid storage. This may partly explain the ability of glucocorticoid excess to promote intra-abdominal adiposity. Moreover, the glucocorticoid effect to stimulate leptin secretion was reported to be more pronounced in visceral as compared with subcutaneous fat (12, 13), and the effect to impair glucose uptake appears to be more prominent in visceral fat (14). Accordingly, a down regulation of the critical signalling proteins insulin receptor substrate-1 and protein kinase B has been demonstrated in visceral fat (14, 15). It is also of interest that the glucocorticoid-activating enzyme 11β-hydroxysteroid dehydrogenase 1, that converts inactive cortisone to active cortisol within tissues, displays a higher expression in visceral than in subcutaneous adipose tissue, and this might be of relevance in the pathogenesis of visceral adiposity and insulin resistance (16). However, the molecular mechanisms for the underlying differential regulation of subcutaneous and visceral adipose tissue by glucocorticoids are still unknown.

Recently, key metabolic pathways were identified to be regulated by dexamethasone incubation in human subcutaneous and omental adipose tissue from severely obese individuals (17) and in human skeletal muscle and subcutaneous adipose tissue after short-term dexamethasone treatment

(18). Identification of biomarkers and possible new pharmacological targets would be valuable in the prevention and treatment of glucocorticoid-induced metabolic dysregulation and could potentially also be of relevance for other metabolic conditions.

The aim of this study was to explore effects of dexamethasone on gene expression in subcutaneous and omental adipose tissue, in order to identify potential novel mechanisms and biomarkers for adipose tissue insulin resistance caused by glucocorticoids.

SUBJECTS AND METHODS

Subjects

Paired biopsies from human abdominal subcutaneous and omental adipose tissue were obtained from subjects going through elective surgery (n=25, 10 men, 15 women), mainly kidney donation. Adipose tissue was used to study the effects of dexamethasone on glucose uptake, gene expression and protein levels. Anthropometric measurements were obtained from all subjects and fasting blood samples were collected for analysis of glucose, insulin and lipids by routine methods at the Department of Clinical Chemistry, Sahlgrenska University Hospital. Clinical and biochemical characteristics of subjects are shown in table 1.

For additional analyses of the effect of the immunosuppressive agents tacrolimus (also known as FK506) and rapamycin (also known as sirolimus) on *FKBP5* (FK506-binding protein 5) gene expression in human subcutaneous and omental adipose tissue, biopsies (n=22, 5 men, 17 women) were obtained either from the lower part of the abdomen after dermal local anaesthesia with lidocaine (subcutaneous, n=7, Xylocain; AstraZeneca, Södertälje, Sweden), or by elective abdominal surgery after induction of general anaesthesia (subcutaneous and/or omental, n=15). The clinical and biochemical characteristics of these subjects are shown in supplemental table 1.

Subjects with type 2 diabetes, hypertension, endocrine disorders, cancer or other major illnesses as well as ongoing medication with systemic glucocorticoids and immune modulating therapies were excluded from this study. The study was approved by the Regional Ethics Review Board in Gothenburg. All participants gave their written informed consent.

Adipose tissue incubations

Dexamethasone

Abdominal subcutaneous and omental adipose tissue obtained from surgery was cut into small pieces, approximately 5-10 mg (1-2 mm^3) and was incubated in DMEM containing 6 mM

glucose (Sigma Chemical Co., St. Louise, MO, USA), 10% BSA (Sigma) and 1% penicillin-streptomycin (PEST, Invitrogen Corporation, Paisley, UK) in the absence or presence of dexamethasone (0.003-3 µM, Sigma) for 24 h in 37C, 5% CO_2. Adipose tissue was thereafter snap-frozen for gene expression (n=25) and protein (n=10) analysis or treated with collagenase for adipocyte isolation and glucose uptake (n=12). Adipocytes were also used for measuring cell size as previously described (19). Due to limited amount of tissue, not all experiments were done in every subject's adipose samples.

After adipose tissue incubation in the absence or presence of dexamethasone (0.01-3 µM; unless otherwise indicated 3 µM as a maximally effective concentration), adipocytes were isolated and glucose uptake was performed with or without insulin (1000 µU/ml), as previously described (20). Moreover, immunoblot analysis of FKBP51 was performed. See Supplemental Methods for details.

Tacrolimus and rapamycin

Subcutaneous and omental adipose tissue (n=18 and 13, respectively) was incubated for 20 h in absence or presence of either tacrolimus (0.1 µM, Sigma) or rapamycin (0.01 µM, Sigma), as previously described for dexamethasone.

Gene expression

RNA preparation

Total RNA was prepared from subcutaneous and omental adipose tissue using the Lipid Tissue Kit (Qiagen, Hilden, Germany). The RNA was used for microarray analysis as well as real-time PCR analysis.

Microarray Analysis

Gene expression in subcutaneous and omental adipose tissue was assessed by mRNA measurement using microarray analysis (Affymetrix, Human Exon 1.0 ST Array, Santa Clara, CA, USA; n=4) in accordance with the guidelines detailed by the manufacturer. Background adjustment, quantile normalization and summarization were computed in the Array Studio software (Omicsoft Corporation) to generate RMA (Robust Multichip Average, (21)) expression values. The core set of transcripts (exon-level probe sets that map to BLAT alignments of mRNA with annotated full-length CDS regions) were summarized using the mean value of all probe sets. See Supplemental Methods for further details.

To determine the tissue distribution of FKBP5 expression, microarray expression profiles (Human U133 plus 2.0 DNA microarray, Affymetrix, Santa Clara, CA) from skeletal muscle, omental and subcutaneous adipose tissue, kidney cortex, liver, heart ventricle and cerebral cortex were downloaded from the GEO database (data set GSE3526). FKBP5 expression was determined using the 224840_at probeset.

Real-time PCR

Real-time PCR was used for validation of microarray results. RNA samples of an additional 21 subjects and 2 of the subjects used for microarray analysis were used. Reagents for real-time PCR analysis were purchased from Applied Biosystems (Foster City, CA) and used according to the manufacturer's protocol. cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase kit and used for real-time PCR amplification and detection by the ABI Prism 7700HT Sequence Detection System (Applied Biosystems) using default parameters. A standard curve for each primer-probe set of pooled adipose tissue cDNA was used. The gene expression levels were normalized to the housekeeping gene 18S rRNA (Applied Biosystems).

Statistical Analysis

All data are presented as mean ± SEM unless stated otherwise. Comparisons between treated and untreated adipose tissue were performed pairwise within the same individual to minimize confounding variables. Wilcoxon signed-rank test, Kruskal-Wallis test or paired *t* test were used to analyse differences in gene expression, protein levels and glucose uptake between treated and untreated adipose tissue/adipocytes, as appropriate. Spearman's correlation test was used to assess correlations between *FKBP5* gene expression and effects of dexamethasone on *FKBP5* gene expression and metabolic variables (BMI, WHR, % body fat mass, adipocyte diameter, HbA1c, HOMA and serum insulin and lipids). Only significant associations are shown. Significant variables in the bivariate regression were subsequently included in a multivariate step-wise regression analysis. A P-value <0.05 was considered statistically significant. Statistical analyses were performed using the SPSS package version 18 (SPSS Inc., Chicago, IL).

RESULTS

Dexamethasone reduced the glucose uptake capacity in human subcutaneous and omental adipocytes

Dexamethasone (3 µM) decreased both basal (subcutaneous 30%; omental 41%) and maximal insulin stimulated (both depots ~20%; 1000 µU/ml) glucose uptake in subcutaneous (P<0.05; n=12) and omental adipocytes (P<0.01; n=12) (Fig. 1). Both basal and maximal insulin stimulated glucose uptake was significantly higher in omental compared to subcutaneous adipocytes (P<0.05). Dexamethasone (0.01-3 µM) had a dose-dependent effect to inhibit basal and maximal insulin-stimulated glucose uptake in both subcutaneous and omental adipocytes (n=6, data not shown). EC50 for dexamethasone to inhibit insulin-stimulated glucose uptake was about 5-fold higher in subcutaneous compared to omental adipocytes (P<0.05).

Effects of dexamethasone on gene expression

Microarray Analysis

In total, 527 genes, represented by 543 probe sets, were changed in dexamethasone-treated adipose tissues compared to non-treated tissues (false discovery rate of 0.05). Pathway analysis of the 527 dexamethasone-regulated genes showed a clear over-representation of functions and pathways related to immune/inflammatory responses (Supplemental Table 2). Single genes affecting lipolysis, glucose uptake and oxidation or adipocyte differentiation were changed after dexamethasone incubation. The two genes with the greatest increase in gene expression after dexamethasone incubation were *FKBP5* and cannabinoid receptor 1 (*CNR1*) with more than 7-fold higher expression compared to non-treated tissues (Fig. 2A and B and Supplemental Table 3). Dexamethasone also increased the expression of the secreted proteins leptin and *TIMP4* (metallopeptidase inhibitor 4) in both fat depots (Fig. 2C and D). No genes responded in opposite directions to dexamethasone treatment. However, *SERTM1* (serine-rich and transmembrane domain containing 1) displayed the most differential response to dexamethasone and was clearly down-regulated in omental adipose tissue, but unchanged in subcutaneous adipose tissue (Fig. 2E).

Real-time PCR

We used real-time PCR in a larger cohort (n=23) to verify effects of the following genes: *FKBP5* and *CNR1*, the two genes with highest increase in expression after dexamethasone incubation; leptin and *TIMP4*, that are possible to measure in blood; and *SERTM1* that was differently regulated by dexamethasone between the adipose tissue depots (Fig. 2A-E). The expression

pattern for all the genes analyzed by real-time PCR analysis very well matched the microarray analysis data, and the expression in both subcutaneous and omental adipose tissue were significantly different for all genes analyzed between control and dexamethasone incubated adipose tissue (P=0.011 to P<0.0001).

FKBP5 tissue distribution

To verify whether expression of FKBP5 may have potential implications for metabolism, its tissue distribution was determined in metabolically active tissues. FKBP5 is widely expressed in metabolically active tissues with the highest expression in muscle and adipose tissue (Supplemental Fig. 1).

Dexamethasone effects on *FKBP5* expression

To further investigate the effects of glucocorticoids on the expression of the *FKBP5* gene in human subcutaneous and omental adipose tissue, we assessed the concentration-response for dexamethasone effects on the expression of *FKBP5* gene and its protein product, FKBP51 (51 KDa FK506-binding protein 5), in both depots. Incubation of subcutaneous and omental adipose tissue with dexamethasone (0.003-0.3 μM) increased *FKBP5* gene and protein expression in a dose depended manner (Fig. 3A and B). The basal FKBP51 protein levels were ~10 fold higher in omental than in subcutaneous adipose tissue (P=0.005, Fig. 3C). Dexamethasone (3 μM) significantly increased FKBP51 protein levels in subcutaneous by ~4-fold (P=0.005), while in omental by ~2-fold (P=0.028) (Fig. 3C).

Effects of tacrolimus and rapamycin on *FKBP5* gene expression in human adipose tissue

To study whether *FKBP5* gene expression could be regulated by other immune-modulators drugs, effects of tacrolimus and rapamycin on *FKBP5* gene expression were explored. Incubation of subcutaneous and omental adipose tissue with rapamycin (0.01 μM) decreased *FKBP5* gene expression by 20-30% (P<0.01, Fig. 4), while tacrolimus had no effect.

Correlation between *FKBP5* gene expression and metabolic variables

FKBP5 gene expression correlated positively with insulin, HOMA-IR and cell diameter in subcutaneous but not in omental adipose tissue (Table 2). After entering serum insulin, HOMA-IR, and cell diameter in a step-wise multivariate analysis only HOMA-IR remained a significant predictor of *FKBP5* gene expression (r^2 = 0.31, P=0.01). Furthermore, the dexamethasone effects on *FKBP5* gene expression (calculated as delta change) correlated negatively with HbA1c, BMI, HOMA-IR and insulin in subcutaneous but not in omental adipose tissue. When included in a

step-wise multivariate analysis to predict the dexamethasone increase on *FKBP5* gene expression, all variables were significant (r^2 = 0.82, *P*<0.001 for model with HbA1C, *P*<0.001; HOMA, *P*<0.05 and BMI, *P*<0.05; and r^2 = 0.81, *P*<0.001 for model with HbA1C, *P*<0.001; insulin, *P*<0.05 and BMI, *P*<0.05). Interestingly, effects of dexamethasone on *FKBP5* gene expression did not correlate with the dexamethasone-induced inhibition of adipocyte glucose uptake of the fat depots.

DISCUSSION

Dexamethasone changed the expression of 527 genes in both subcutaneous and omental adipose tissue at a supra-physiological and maximally effective concentration (3 μM), according to microarray analysis. Pathway analysis showed a clear over-representation of functions and pathways related to inflammation. Single genes affecting lipolysis, glucose uptake and oxidation or adipocyte differentiation were changed after dexamethasone incubation. *FKBP5* was identified for the first time as one of the genes with the highest increase in gene expression after dexamethasone incubation in subcutaneous and omental adipose tissue. Moreover, *FKBP5* gene expression in subcutaneous adipose tissue appears to be positively correlated to biomarkers of insulin resistance, and its basal protein levels were higher in omental than in subcutaneous adipose tissue.

These results are in accordance to a previous microarray study (17), that reported that dexamethasone changed the gene expression of 535 genes in both subcutaneous and omental depots and that most genes were commonly regulated by dexamethasone in both depots.
Dexamethasone reduced both basal and insulin stimulated glucose uptake capacity in omental adipocytes, which was in line with previous data (14). In contrast to previous data (14), dexamethasone also reduced glucose uptake in subcutaneous adipocytes. However, omental adipocytes had lower EC50 compared to subcutaneous, supporting greater sensitivity to the dexamethasone effects.

The concentration of dexamethasone used in this study (3 μM) was at supra-physiological level in relation to the normal cortisol range (140-700 nM), since dexamethasone potency has been reported to be nearly 5 times higher than cortisol, and EC50 was 4.8 nM for dexamethasone and 24 nM for cortisol when assessed as effects on β-adrenergic receptor expression (22).

The *FKBP5* gene was first characterized as coding for a protein product, denoted 51 KDa FK506-binding protein 5 (FKBP51), which is a member of a family of immunophilins with peptidyl-propyl cis-trans isomerise activity (23). The designation of immunophilin refers to the capacity to bind two immunosuppressant drugs, tacrolimus (also known as FK506) and rapamycin (24). The immunosuppressive properties of tacrolimus and rapamycin arise from the formation of the immunophilin-immunosuppressant complex that inhibits two distinct T cell activation signalling pathways, T cell activation and T cell proliferation, respectively (25, 26). In addition to the capacity to bind to immunosuppressant drugs, FKBP51 was shown to inhibit glucocorticoid action (27, 28). FKBP51 has co-chaperone activity by participating in the heat shock protein 90 (Hsp90)-steroid receptor complex and regulating the glucocorticoid receptor activity (28). During maturation of the glucocorticoid receptor, FKBP51 binds to Hsp90, and the receptor complex has lower affinity for cortisol (27). Upon hormone binding FKBP51 is replaced by FK506-binding protein 4, which recruits dynein into the complex, allowing its nuclear translocation and transcriptional activity (27, 29). In addition to *FKBP5* functions through molecular interactions with receptors or proteins, no other mechanism of immunomodulation is known.

Induction of *FKBP5* by glucocorticoids has been shown in several studies (28, 30, 31), but to the best of our knowledge this is the first study to show that *FKBP5* gene expression and protein levels are directly regulated by dexamethasone in both subcutaneous and omental adipose tissue. In contrast to the dexamethasone effect, we show that rapamycin modestly inhibited *FKBP5* gene expression in adipose tissue whereas tacrolimus had no effect. Thus, the glucocorticoid up-regulation of *FKBP5* gene expression in adipose tissue is not a general effect related to immunosuppressive drugs.

Furthermore, *FKBP5* gene expression was similar in both subcutaneous and omental depots, but omental adipose tissue had a larger increase in FKBP51 protein levels compared with subcutaneous. This may suggest that *FKBP5* has different post-translational regulation, e.g. involving protein stability, in subcutaneous and omental adipose tissue, respectively. Since FKBP51 appears to inhibit nuclear translocation of the glucocorticoid-receptor following ligand-binding (27, 29), excess of FKBP51 might contribute to a desensitization for glucocorticoids. It is interesting that obese individuals with elevated insulin resistance are less sensitive to the effects of dexamethasone on *FKBP5*. It is conceivable that the lesser increase in *FKBP5* gene expression in adipose tissue in obese subjects, after incubation with dexamethasone, may be due to an already pre-existing high level of *FKBP5* on these tissues.

We also show that *FKBP5* gene expression in subcutaneous adipose tissue correlates positively with HOMA-IR, subcutaneous adipocyte diameter and serum insulin, which suggests that *FKBP5* expression, is linked to insulin resistance. FKBP51 has been reported to function as a negative regulator of the PKB pathway by acting as a scaffolding protein for PKB and its phosphatase, the PH domain leucine-rich repeat protein phosphatase (PHLPP) (32). Therefore, the FKBP51 up-regulation caused by glucocorticois, may have important implications on the negative regulation of the PKB pathway and thus contibute to desensitization of the insulin signalling.

Both the syndrome of glucocorticoid excess, i.e. Cushing's syndrome, and the metabolic syndrome are characterized by alterations in glucose and lipid metabolism, body fat accumulation and redistribution and immune function (6, 7). Obesity is associated with activation of inflammatory/immune system, including elevation in cytokine levels (e.g. interleukin-6, and tumor necrosis factor-α), that are known to be associated with insulin resistance (33). Thus, it would be of particular value to identify genes that are dysregulated in these two conditions and that may contribute to the perturbations described. Such studies might provide unique pharmacological targets and/or biomarkers for common metabolic disorders. Our present findings on regulation of *FKBP5* can be of importance in this context, since its expression in subcutaneous adipose tissue appears to be correlated to insulin resistance and adiposity and since it is also regulated by dexamethasone and the immunosuppressive drug rapamycin. This suggests that *FKBP5* can be involved in inflammation as well as in metabolic dysregulation, and *FKBP5* overexpression could possibly be a common mechanism linking these two phenomena, e.g. in obesity.

CNR1 was, together with *FKBP5*, the gene with the greatest increase in gene expression after dexamethasone incubation. *CNR1* is most abundantly expressed in the central nervous system, but it is also expressed in other tissues for example liver, muscle and adipose tissue. Activation of the central cannabinoid system promotes food intake and weight gain. The *CNR1* gene expression has previously been shown to be higher in visceral compared to subcutaneous adipose tissue (34). The *CNR1* expression has also been shown by some to be reduced with obesity in subcutaneous and visceral adipose tissue (34, 35), whereas others found an increased *CNR1* expression with obesity in subcutaneous and visceral adipose tissue (36) or did not find any association between *CNR1* expression and measures of body fat, metabolic parameters or fat cell function (37).
We show that leptin and *TIMP4* expression, two regulatory genes encoding for secreted factors, are increased after dexamethasone treatment of both depots. Both leptin expression in adipose

tissue and concentrations of leptin in serum has previously been shown to be increased by dexamethasone (13, 38). However, differences in leptin secretion in both sexes (38) and fat depots (12, 13) and the fact that serum level is tightly linked to BMI and fat mass, argues against leptin as a suitable biomarker to study dexamethasone responses. *TIMP4* has not previously been studied in the context of glucocorticoids and adipose tissue. *TIMP4* is an inhibitor of the matrix metalloproteinases and microarray based tissue distribution datasets (GEO database GSE3526 data set) show that adipose tissue is a major site of *TIMP4* expression. This suggests that *TIMP4* is a putative dexamethasone-response biomarker that warrants further investigation.

One additional aim of this study was to search for depot differences in gene expression in response to dexamethasone treatment. Genes differentially regulated in the two adipose depots, may provide insights into accumulation of omental adipose tissue seen after glucocorticoid treatment. In this study, only *SERTM1* displayed a clearly different response to dexamethasone in the two fat depots. *SERTM1* is a gene with unknown function and therefore these findings provides no mechanistic insight into processes that are related to metabolic disease. Otherwise, we could not find any clear depot differences in the gene expression response to dexamethasone.
This study has several limitations. We report effects of dexamethasone in adipose tissue *in vitro*, which may not reflect measurements *in vivo*. We mainly used a very high concentration of dexamethasone, corresponding to supraphysiological glucocorticoid levels, and therefore we cannot exclude that some genes may respond differentially in the two depots with exposure to more physiological levels. We only studied 24 h of dexamethasone incubation, which does not identify genes that might be regulated only during shorter- or longer-term treatment. Thus, further studies could be of value in order to identify genes differentially regulated between the depots across a range of glucocorticoid concentrations and duration of treatment.

In conclusion, *FKBP5* is a novel gene regulated by dexamethasone in both subcutaneous and omental adipose tissue, and its expression in subcutaneous adipose tissue appears to be correlated to markers of insulin resistance and adiposity. However, the gene product was present at markedly higher levels in the omental than in the subcutaneous depot. *FKBP5* is a potential mechanism linking insulin resistance with alterations in immune function and inflammatory responses. Further studies should address whether FKBP5 can provide novel pharmacological targets for the treatment of insulin resistance in the context of glucocorticoid excess as well as in the metabolic syndrome and type 2 diabetes.

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DISCLOSURE STATEMENT

JWE and MH are employed by AstraZeneca R&D. All other authors have nothing to disclose.

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TABLES

Table 1. Clinical and biochemical characteristics of study participants, in whom the effects of dexamethasone on adipose tissue was evaluated (n=25).

Variable	Value
Sex (male/female; n)	10M/15F
Age (years)	45±10
BMI (kg/m^2)	27±3
WHR	0.95±0.05
Fat mass (%)	28±7
Subcutaneous adipocyte diameter (μm)	94.9±10.4
Omental adipocyte diameter (μm)	84.2±14.7
HbA_{1c}, IFCC (mmol/mol)	32±4
Serum insulin (mU/L)	10.2±6.9
Plasma glucose (mmol/L)	4.8±0.4
HOMA-IR	2.2±1.4
Serum triglycerides (mmol/L)	1.5±0.7
Serum total cholesterol (mmol/L)	5.4±0.9
Serum LDL-cholesterol (mmol/L)	3.4±0.7
Serum HDL- cholesterol (mmol/L)	1.4±0.3

Data are mean ± SD; n for each variable=20-25; BMI, body mass index; WHR, waist to hip ratio; HbA_{1c}, glycosylated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance index (fasting blood glucose × fasting insulin/22.5); LDL, low density lipoprotein; HDL, high density lipoprotein.

Table 2. Correlations between *FKBP5* gene expression in subcutaneous and omental adipose tissue (control and dexamethasone-induced increase) and metabolic parameters.

	FKBP5 mRNA (control)				**Δ - FKBP5 mRNA (dexa treated-control, %)**			
	Sc		**Om**		**Sc**		**Om**	
	r	***P***	**r**	***P***	***r***	***p***	***r***	***P***
Serum -insulin	**0.58**	**0.008**	0.06	0.813	**-0.67**	**0.001**	-0.21	0.384
HbA_{1c}	0.28	0.248	-0.26	0.277	**-0.60**	**0.007**	0.10	0.690
HOMA-IR	**0.59**	**0.006**	0.09	0.704	**-0.66**	**0.002**	-0.25	0.279
BMI	0.09	0.672	-0.03	0.911	**-0.41**	**0.050**	-0.17	0.449
Fat cell diameter	**0.48**	**0.020**	0.16	0.464	-0.30	0.165	-0.23	0.293
Δ - basal glucose uptake (dexa treated-control, %)	-	-	-	-	-0.22	0.533	0.25	0.489
Δ - insulin-stimulated glucose uptake (dexa treated-control, %)	-	-	-	-	0.30	0.405	0.37	0.293

Sc, subcutaneous; Om, omental; dexa, dexamethasone; HbA_{1c}, glycosylated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance index; BMI, body mass index. r-values are Spearman correlation coefficients.

FIGURES

Figure 1.

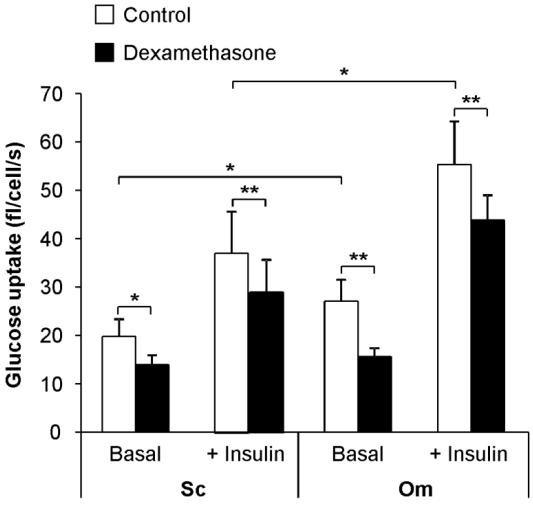


Figure 1. Effects of dexamethasone incubation on glucose uptake in human subcutaneous and omental adipocytes. Dexamethasone (3 μM) decreased both basal and maximally insulin (1000 μU/ml) stimulated glucose uptake in subcutaneous and omental adipocytes after 24 h incubation (n=12). Sc, subcutaneous; Om, omental. * $P<0.05$; ** $P<0.01$.

Figure 2.

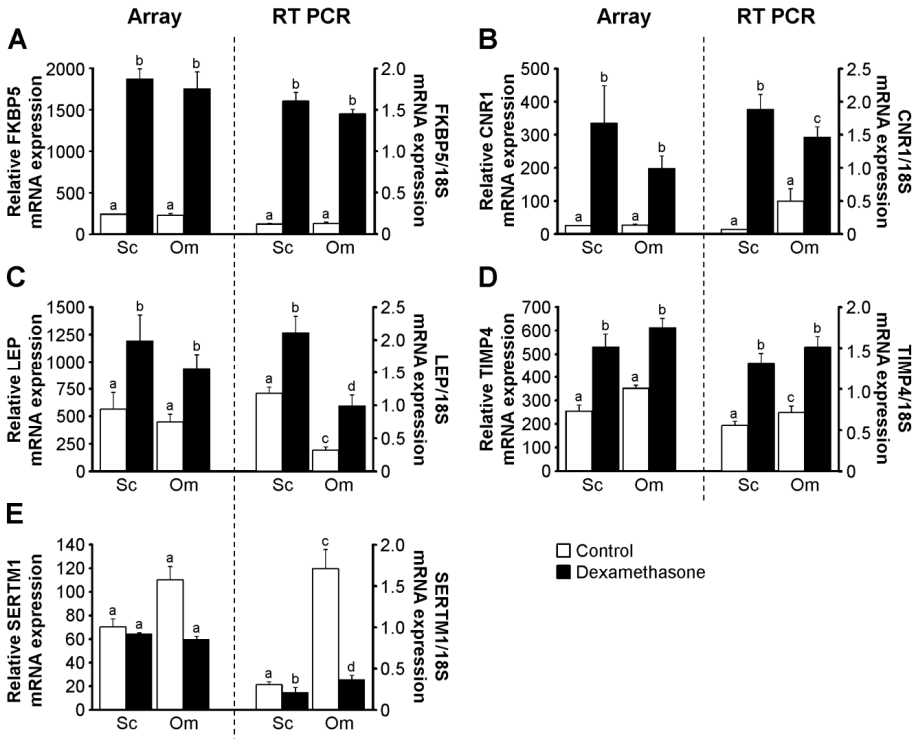


Figure 2. Gene expression in subcutaneous and omental adipose tissue after incubation in absence or presence of dexamethasone (3 μM). Array, microarray analysis (n=4); RT PCR, real-time polymerase chain reaction (n=20-23); FKBP5, FK binding protein 5; CNR1, cannabinoid receptor type 1; LEP, leptin; TIMP4, metalloproteinase inhibitor 4, SERTM1, serine-rich and transmembrane domain containing 1; Sc, subcutaneous; Om, omental. Means not sharing the same letter are significantly different from each other (P<0.05).

Figure 3.

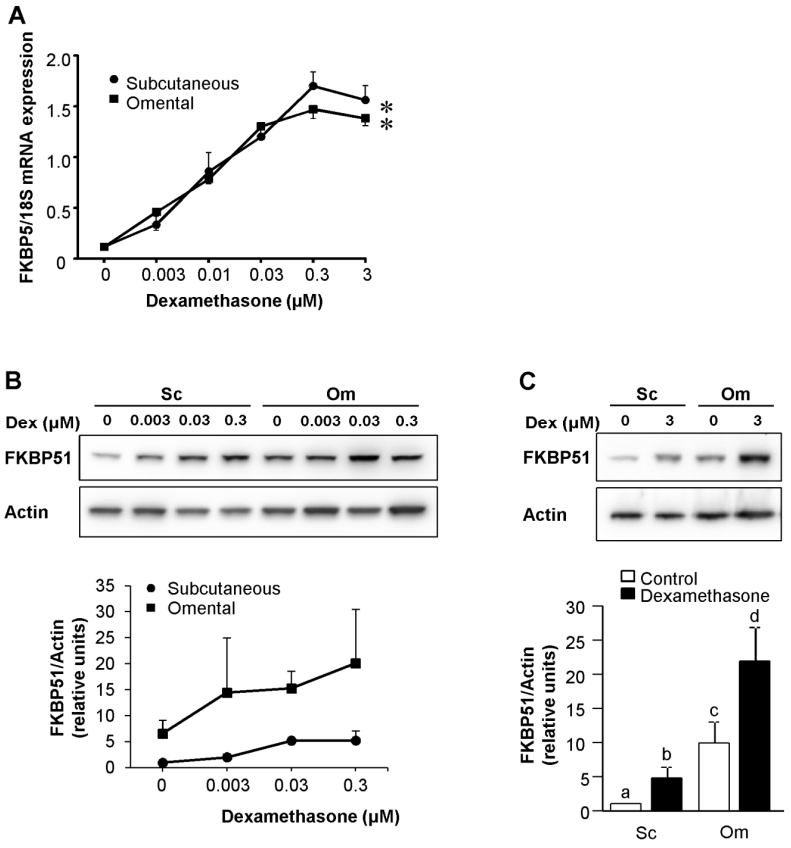


Figure 3. Dose-response curve for the effects of dexamethasone on FKBP5 gene and protein expression in subcutaneous and omental adipose tissue. (A) Dexamethasone (0.003-3 μM, n=3-10) increases in a dose dependent manner the FKBP5 gene expression in subcutaneous and omental adipose tissue vs. untreated cells (n=3-10), $P<0.05$. (B) Dexamethasone (0.003-0.3 μM) increases in a dose dependent manner the FKBP51 protein levels in subcutaneous and omental adipose tissue. Graph represents FKBP51 quantification data (n=3). (C) FKBP51 protein levels in subcutaneous and omental adipose tissue incubated without or with dexamethasone (3 μM, n=10), and quantification of protein levels. Actin was used as a loading control protein. Results show representative gels and mean ± SEM of densitometry analysis. Densitometry measures of FKBP51 were normalized for the respectively actin protein levels. Sc, subcutaneous; Om, omental. Means not sharing the same letter are significantly different from each other ($P<0.05$).

Figure 4.

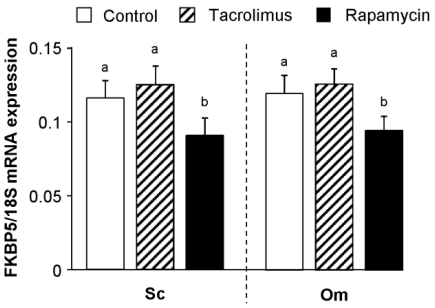


Figure 4. Effects of tacrolimus and rapamycin on FKBP5 gene expression in subcutaneous and omental adipose tissue after 20 h incubation. n= 18 and 13 for subcutaneous and omental adipose tissue, respectively. Sc, subcutaneous; Om, omental. Means not sharing the same letter are significantly different from each other (P<0.05).

Supplemental Methods

Glucose uptake

After collagenase type II digestion (from *Clostridium histolyticum*, Sigma) the adipocytes were washed four times with medium without glucose and pre-incubated for 15 minutes in absence and presence of human insulin (1000 µU/ml; Actrapid, Novo Nordisk A/S, Copenhagen, Denmark). Thereafter, D-[U-^{14}C]-glucose was added and the cells were incubated for an additional 45 minutes before the glucose uptake was terminated and the cell-associated radioactivity was measured by scintillation counting. The cellular clearance of glucose from the medium was taken as an index of the rate of glucose uptake and calculated according to the following formula: cellular clearance of medium glucose = (cell associated radioactivity x volume)/(radioactivity in medium x cell number x time).

Microarray Analysis

Array data was subjected to quality control assessments for GeneChip arrays and data integrity which indicated that all samples showed high quality profiles and passed data integrity controls.

Target was prepared for hybridization to Affymetrix GeneChip® Human Exon 1.0 ST arrays in accordance with the guidelines detailed by the manufacturers: Total RNA was amplified using the NuGEN™ WT-Ovation™ Pico RNA Amplification System. The NuGEN WT-Ovation™ Exon Module was used to generate sense-strand cDNA targets ready for fragmentation and labelling with NuGEN's FL-Ovation™ cDNA Biotin Module V2. The resultant fragmented and labelled cDNA was added to the hybridization cocktail in accordance with the NuGEN™ guidelines for hybridization onto Affymetrix GeneChip® arrays. Following the hybridization for 16-18 hours at 45°C in an Affymetrix GeneChip® Hybridization Oven 640, the arrays were washed and stained on the GeneChip® Fluidics Station 450 using the appropriate fluidics script, before being scanned using the GeneChip® Scanner 3000.

The General Linear Model implemented in Array Studio (Omicsoft Corporation) was used to compare the effects of dexamethasone treatment on subcutaneous and omental adipose tissue. The model included the factors Subject (random), Tissue (with levels subcutaneous

and omental adipose tissue), and Treatment (with levels control and dexamethasone). Pair wise comparisons were performed within the model and multiple test correction was applied using False Discovery Rate (FDR, Benjamini and Hochberg) on a per-test basis. Multiple test correction-adjusted p-values with an alpha level of 0.05 were used to filter for genes changed in any tissue in response to treatment. When each tissue was compared separately (controlling for Subject and Treatment) this proved overly stringent, therefore the data was filtered using an unadjusted p-value threshold.

Genes that were significantly changed in response to dexamethasone (controlling for tissue type, i.e. in the general linear model described in the supplement) were imported to the Ingenuity Pathway Analysis software (Ingenuity Systems).

Adipose tissue lysates and immunoblotting

Adipose tissue was homogenized in ice-cold lysate buffer (25 mM Tris-HCl pH 7.4, 0.5 mM EGTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine, 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochlorine and 0.1 µM okadaic acid) with the TissueLyserII (Qiagen). The homogenates were lysed at 4ºC for 2 h, before being centrifuged (12 000 *g*, 15 min, 4ºC), and the supernatant was collected and saved at -80ºC. Protein content was measured with the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Total lysate (20 µg/lane) were subjected to SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-FKBP51 (1:1000, Cell Signaling Technologies, Beverly, MA, USA) and anti-actin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and thereafter with secondary antibody linked to horseradish peroxidase (1:2000, Cell Signaling Technologies). Detection was made with chemiluminescence reagent (ECL, Amersham Biosciences GE Healthcare, Buckinghamshire, UK) using a ChemiDoc XRS detection system (Bio-Rad, Richmond, CA, USA). Bands were quantified using Quantity One Imaging software (version 4.6.6. from Bio-Rad).

SUPPLEMENTAL RESULTS

Microarray Analysis

Changes in gene expression among 18 686 well annotated transcripts of the "core" subset were investigated using Affymetrix GeneChip Exon 1.0 ST arrays.

147 probe sets representing approximately 144 genes differed in their expression levels in subcutaneous versus omental adipose tissue when controlling for treatment with dexamethasone and whiten subject (false discovery rate of 0.05). 121 probe sets (representing 118 genes) were expressed at higher levels and 26 genes at lower levels in omental compared to subcutaneous tissue. Something more, or just delete

SUPPLEMENTAL TABLES

Supplemental Table 1. Clinical and biochemical characteristics of study participants in whom the effect of the immunosuppressive agents, tacrolimus and rapamycin on adipose tissue was evaluated (n=22).

Variable	Value
Sex (male/female, n)	5M/17F
Age (years)	51±12
BMI (kg/m^2)	26±3
WHR	0.90±0.08
Fat mass (%)	30±8
Subcutaneous adipocyte diameter (sc)	97.3±11.6
Omental adipocyte diameter (om)[a]	87.3±14.2
HbA_{1c} (mmol/mol)[b]	34±3
Serum insulin (mU/L)	7.3±3.5
Plasma glucose (mmol/L)	5.1±0.6
HOMA-IR	1.7±0.8
Serum triglycerides (mmol/L)	1.1±0.5
Serum total cholesterol (mmol/L)	5.7±1.1
Serum LDL-cholesterol (mmol/L)	3.4±0.9
Serum HDL-cholesterol (mmol/L)	2.0±0.7

[a] n=11

[b] Normal range 27-46 mmol/mol (IFCC standard)

BMI, body mass index; WHR, waist to hip ratio; HbA_{1c}, glycosylated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance index (fasting blood glucose × fasting insulin/22.5) (39); LDL, low density lipoprotein; HDL, high density lipoprotein. Data are means ± SD.

Supplemental Table 2. List of the 20 genes with the greatest decrease in gene expression after dexamethasone incubation of subcutaneous and omental human adipose tissue as analyzed by microarray analysis (Affymetrix, n=4).

Subcutaneous	Fold change	Omental	Fold change
CCL8[1,2]	-19,05	CXCL10[1,2]	-22,76
CXCL10[1,2]	-18,51	CXCL11[1,2]	-19,45
CXCL11[1,2]	-17,35	CCL8[1,2]	-13,12
IFIT1[1,2]	-12,93	CXCL9[1,2]	-10,31
RSAD2[1,2]	-12,31	IL1B[1,2]	-8,59
IFIT2[1]	-11,65	IFIT3[1,2]	-7,72
IFI44L[1,2]	-9,11	LOC100288077 // MMP1[1]	-7,55
PTGS2[1,2]	-9,09	PTGS2[1,2]	-7,43
IFIT3[1,2]	-9,00	IFIT1[1,2]	-6,96
LOC100288077 // MMP1[1]	-8,30	RSAD2[1,2]	-6,84
IL13RA2	-8,10	IFIT2[1]	-6,43
IL1B[1,2]	-7,48	IFI44L[1,2]	-6,33
CCL3 // CCL3L1 // CCL3L3[2]	-6,72	SFRP4	-5,8
CXCL9[1,2]	-6,42	LOC100131190 // MX1 // MX2	-5,29
MX1	-6,41	GBP1 // GBP2 // GBP3 // LOC400759	-4,78
FST	-6,33	OAS1[1,2]	-4,76
IL6[1,2]	-6,12	IL6[1,2]	-4,58
OAS1[1,2]	-6,09	CCL7[2]	-4,35
TNIP3[2]	-5,94	INHBA	-4,35
SAMD9L	-5,91	OAS2[2]	-4,31

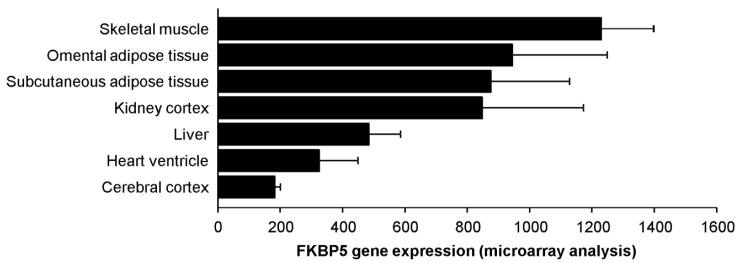
[1] Common genes in both subcutaneous and omental adipose tissue. [2] Genes known to be involved in immune/inflammatory pathways.

Supplemental Table 3. List of the 20 genes with the greatest increase in gene expression after dexamethasone incubation of subcutaneous and omental human adipose tissue as analyzed by microarray analysis (Affymetrix, n=4).

Subcutaneous	Fold change	Omental	Fold change
CNR1[1]	10.82	FKBP5 // LOC285847[1]	7.69
FKBP5 // LOC285847[1]	7.76	CNR1[1]	6.95
PPP1R3C[1]	6.21	POSTN	4.39
F8[1]	4.42	FBLN5[1]	3.88
FBLN5[1]	4.25	LAMA2[1]	3.75
PDK4[1]	4.25	STEAP4[1]	3.76
LAMA2[1]	3.97	PPP1R3C[1]	3.51
CIDEC // CIDECP[1]	3.88	MS4A4A[1]	3.35
HSPB7[1]	3.82	F8[1]	3.31
KIAA0040	3.80	HSPB7[1]	3.18
VSIG4[1]	3.78	CPM[1]	3.14
CYP4B1	3.63	ZBTB16[1]	3.12
CD163[1]	3.58	CD163[1]	3.11
CPM[1]	3.50	SRPX	3.09
MS4A4A[1]	3.30	ADAMTS5	3.10
MAOA	3.29	CIDEC // CIDECP[1]	3.06
LOC648149 // MAP2	3.04	VSIG4[1]	3.00
STEAP4[1]	3.01	LMO3	3.01
ZBTB16[1]	3.01	PDK4[1]	2.96
SERPINA3	3.00	COPS8	2.90

[1] Common genes in both subcutaneous and omental adipose tissue.

SUPPLEMENTAL FIGURES



Supplemental Figure 1. Gene expression of FKBP5 in metabolically active tissues, skeletal muscle, adipose tissue (omental and subcutaneous), kidney, liver, heart and brain measured by microarray analysis. The relative expression in each tissue was determined as described in Methods.