DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Mechanisms of Insulin Resistance after Immunosuppressive Therapy in Brown Adipose Tissue

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Eugénia Carvalho (Universidade de Coimbra) e da Professora Doutora Angela M. Valverde (Instituto de Investigationes Biomedicas Alberto Sols CSIC/UAM, Espanha)

Carlos Manuel de Moura Ferreira

2013

Abstract

Mechanisms of insulin resistance after immunosuppressive therapy in brown adipose tissue

Post transplant diabetes mellitus (PTDM) is a metabolic syndrome that affects a large number of patients with chronic treatment with immunosuppressive agents (IAs). It is known that IAs modulates negatively insulin sensitive-tissues such as skeletal muscle, liver and white fat. However, the effects of IAs on insulin action and thermogenesis in brown adipose tissue have not been studied. In this study we have analyzed the impact of rapamycin, cyclosporine A (CsA) and tacrolimus (FK506) in the insulin signaling cascade in differentiated brown adipocytes from rat. We found that the three compounds inhibits insulin-mediated protein kinase B (PKB/Akt) phosphorylation as well as glucose transporter 4 (GLUT4) translocation to the plasma membrane and glucose uptake, with the effect of the immunosuppressor rapamycin being more evident ($p<0.001$). Moreover, degradation of IRS-1 was only observed in rapamycin and CsA-treated cells. Rapamycin induced an early activation of N-terminal Janus activated kinase (JNK) and, as a consequence, increased serine phosphorylation of insulin receptor substrate (IRS-1) at serine 307 residue. However, no effects of rapamycin were found in other stress kinases such as p38 mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK). Rapamycin also inhibited basal ($p<0.05$) and norepinephrine-induded ($p<0.05$) mRNA levels of uncoupling protein 1 (UCP1) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α). Conversely, CsA and FK506 induced the opposite effect by increasing both UCP1 and PGC1α mRNAs. Our results have demonstrated for the first time the unique role of brown adipose tissue as a target of IAs suggesting that insulin resistance in BAT might contribute to PTDM.

Keywords: Immunosuppressive agents, insulin resistance, post-transplant diabetes mellitus, brown adipose tissue

Resumo

Mecanismos de resistência à insulina após terapia de imunossupressão em tecido adiposo castanho

Diabetes mellitus pós-transplante (PTDM) é uma síndrome metabólica que afecta um grande número de doentes em tratamento crónico com agentes imunossupressores (IAs). É sabido que os IAs modulam negativamente tecidos sensíveis à insulina como são o músculo-esquelético, o fígado e o tecido adiposo branco. Contudo, os efeitos dos IAs na acção da insulina e na termogénese no tecido adiposo castanho ainda não são conhecidos. Neste estudo, analisámos o impacto da rapamicina, da ciclosporina A (CsA) e tacrolimus (FK506) na cascada da sinalização de insulina em adipócitos castanhos diferenciados de ratos. Verificámos que os três compostos inibem a proteína cinase B (PKB / Akt) mediada por insulina assim como inibe a translocação do transportador de glucose 4 (GLUT4), sendo que o efeito da rapamicina é mais evidente ($p<0.001$). Além disso, observámos degradação de IRS-1 apenas em células tratadas com os compostos rapamicina e CsA. A rapamicina induziu uma activação inicial da cinase N-terminal Janus activada (JNK) e, como consequência, aumentou a fosforilação do substrato do receptor de insulina 1 (IRS-1) no resíduo de serina 307. No entanto, não verificámos efeitos da rapamina em outras cinases de stress como a proteína cinase activada por mitógeno (MAPK) p38 ou a cinase regulada por sinal extracelular (ERK). A rapamicina inibiu também os níveis basais ($p<0.05$) e induzidos por norepinefrina ($p<0.05$) de mRNA da proteína desacopladora 1 (UCP1) e "peroxisome proliferator-activated receptor gamma coactivator 1 alpha" (PGC1α). Por outro lado, CsA e FK506 induziram o efeito oposto, aumentando ambos os níveis de mRNA de UCP1 e PGC1α. Estes resultados demonstraram pela primeira vez o papel único do tecido adiposo castanho como sendo um alvo dos IAs, sugerindo que a resistência à insulina em BAT pode contribuir para a PTDM.

Palavras-chave: Agentes imunossupressores, insulino-resistência, diabetes mellitus pós-transplante, tecido adiposo castanho

Table of contents

Abbreviations

4EBP1 – 4E binding protein 1

BAT – brown adipose tissue

C/EBPβ – CCAAT-enhancer binding protein β

CaM – calmodulin

CBG – corticosteroid-binding globulin

CnA – Calcineurin A subunit

Cnb1 – Calcineurin b1 subunit

CREB – cAMP response element-binding protein

CRTC2 – CREB-regulated transcription coactivator 2

CsA – cyclosporine A

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

ERK – extracellular signal-regulated kinase

EDTA – ethylenediaminetetraacetic acid

FAS – fatty acid synthase

FBS – fetal bovine serum

FFA – free fatty acid

FK506 – tacrolimus

FoxO1 – forkhead box O1

G6Pase – glucose-6-phosphatase

GCs – glucocorticoids

GLUT4 – glucose transporter type 4

HMG CoA reductase – 3-hydroxy-3-methyl-glutaryl-CoA reductase

IAs – immunosuppressive agents

IL – interleukin

INF-γ – gamma interferon

IR – insulin receptor

IRS-1 – insulin receptor substrate 1

JKN – c-Jun N-terminal kinase

LDL-C – low density lipoprotein cholesterol

LPL – lipoprotein lipase

mTOR – mammalian target of rapamycin

mTORC1 – mTOR complex 1

mTORC2 – mTOR complex 2

NE – norepinephrine

NFATc – nuclear factor of activated T cells

NFATn – nuclear binding partner of NFATc proteins

p70S6K1 – p70S6 kinase-1

PEPCK – phosphoenolpyruvate carboxykinase

PGC1α – PPARγ coactivator 1-alpha

PI3-K – phosphatidylinositol 3-kinase

PKB/Akt – protein kinase B

PPARγ – peroxisome proliferator-activated receptor γ

PRDM16 – PRD1-BF-1-RIZ1 homologous domain containing protein-16

PTDM – post-transplant diabetes mellitus

PTP – permeability transition pore

T3 – triiodothyronine

TTBS – Tris-Buffered Saline and 0.05% Tween-20

UCP – uncoupling protein

WAT – white adipose tissue

I. Literature Review

1.1. Insulin resistance and type 2 diabetes

Type 2 diabetes (T2D) is a chronic metabolic disease that has become increasingly common over the last decades. Each year the number of new outcomes for this epidemic disease continues to rise and there are about 346 million people suffering with T2D. It is predicted that by 2030 over 439 million people worldwide will suffer from T2D, and, therefore its socioeconomic impact is experienced globally (Shaw JE. et al. 2010, Zhang P. et al. 2010). T2D is caused by a combination of genetic and environmental factors. It begins with an impairment of the insulin signaling cascade in the target tissues, skeletal muscle, liver and adipose tissue leading to a metabolic condition known as insulin resistance. When insulin resistance develops, insulin, secreted by the beta-cell in the pancreas, is needed at much higher levels in order to maintain the normal homeostasis of glucose in the peripheral tissues. This disorder may lead to beta-cell failure which may culminate in the development of T2D.

1.2. Immunosuppressive agents and "secondary diabetes"

Immunosuppressive agents (IAs) inhibit the immune system in order to terminate an immune response (Subramanian S. et al., 2007). They are commonly used for prevention of allograft rejection and autoimmune diseases. However, in long-term treatments, secondary complications can be developed including osteoporosis, hypertension, dyslipidaemia, insulin resistance and T2D (Coutinho AE. et al., 2011).

IAs are often used after solid organ transplantation, including kidney, heart, liver, and pancreas in order to prevent rejection episodes. However, previously non-diabetic subjects, undergoing transplantation develop post-transplant diabetes mellitus (PTDM) in response to immunosuppressive therapies (Hjelmesaeth J. et al., 2005). There are some contributing factors to the development of PTDM including increasing dose use, older age, positive family history of diabetes mellitus, and ethnicity (Subramanian S. et al., 2007).

Agents commonly employed for immunosuppression include glucocorticoids such as prednisone and dexamethasone, calcineurin inhibitors such as cyclosporine A (CsA) and

tacrolimus (FK506), and mTOR inhibitors such as rapamycin (also known as sirolimus) and everolimus.

1.2.1. Glucocorticoids as immunosuppressive agents - endogenous and exogenous Glucocorticoids

Glucocorticoids (GCs) are used for their potent anti-inflammatory effects. Indeed, these agents are often used to stop undesirable immune responses like in autoimmune diseases and in post-transplant therapy. Endogenous GCs can modulate the immune system and shape both adaptive and innate immune responses (Kauh EA. et al., 2011).

Cortisol is the main endogenous GC and the secretion of this steroid hormone secretion is controlled by a complex series of feedback loops. However, the principal triggers are the free-circulating levels of cortisol, the sleep-wake cycle (diurnal variation) and corticotrophin-releasing hormone. Cortisol levels increase quickly in stress environments, whether physical (trauma, surgery, exercise), psychological (anxiety, depression), or physiological (hypoglycemia, fever) (Bujalska IJ. et al., 1999). However, free endogenous GCs are confined to a great percentage within a floating glycoprotein called corticosteroid-binding globulin (CBG) and thus, most of the cortisol is restricted from binding to the GC receptor, preventing its intrinsic function. Differently from cortisol, exogenous or synthetic GCs have low affinity for CBG and thus, this protein seems to play no role in insulin resistant models that had exogenous GC treatment (Qi D. et al., 2006).

GC therapies occur mostly by the use of exogenous GCs, named prednisone (inactive form), prednisolone (active form) and dexamethasone which is known to be the most potent GC immunosuppressor (Kauh EA. et al., 2011). Dexamethasone is commonly used in severe inflammatory conditions and autoimmune disorders like rheumatoid arthritis (Hasan EA. et al., 2009). However, because of the numerous side effects in different types of tissues, dexamethasone can't be used in a full dose range or for long-term treatment periods (Kauh EA. et al., 2011).

GCs can lead to secondary diabetes by the development of insulin resistance followed by enhanced gluconeogenesis in the liver and decreased glucose uptake and glycogen synthesis in skeletal muscle (Hjelmesaeth J. et al., 2005). In addition, GCs also induce degradation of proteins to free amino acids in the muscle, lipolysis in adipose tissue and inhibition of pancreatic insulin production and secretion (Subramanian S. et al. 2007; Kim JW. et al. 2012). Moreover, treatment

with GCs represents the higher risk to develop PTDM with reported incidence rates up to 40% in renal and liver transplant recipients (Subramanian S. et al. 2007).

GCs therapy also promote hyperlipidemia (elevated blood lipid levels) in the post-transplant period, with incidence rates up to 60% for kidney transplants, 80% for cardiac transplant, and 45% for liver transplant. Evidence shows that GCs can disrupt the activity of several key enzymes, including increased activity of acyl coenzyme A carboxylase, fatty acid synthase and HMG CoA reductase, and decreased activity of lipoprotein lipase (LPL), contributing to the undesired effect of hiperlypidemia and dyslipidaemia (Subramanian S. et al. 2007).

For a long time, exogenous GCs, have been playing an important role in immunosuppression. In fact, prednisone and dexamethasone were the main IAs for organ transplantation until the introduction of new IAs such as calcineurin inhibitors, that show less adverse effects (Hjelmesaeth J. *et al.* 2005). In recent years, GCs, such as the steroid prednisolone, are given to patients in combination therapies with calcineurin inhibitors such as CsA or FK506 in order to reduce and prevent relapse of steroid use because of the serious health problems such as PTDM when these GCs are used chronically (Hjelmesaeth J. et al. 2005; Eguchi A. et al. 2010)

Many reports show that subjects treated with these drugs have a propensity to develop insulin resistance and insulin signaling impairment (Sakoda H. et al. 2000; Burén J. et al. 2002) However, the mechanisms through which the metabolic syndrome is developed remain poorly understood. Dexamethasone treatment decreases insulin-stimulated glucose uptake in adipocytes in a time-dependent manner and these results appears to be due to depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB/Akt) (Burén J. et al. 2002). In addition, dexamethasone treatment has been shown to decrease p70S6K phosphorylation by about 50% (Burén J. et al. 2002). This protein kinase has an important role in insulin signaling involving the PI3-K/PKB pathway for glucose uptake and to terminate insulin response mechanisms (Figure 1).

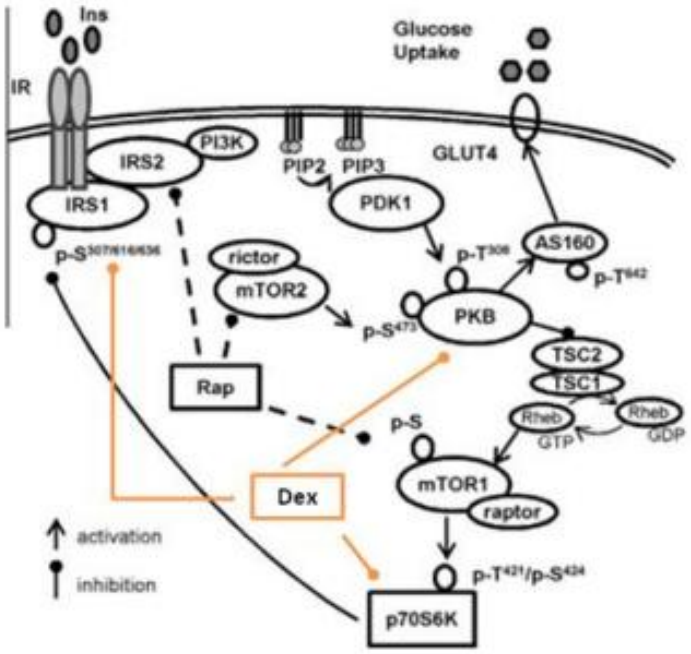

Figure 1. Schematic ilustration of the insulin signaling cascade in adipose tissue and the action of both dexamethasone (orange arrows) and rapamycin (black arrows) on the IRS,PI3-K,PKB/Akt pathway (Adapted from Pereira MJ. *et al,* 2012).

1.2.2. Calcineurin inhibitors

Calcineurin inhibitors play a major role in immunosuppressive regimens since their introduction in 1980 with CsA and a decade later with FK506 (Subramanian S. et al*.,* 2007; Hjelmesaeth J. et al., 2005). Calcineurin is a calmodulin-dependent serine/threonine phosphatase that consists of a regulatory subunit, calcineurin b1 (Cnb1), and a catalytic subunit, calcineurin A (CnA), both of which are required for calcineurin function (Heit JJ. 2007). The calcineurin complex is activated by increased levels of intracellular Ca^{2+}, leading to dephosphorylation of its substrates, which include the cytoplasmatic subunits of nuclear factor of activated T cells (NFATc) transcription complexes. NFATc proteins are then translocated into the nucleus where they bind sequences in target genes with partner transcription factors (NFATn) and activate transcription of genes (figure 2) (Heit JJ. 2007).

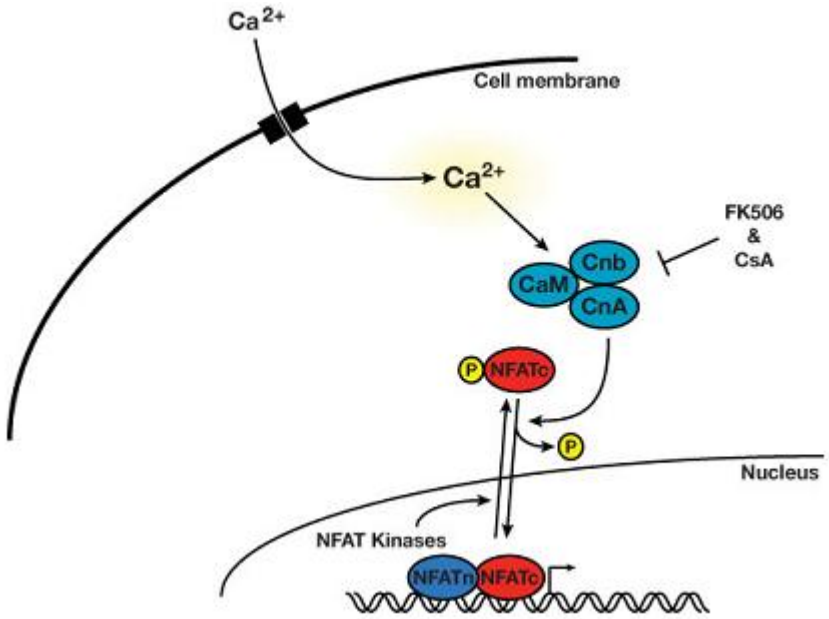

Figure 2. Representation of calcineurin/NFAT signaling pathway. Abbreviations: CnA, Calcineurin A; Cnb1, Calcineurin b1; CaM, calmodulin; NFATn, nuclear binding partner of NFATc proteins; P, phosphate group,; CsA, cyclosporine A; FK506, tacrolimus (Adapted from Heit JJ, 2007).

Activation of the calcineurin/NFAT pathway in response to increased intracellular Ca^{2+} levels, results in coordination of gene expression, growth, terminal differentiation and cellular response to environmental cues (Heit JJ. 2007; Heit JJ. et al. 2006). In the beta-cell, calcineurin/NFAT signaling induces activation of *insulin* transcription, thereby increasing insulin production (Heit JJ. 2007). Of most importance, calcineurin/NFAT signaling mediates T-cell activation in lymphocytes through transcriptional activation of IL-2 and other important factors (Heit JJ. 2007). Thus, after characterization of this pathway in lymphocytes, there was the development of IAs such as CsA and FK506, an important step in immunssuppressive therapy (Heit JJ. 2007; Øzbay LA. et al. 2011). CsA and FK506 inhibit calcineurin function by binding to the regulatory proteins of the enzyme, cyclophilin A and 12 kDa FK506-binding protein (FKBP-12), respectively (Heit JJ. 2007). Both drugs also inhibit the actions of prolactin, an immune activator, thereby providing a synergistic effect on immunomodulation (Subramanian S. et al. 2007).

It is known that both calcineurin inhibitors are associated with elevation in blood glucose levels (hyperglycemia) and blood lipid levels (hyperlipidemia), a condition that results in impaired beta-cell function (Subramanian S. et al. 2007; Böhmer AE. et al. 2009). Although these two calcineurin inhibitors have some differences in their mechanisms of action and metabolism,

both show identical adverse effects if used for long-term periods. FK506 shows fewer side effects regarding lipid metabolism compared to the administration of CsA (Deleuze S. et al. 2006). In addition, FK506 also demonstrates fewer effects on total cholesterol and low density lipoprotein cholesterol (LDL-C) levels, compared to CsA that showed increased levels of these lipid components increasing the risk of cardiovascular diseases (Deleuze S. et al. 2006). Many studies have been made in order to understand the mechanism by which calcineurin regulates *insulin* transcription and whether other important beta-cell factors are similarly affected by calcineurin inhibitors, however, much is still unclear regarding their mechanism of action. (Heit JJ. 2007; Böhmer AE. et al. 2009).

1.2.2.1. Cyclosporine A

CsA is a hydrophobic cyclic polypeptide consisting of 11 amino acids (figure 3). It has been widely used as an immunosuppressant in organ transplantation, as well as in the therapy of autoimmune disorders (Böhmer AE. et al. 2009; Wolf A. et al. 1997). Currently, CsA is commercially manufactured from the fungi culture *Tolypocladium inflatum* (Borel et al, 1974).

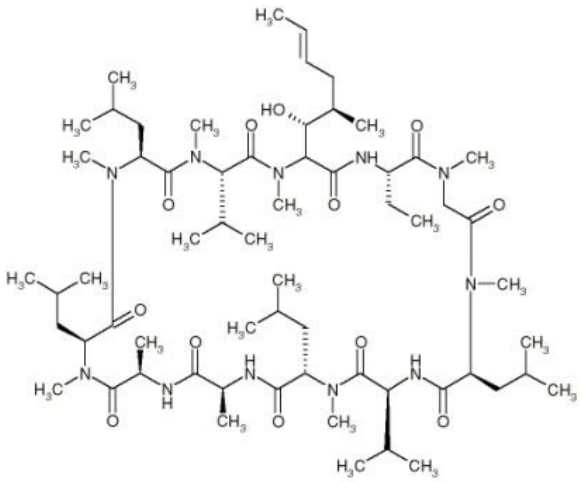

Figure 3. The structure of Cyclosporine A. Its molecular formula: $C_{62}H_{111}N_{11}O_{12}$ and molecular weight: 1202.61 g/mol.

The use of CsA is associated with impaired glucose metabolism and PTDM in 5% to 35% of renal transplant recipients (Subramanian S. et al. 2007). Differently from other IAs, the mechanism by which CsA impairs glucose metabolism appears to be mainly due to a decrease in insulin secretion (Subramanian S. et al. 2007; Heit JJ. 2007; Heit JJ. et al. 2006).

CsA inhibits the production of interleukins, gamma-interferon (INF-γ) and other lymphokines, acting as an immunomodulator of immune and inflammatory reactions (Heit JJ. 2007; Böhmer AE. et al. 2009). *In vivo*, an adverse effect is that, CsA interferes with oxidative stress in the rat kidney and liver by increasing lipid peroxidation, depleting the hepatic and renal pools of glutathione and impairing antioxidant defense system, causing toxicity in cells (Wolf A. et al. 1997).

Studies performed in rats show that CsA treatment decreases the serum levels of IL-1alpha/beta and IL-2 by treatments with either 5 or 15mg/kg/day and no significant changes were detected in IL-6 and INF-γ with either concentration (Böhmer AE et al. 2009). In addition, with a 15mg/kg/day CsA treatment, a mild hepatocellular vacuolinization with scattered cell infiltration was observed in the liver, but the metabolic functions were preserved (Böhmer AE et al. 2009). This information demonstrates that the nephrotoxicity and hepatoxicity are dependent of the CsA concentration and further investigation is needed to reveal the toxicity of CsA upon either renal or liver functions.

Knock out studies for the regulatory subunit, Cnb1 show that this deletion decreases beta-cell proliferation and reduces pancreatic insulin content, resulting in diabetes-like symptoms (Heit JJ. et al. 2006). Thus, it is demonstrated that calcineurin plays a major role in the regulation of pancreatic cell growth and proliferation by controlling multiple factors involved in gene transcription (Heit JJ. 2007; Heit JJ. et al. 2006).

1.2.2.2. Tacrolimus

FK506 is a powerful macrolide immunosuppressor and selective anti-T-lymphocyte agent (figure 4) (Pierre E. et al., 1993). This immunosuppressive agent discovered in 1984 was isolated from the fungus *Streptomyces tsukubaensis,* and similar to CsA, it also targets calcineurin, inhibiting calcineurin/NFAT signaling (Thomson AW. et al. 1993).

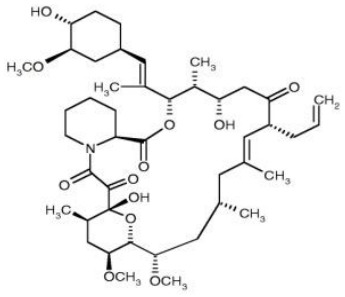

Figure 4. FK506 structure. Molecular formula: $C_{44}H_{69}NO_{12}$. Molecular weight: 804.018 g/mol.

The effects of CsA and FK506 on insulin signaling and beta cell growth have been studied in order to compare the side effects of both calcineurin inhibitors. Some studies have reported that FK506 is more diabetogenic than CsA and therefore the incidence of "insulin dependent" PTDM has been reported to be higher in subjects taking FK506 (Hjelmesaeth J. et al. 2005). The mechanism regarding this evidence might be explained by a greater inhibition on the transcription of the *insulin 2* gene and its release, and also a higher decrease on beta-cell proliferation by the calcineurin inhibitor FK506 (Hjelmesaeth J. et al. 2005; Øzbay LA. et al. 2011, Rodriguez-Rodriguez AE. et al. 2013).

Regarding lipid homeostasis, recent studies show that incubation with FK506 increases both basal and isoproterenol-stimulated lipolysis in human subcutaneous adipocytes; an effect shared by other IAs such as CsA and rapamycin (Pereira MJ. et al 2013).

1.2.3. mTOR inhibitors as immunosuppressors

The mammalian target of rapamycin (mTOR) is part of two multiprotein complexes, mTORC1 (or mTOR-raptor) and mTORC2 (or mTOR-rictor), which have different roles in the cell (Di Paolo S. et al. 2006). These kinases are important for the insulin signaling by acting in a negative feedback mechanism, terminating/inhibiting insulin effects (Veilleux A. et al. 2010, Di Paolo S. et al. 2006). mTORC1 acts as a cellular nutrient sensor and regulates cell growth through effectors such as p70S6 kinase-1 (p70S6K1) and 4E binding protein 1 (4EBP1), while mTOR2 controls cell survival and cytoskeleton regulation (Di Paolo S. et al. 2006). mTORC1 regulates other cellular effects including mitochondrial oxidative function through the transcription factor peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α;) protein synthesis through activation of p70S6K effector; and autophagy in starvation tolerance (figure 5) (Di Paolo S. et al. 2006, Ost A. et al. 2010). It is suggested that mTORC2 is insensitive to rapamycin, however, long-term treatment seems to disrupt the appropriate assembly of mTORC2 and its associated proteins (Sarbassov DD. et al. 2006). The mTOR signaling pathway can be activated by different agents such as growth factors, hormones (insulin), nutrients (glucose) and the cellular energy levels as well (Di Paolo S. et al. 2006).

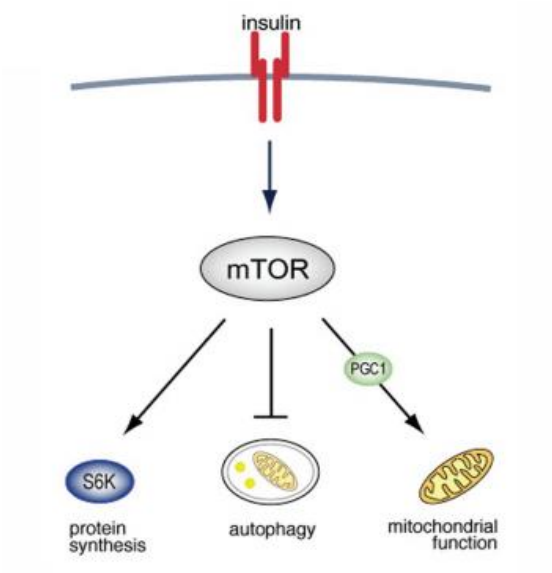

Figure 5. Major mTOR functions: protein synthesis, protection from autophagy and mitochondrial function (Adapted from Öst A. *et al*, 2010).

mTOR inhibitors such as rapamycin (also known as sirolimus) have been extensively used in immunosuppressive therapy after transplantations, in more recent years. However, the inhibition of the mTOR signaling pathway is also associated with diverse side effects at the lipid and glucose metabolism levels in different tissues (Di Paolo S. et al. 2006). Some reports show evidence that chronic rapamycin administration causes hyperlipidemia, reduces fat mass and promotes glucose intolerance that can result in PTDM (Di Paolo S. et al. 2006, Houde VP. et al. 2010). These reports are in accordance with other studies where inhibition of the mTOR signaling pathway blocks insulin-induced phosphorylation of insulin receptor substrate 1 (IRS-1) on serine and tyrosine residues, resulting in decrease insulin sensitivity (Øzbay LA. et al. 2011, Di Paolo S. et al. 2006 and Takano A. et al. 2001).

The mechanism by which mTOR inhibitors affect insulin action still needs further investigation, but most investigators speculate that the long-term inhibition of the mTORC1 blocks the effector p70S6 kinase impairing IRS-1 phosphorylation, thus preventing its degradation (Øzbay LA. et al. 2011, Di Paolo S. et al. 2006 and Takano A. et al. 2001). This leads to partial inhibition of mTORC2 activity which decreases activation of Akt/PKB that is associated in glucose transporter type 4 (GLUT4) translocation, resulting in decreased glucose uptake in the insulin target cells, particularly, in skeletal muscle and adipose tissue (Paolo S. et al. 2006 and Takano A. et al. 2001). In the liver, there is evidence that chronic rapamycin treatment stimulates gluconeogenesis by upregulating gluconeogenic key enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) and thereby promoting insulin resistance (Houde VP. et al. 2010). Other proteins regarding gluconeogenesis

are also upregulated such as PGC1α, forkhead box O1 (FoxO1), cAMP response element-binding protein (CREB) and CREB-regulated transcription coactivator 2 (CRTC2). These alterations allow increasing plasma glucose levels and, therefore, causing glucose intolerance that might leads to insulin resistance (Houde VP. et al. 2010). The pathogenic effects of IAs are summarized on Table 1.

Table 1 – Summary of potential pathogenic mechanism(s) of drug-induced PTDM:

Immunosuppressive agent	Proposed mechanism(s)	Comments
Glucocorticoids	• ↓ Peripheral insulin sensitivity • Promote protein degradation to free amino acids in muscle, lipolysis • Inhibit pancreatic insulin production and secretion • ↑ Hepatic gluconeogenesis	• Dose-dependent • Impact of complete withdrawal of chronic low-dose steroids unclear • Potential ↓ NODAT risk in steroid-free regimens
Cyclosporine A	• ↓ insulin secretion (CsA < FK506) • ↓ insulin synthesis • ↓ beta-cell density	• Dose-dependent, • Diabetogenic effect ↑ with ↑ steroid dose*
Tacrolimus	• ↓ insulin secretion (FK506 > CsA) • ↓ insulin synthesis	• Dose-dependent, • Diabetogenic effect ↑ with ↑ steroid dose*
Rapamycin	• ↑ Peripheral insulin resistance • Impair pancreatic beta-cell response	• Diabetogenicity when use with CNIs

Note: * Demonstrated in some but not all studies.
Abbreviations: CsA, cyclosporine A; FK506, tacrolimus; CNIs, calcineurin inhibitors; ↑increased; ↓, decreased. (Pham PT. et al. 2011)

1.3. Adipose tissue

Adipose tissue is a peripheral tissue that is mainly composed of two different subtypes: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is widely distributed and it represents the primary site of fat metabolism and storage, whereas BAT is relatively scarce and is specialized in thermogenesis. White adipose tissue is composed of mostly adipocytes, surrounded by connective tissue that is highly vascularised and innervated; it contains macrophages, fibroblasts and adipocyte precursors. Brown adipose tissue is a major site for nonshivering thermogenesis in mammals and consisting essentially of brown adipocytes (Valverde AM. et al., 2005). Morphologically, brown adipocytes are characterized by the presence of multiple lipid droplets with a high number of mitochondria (Figure 6) (Smorlesi A. et al. 2012). We will focus on brown adipose tissue, since it is most relevant for the context of this work.

In addition to muscle and liver, adipose tissue is a relevant site of insulin action. Insulin resistance in adipose tissue leads to an increase in lipolysis, with subsequent release of glycerol and free fatty acids (FFA) into the circulation where its high availability and utilization contributes to the development of skeletal muscle insulin resistance, as well as to increased hepatic glucose production and impair beta-cell function (Foley, 1992; Yuan, M. et al., 2001).

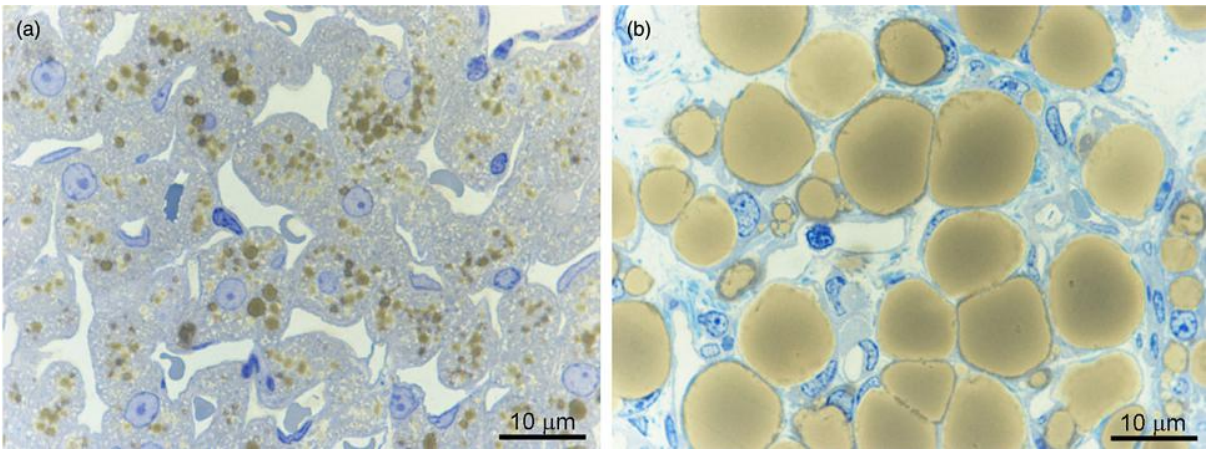

Figure 6: Morphology features of brown (a) and white fat cells (b). Light microscopy of mouse anterior subcutaneous fat depot in an area where white and brown adipose tissues are close to each other. (Extracted from Smorlesi A. et al., 2012.

1.3.1. Brown Adipose tissue

BAT has its primary development during the fetal formation and is found in all mammals at their time of birth and also especially abundant in hibernating mammals (Gesta S. et al. 2007).

The relevance of BAT in adult humans has been a subject of controversy for many years due to a great loss of phenotype and function in the first years after birth. However, after the (re)discovery of BAT (Cohade C. et al. 2003; Zingaretti MC. et al. 2009), the scientific community is now challenged to evaluate the real relevance of BAT in adult humans. Interestingly, the presence of BAT was revealed during the diagnosis for cancers by positron emission tomography / computed tomography (PET/CT). Small amounts of BAT can be found in the neck, in the supraclavicular and axillary regions; in the paravertebral, perirenal/adrenal, and in paraventral regions; and around the major vessels (the aorta and its main branches: carotids, subclavian, intercostals, and renal arteries) (Figure 7). BAT can also be found within WAT and skeletal muscles (Cypess et al. 2010, Charmaine S. Tam et al. 2012; Richard D., Picard F. 2011).

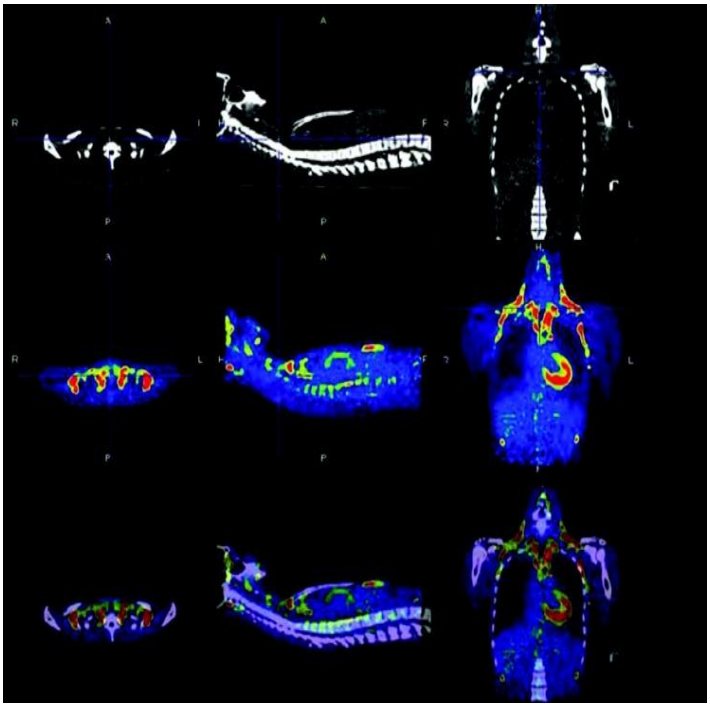

Figure 7. Localization of brown adipose tissue in an adult lean human measured by emission tomography. Computed tomographic (CT; top) and positron emission tomographic (PET; with ^{18}F-fluorodeoxyglucose; middle) images from the neck and thoracic region. Bottom, The superimposition of the CT and PET scans. In the 3 panels, shown from left to right are a transverse slice at the level of the clavicles, a sagittal slice at the level of the spine, and a coronal slice of the thorax. Activated brown adipose tissue areas (red and green) are in the cervical-supraclavicular (most common), perirenal/adrenal, and paravertebral regions. (Extracted from Charmaine S. Tam *et al.*, 2012)

BAT has characteristically dark brown color due to the high mitochondrial content and an elevated number of lipid droplets in opposition to WAT, which has characteristically white color, with few mitochondria and with only one large lipid droplet. When inactivated, BAT loses its phenotype, probably due to apoptotic phenomena and turning into the appearance of the

WAT, losing its specific characteristics gradually. Functionally, BAT is involved in the dissipation of energy via heat generation. This unique thermogenic capacity results from the expression of uncoupling protein-1 (UCP-1), a protein existing in the inner mitochondrial membrane, which uncouples fatty acid oxidation from ATP synthesis, allowing dissipation of energy from substrate oxidation as heat (Miranda S. et al., 2009).

The development of brown adipocytes are mainly controlled by two essential transcriptional factors known as peroxisome proliferator-activated receptor γ (PPARγ) and PGC1α, which induce adipogenesis and enhance the thermogenic machinery of brown adipocytes (Tontonoz P, et al. 2008). Despite the ultrastructural and metabolic morphology differences between BAT and WAT, it is believed that both fat tissues derive from the same precursors relying on the role of PPARγ during evolution and maturity of the cells (Tontonoz P, et al. 2008, White UA. et al. 2010). Moreover, recent data show that a specific stimulus such as chronic cold exposure or β-adrenergic stimulation in mice or rat induces the transdifferentiation of white adipose depots into brown-appearing depots in a process called "browning" (Ohno H. et al. 2012, Smorlesi A. et al. 2012; Bonet ML. et al. 2012). The brown-like adipocytes that appear in classical WAT depots have been called "brite" (brown-in-white) or "beige" adipocytes (Charmaine S. Tam et al. 2012; Ohno H. et al. 2012). They express low levels of UCP1 in comparison to classic brown adipocytes but can be highly inducible in response to appropriate stimuli as mentioned, allowing increasing energy expenditure by the thermogenic mechanism of UCP1 (Charmaine S. Tam et al. 2012). The mechanisms regarding the browning process are associated with enhanced expression of transcriptional components that controls the development of classical brown adipocytes such as PRD1-BF-1-RIZ1 homologous domain containing protein-16 (PRDM16), PGC1α, PPARγ, CCAAT-enhancer binding protein β (C/EBPβ) among others (Kajimura S. et al. 2010). Understanding the biological processes controlling brown adipocyte activity and differentiation might help to design strategies involving transdifferentiation of brown-like adipocytes to increase energy expenditure and therefore, fight against obesity and diabetes.

1.3.2. The uncoupling protein UCP1

UCP1 was first discovered and isolated at the end of the seventies (Nicholls DG. et al. 1978; Lin CS. et al. 1980) and it was further fully characterized in the following decade (Ricquier D. et al. 1983; Bouillaud F. et al. 1985; Bouillaud F. et al. 1986). UCP1 has a molecular weight that ranges between 32 and 33 kDa and it was first referred as thermogenin before the discovery

of others UCPs. But despite being a member of the UCP superfamily, which also includes UCP2, UCP3, UCP4, UCP5 brain mitochondrial carrier protein 1 (BMCP1), UCP1 evolved to be the only UCP with a recognized thermogenic capacity (Richard D., Picard F. 2011).

Functionally, when activated, UCP-1 causes a leak of the electrochemical proton gradient that is generated by the respiratory chain and hence, uncoupling the mitochondrial respiration from ATP synthesis (Richard D., Picard F. 2011). UCP1 then enables the re-entry of protons to the mitochondrial matrix and, as a consequence, energy liberated from substrate oxidation is dissipated as heat (figure 8).

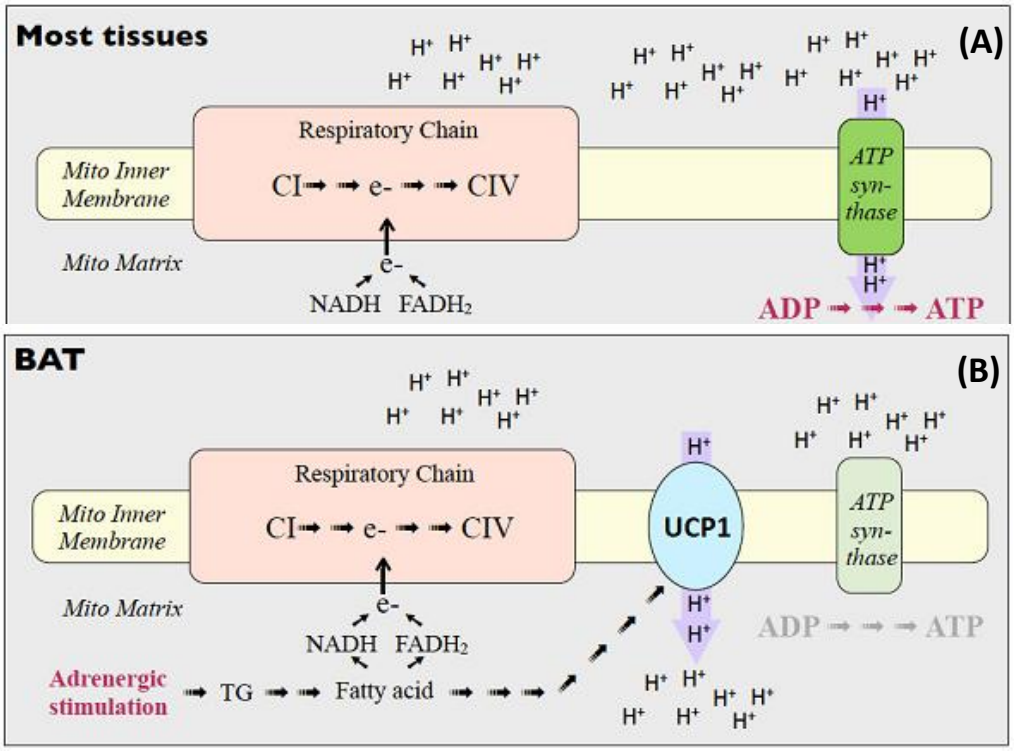

Figure 8. Molecular mechanism implicated in termogenesis. The electron transport chain produces a proton flux into the intermembrane space through the mitochondrial inner membrane. These protons can re-entry to the mitochondrial matrix by the ATP synthase, thus producing ATP **(A)**, or alternatively, can cross the mitochondrial inner membrane by UCP1 proteins, leading to production of heat **(B)**. (Adapted from Richard D., Picard F. 2011)

1.4. Immunosupressors and mitochondrial function

Mitochondria plays an essential role in the viability of eukaryotic cells producing its energy for the different metabolic functions and IAs are known to disturb these functions. However, the effects of these agents on mitochondrial respiration and function are not well known and should be further investigated.

Indeed, it is known that mitochondria activity is more sensitive to glucose in the presence of the hormone steroid dexamethasone in pancreatic islets of rats, resulting in increased NAD(P)H production which affects K_{ATP} channel activity leading to insulin hypersecretion (Rafacho A. et al. 2010).

Mitochondria is an organelle that plays important roles regarding the life time of a cell by many different ways, imposing its mechanisms of cell death, cell cycle and cell growth. One of the mechanisms of cell death is by the mitochondrial permeability transition pore (PTP), which is a mitochondrial channel involved in cell death when it stays open for long periods of time (Lablanche S. et al. 2011). PTP is also involved in important mechanisms that trigger the oscillatory activity of the beta-cells (Düfer M. et al 2001). This pore is inhibited by the immunosuppressor CsA and a study performed in pancreatic islet cells of fed female NMRI mice showed that CsA acts on PTP, inhibiting the oscillations in the electrical potential (Düfer M. et al 2001). These results are accompanied by the reduction of the cytoplasmatic free-Ca^{2+} leading to reduced insulin secretion which is independent of calcineurin inhibition (Düfer M. et al 2001).
It has been shown that inhibition of mTOR with rapamycin enhances autophagy in adipocytes from patients with T2D (Ost A. et al. 2010). This study has shown that the inhibition of mTOR signaling can potentiate autophagy at the level of mitochondria in adipose tissue of these patients, which may be the basis of some of the adverse effects in mTOR inhibition (Ost A. et al. 2010).

Glucose-induced insulin secretion from beta-cells is often impaired due to the reduced glucose-induced ATP elevation by exposure to high metabolic fuels and after exposure to diabetogenic pharmacological agents (Fujimoto S. et al 2007). In pancreatic beta-cells, mitochondrial ATP production via glycolysis results from the entry of glucose in cells which is fundamental for the mechanism of insulin secretion (Maechler P. et al. 2001). Thus, experiments performed in pancreatic beta-cells on Wistar rats exposed to chronic rapamycin exhibited reduced mitochondrial ATP production compared to controls. However immunoblotting assays revealed that rapamycin did not affect expression of complex I, III, IV and V of the mitochondrial respiratory chain proteins (Shimodahira M. et al. 2010). These observations show that rapamycin suppresses high glucose-induced insulin secretion on pancreatic islets, and the

process involved is believed to be due by the reduction of mitochondrial ATP production through decreased alpha-ketaglutarate activity that limits the velocity of carbohydrate metabolism in the Krebs cycle (Shimodahira M. et al. 2010).

Further information is needed to clarify the role of the diabetogenic pharmacological agents, such as immunosuppressors in insulin signaling and in mitochondrial function on the different insulin target tissues.

II. Aims of study

a) To investigate the effect of IAs on the insulin signaling cascade in differentiated brown adipocytes.

b) To verify whether or not IAs inhibits GLUT4 translocation and glucose uptake in insulin-stimulated differentiated brown adipocytes.

c) To verify the effect of IAs on the mRNA levels of UCP1 and PGC1α in differentiated brown adipocytes after stimulation or not with norepinephrine.

III. Materials and Methods

1. Materials

1.1. Reagents

- Fetal bovine serum (FBS), Medium 199, Dulbecco's Modified Eagle Medium (DMEM), 0.25% Trypsin-0.02% EDTA, and Trizol were from Gibco Life Technologies, Gaithersburg, MD, USA (www.invitrogen.com).
- Insulin, penicillin, streptomycin, FK506, norepinephrine, dimethyl sulfoxide (DMSO) and Tris were from Sigma-Aldrich, St Louis, MO, USA (www.sigma-aldrich.com).
- Leupeptin, and aprotinin were from Roche Molecular Biochemicals, Barcelona, Spain (www.roche-applied-science.com).
- Reagents for protein electrophoresis were from Bio-Rad, Hercules, CA, USA (www.bio-rad.com).
- Rapamycin was from Santa Cruz Biotechnology (www.sbtc.com)
- CsA was from EMD Millipore chemicals (http://www.emdmillipore.com)

1.2. Table 1. Primary antibodies

Antibody	Provenance	Reference
Anti-PKB/Akt	Cell signalling technology (MA, USA)	#9272
Anti-phospho JNK	Cell signalling technology	#4668
Anti-JNK	Santa Cruz	sc-571
Anti-phospho PKB/Akt1/2/3 (Ser 308)	Santa Cruz	Sc-16646-R
Anti-phospho PKB/Akt1/2/3 (Ser 473)	Santa Cruz	Sc-7985-R

Anti-FAS	BD	F72520
Anti-IRS-1	Upstate (Millipore)	06-248
Anti-P85α	Upstate (Millipore)	06-195
Anti phospho IRS-1 Ser 307	Upstate (Millipore)	07-844
Anti Insulin Receptor (IR)	Santa Cruz	sc-711
Anti phospho IR (Ty1162/1163)	Santa Cruz	sc-25103-R
Anti-phospho p70S6K	Cell signalling	9202
Anti-tubulin	Sigma	T5168
Anti-phospho ERK 1/2 (Thr 177/160)	Santa Cruz	sc-23759-R
Anti-PGC1α	Cayman Chemical	101707
Anti-UCP1	Abcam	Ab10983

2. Cellular culture

2.1. Generation of brown adipocyte cell line

2.1.1. Primary cultures of brown adipocytes

Precursor cells were obtained from the interscapular BAT of 20-day-old rats (Sprague-Dawley), isolated as previously described by Néchad et al. (1983), by collagenase digestion (0·2%) and filtration through 250 μm silk filters. Mature cells were allowed to float, and the infranatant was put through 25 μm silk filters and centrifuged. Precursor cells were seeded at a density of 1500–2000 cells/cm2 on day 1, and grown in DMEM supplemented with 10% FBS, 3 nM insulin, 10 mM HEPES, 50 IU penicillin and 50 μg streptomycin/ml, and 15 μM ascorbic acid. Culture medium was changed on day 1 and every second day thereafter. Precursor cells proliferate actively under these conditions, reach confluence at day 4 or 5 after seeding (40 000–

60 000 cells/cm2) and differentiate spontaneously into mature brown adipocytes. All studies and treatments were performed in fully differentiated brown adipocytes (at days 8–9 after seeding).

2.1.2. Immortalization of primary brown adipocytes

Brown adipocytes were obtained from interscapular brown adipose tissue of 20 day-old lactating Wistar rats. Cells were plated in 6-well multiwall tissue culture plates in DMEM supplemented with 20% FBS, 25 mmol/l glucose, 20 mmol/l HEPES, and 100 U/ml penicillin/streptomycin to allow cell attachment and growth to 80% confluence. Retroviral infection with large T antigen particles was performed accordingly with the protocol set up in the laboratory of Dr AM Valverde and detailed in Valverde et al., 1999.

2.1.3. Differentiation of immortalized brown adipocytes

Immortalized brown preadipocytes were grown in DMEM supplemented with 10% FBS, 20 nM insulin and 1 nM triiodothyronine (T3) (differentiation medium) until reaching confluence as describe by Miranda S. et al. (2010). Then, the cells were cultured for 48 hours in induction medium consisting of differentiation medium supplemented with 0.5 μM dexamethasone, 0.125 μM indomethacin and 0.5 mM isobutylmethylxanthine. Thereafter, cells were cultured back in differentiation medium until they exhibited a fully differentiated phenotype (≈ day 8) with numerous multilocular lipid droplets in the cytoplasm. Differentiation medium was renewed each 48 hours (Miranda S. et al. 2010).

2.2. Cell treatments

Cells were treated with CsA, FK506 or rapamycin using three dose ranges, the highest dose is considered a therapeutic concentration, in the clinic. Cells were treated with the respective IA during different times, depending on the aim of the experiment but mostly, cells were treated for 24 hours. Then, for analysis of insulin signaling and glucose uptake, cells were serum-starved for 1 hour followed by stimulation with insulin (10 nM) for 10 minutes. In another experiments, cells were further stimulated with norepinephrine (5 μM) for 8 or 16 hours.

2.3. Preparation of total cell lysates

At the end of each experiment, the cells were firstly washed twice in PBS, and then collected in lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 30 mM disodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin pH 7.6.

3. Analysis of Protein Expression:

3.1. Protein Extracts:

After harvesting brown adipocytes with lysis buffer, homogenates were stored at -20ºC or used immediately to prepare protein extract. For this goal, homogenates were centrifuged twice at 11.200 x g for 7 minutes, first to discard the pellet and second to discard lipids on the surface of the supernatant.

3.2. Protein Quantification

Protein quantification was performed by the Bradford dye method in duplicate, using the Bio-Rad reagent and BSA to prepare the standard curve.

3.3. Western blotting

Western Blot (WB) analyses were used to determine proteins levels and phosphorylation of some mediators of the insulin signaling cascade in brown adipocyte lysates, allowing us to collect information about the expression of proteins as well as their activation levels.

Equal amount of protein (15 μg or 25 μg), mixed with 4x loading buffer (for a final concentration of 1x) were heated at 95ºC for 5 minutes. Then, total protein extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) transfer membranes.

3.4. Protein Transfer

PVDF membranes (Immobilon P, Millipore), were firstly activated for 1 minute in methanol and then washed in distilled water for 5-7 minutes to remove the excess of methanol. Membranes were then maintained wet in transfer buffer until the preparation of the *cassette* and transfer equipment. Protein transfer conditions used were: 300 mA for 2 hours or 100 V for 1 hour.

3.5. Antibody staining

Membranes were blocked for 1 hour at room temperature or overnight at 4°C using blocking solution (3% bovine serum albumin (BSA)). Then, the membranes were incubated with appropriate dilutions of primary antibody in Tris-Buffered Saline and 0.05% Tween-20 (TTBS) overnight at 4°C or for 2 hours at room temperature. The membranes were washed four times with TTBS (10 minutes each). Then, the membranes were incubated with the recommended dilution of labelled secondary antibody in TTBS at room temperature for 1 hour with either alkaline phosphatase-conjugated anti-rabbit antibody (1:15000), alkaline phosphatase-conjugated anti-mouse antibody (1:15000) or alkaline phosphatase-conjugated anti-goat antibody (1:15000). The membranes were washed three times with TTBS (10 minutes each) and a final wash with TBS without Tween-20 for additional 10 minutes. Immunoreactive proteins were visualized by chemiluminescence using the ECL western blotting protocol (Millipore) and Agfa radiographic films.

4. Analysis of gene expression by Quantitative Real-time PCR

Total RNA extraction from brown adipocytes was performed with Trizol. Complementary DNA (cDNA) was synthesized by reverse transcription PCR (RT-PCR) from 1 μg of RNA using iScript cDNA synthesis kit (BioRad, Hercules, CA). For qPCR, each cDNA sample was run in an ABI 7300 in triplicate well, in the following PCR program: 50 °C for 2 minutes, 94 °C for 10 minutes, and 30 cycles of 97 °C for 30 s followed by 60 °C for 1 minute . UCP-1 mRNA was quantified using specific FAM™ dye-labeled TaqMan® probes for UCP-1 (Rn00562126-m1; Gene expression assays, Applied Biosystems, Foster City, CA) and PGC1α (Rn00580241_m1; Gene expression assays, Applied Biosystems, Foster City, CA). Results were

normalized using VIC® dye-labeled TaqMan® probe for ubiquitin (Rn01789812-g1, Applied Biosystems).

5. Measurement of glucose transport

At the end of the incubation without or with compounds, differenciated brown adipocytes were serum-deprived for 2 hours. After that, cells were washed three times with ice-cold Krebs-Ringer-phosphate buffer (KRP) (135 mM NaCl, 5.4 mM KCl, 1.4 mM CaCl2, 1.4 mM MgSO and 10 mM sodium pyrophosphate, pH 7.4) and then incubated with 1 ml KRP buffer with or without insulin for 10 minutes at 37ºC. 2-Deoxy-D[1-^{3}H]glucose (500 nCi/ml) was added to this solution, and the incubation was continued for 5 minutes at 37º C. The cells were then washed three times with ice-cold KRP buffer and solubilized in 1 ml 1% SDS, as previously described (Valverde, AM. et al 1998). The radioactivity of a 200-µl aliquot was determined in a scintillation counter. Glucose transport was determined in triplicate from three independent experiments.

6. Oxygen Consumption

An XF24 Seahorse Bioscience (North Billerica, MA, USA) instrument was used to measure the oxygen consumption rate (OCR) of differentiated brown adipocytes at days 7-8 of differentiation. DMEM growth media was replaced by unbuffered DMEM supplemented with 25 mM glucose, 1 mM pyruvate and 2 mM L-glutamine and cells incubated at 37 °C in a CO2-free incubator for 1 h. Cells were then placed in the instrument and basal oxygen consumption was recorded for 24 minutes and subsequently 1 µg/ml oligomycin and 400 nM FCCP were added. At the end of the run, 1 µM rotenone and 1 µM antimycin A were added to determine the mitochondria-independent oxygen consumption according to Ortega-Molina et al. (2012).

7. Immunofluorescence and confocal imaging

Cells were grown on glass coverslips, fixed in 4% paraformaldehyde and processed for immunofluorescence. Briefly, anti-GLUT4 (sc-56566, Santa Cruz) primary antibody was applied

for 1 h at 37ºC in PBS-1% BSA at 1/50 dilution. The secondary antibody used was Alexa 488 goat anti-mouse (Invitrogen) at 1/500 dilution. Cell nuclei were counterstained with DAPI (Sigma). Immunofluorescence was examined in a Nikon Eclipse 90i microscope with imaging software Nis elements.

8. Statistical analysis

Results are given as mean ± standard error of the mean (SEM) using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA, USA). Statistical analyses using the Student's t-test were performed when two groups were considered. For multiple group comparisons, the One-Way ANOVA test, followed by the post hoc Bonferroni´s Multiple Comparison was used. Differences were considered significant when $*p < 0.05$ or $**p < 0.01$

IV. Results

Effect of rapamycin treatment in the insulin signaling cascade in differentiated brown adipocytes

For the studies of the impact of IAs on insulin-mediated signaling, we used an immortalized rat preadipocyte cell line generated in the laboratories of Drs AM Valverde and MJ Obregón (CSIC, Madrid, Spain) as detailed in Materials and Methods. Brown preadipocytes were seeded in 12 well plastic culture plates and differentiated as described in Materials and Methods. At day 8, we observed that have reached a fully differentiated phenotype as shown by the accumulation of multilocular lipid droplets as well as by the expression of the adipogenic marker fatty acid synthase (FAS) and the thermogenic marker UCP1 (Figure 1A) that assessed terminal differentiation of brown fat cells.

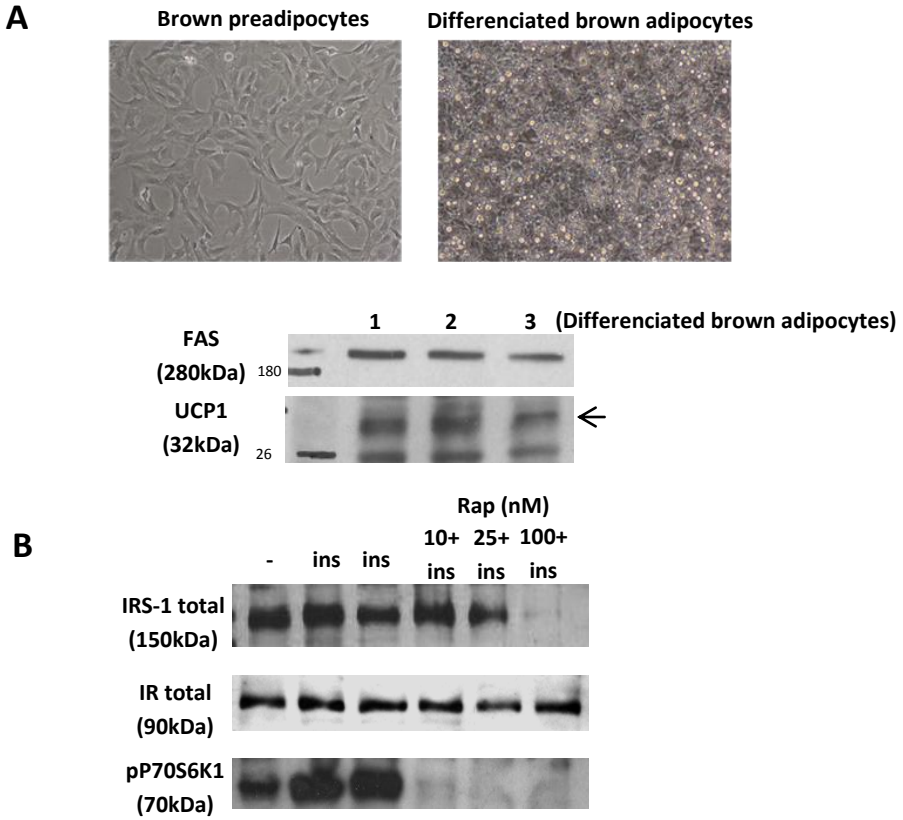

Figure 1. *Upper panel.* Images of immortalized brown preadipocytes generated from lactating rats (left) and differentiated brown adipocytes at day 8 (right). *Lower panel.* Expression of FAS and UCP1 in differentiated brown adipocytes analyzed by western blot with specific antibodies. **B** Expression of insulin receptor substrate 1 (IRS-1), insulin receptor (IR) and phospho p70S6 kinase in immortalized rat brown adipocytes at day 8 of differentiation exposed for 16 h to increasing doses of rapamycin (Rap) and stimulated 10 min with 10 nM insulin. Representative western blot of three independent experiments are shown.

We proposed to investigate total levels of IRS-1 in differentiated brown adipocytes treated with various doses of rapamycin (10-100 nM) for 16 hours because it has been previously reported that IRS-1 is a critical node of the insulin signaling cascade in brown adipocytes and since its deficiency in mice inhibits the signaling pathway that leads to adipogenic and thermogenic gene expression (Valverde AM. et al., 1999; Valverde AM. et al 2003). As shown in Figure 1B, IRS-1 levels decrease in brown adipocytes incubated with rapamycin in a dose-dependent manner. Notably at 100 nM dose of rapamycin, IRS-1 was barely detected whereas the expression of the IR beta chain remained unchanged regardless rapamycin treatment. The efficacy of rapamycin treatment was assessed by the complete inhibition of the phosphorylation of the mTOR substrate p70S6K1 in insulin-stimulated cells as compared with cells that were not pretreated with this compound.

We next investigated the effect of rapamycin in insulin signaling towards the activation of PKB/Akt that has been shown to regulate adipogenic- and thermogenic-related gene expression downstream from IR and IRS-1 in brown adipocytes (Valverde AM. et al., 1999; Fasshauer M et al., 2001, Valverde AM. et al., 2003). Pretreatment of brown adipocytes with rapamycin did not modulate tyrosine phosphorylation of the IR upon insulin stimulation (Figure 2). However, rapamycin decreased insulin-induced PKB/Akt phosphorylation at both serine 473 (within the regulatory domain) and threonine 308 (within catalytic domain) residues in a dose-dependent manner.

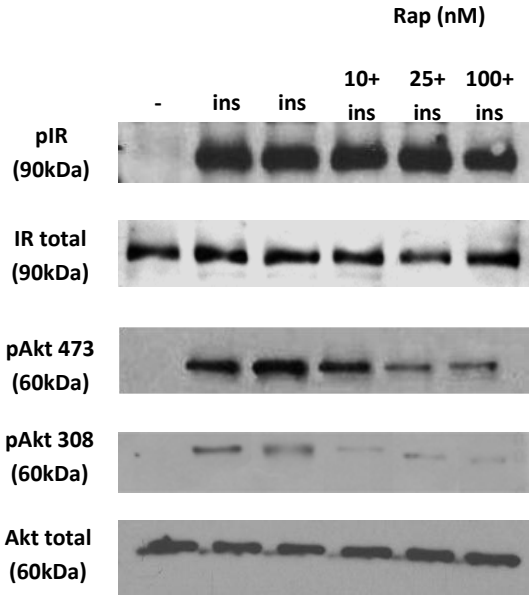

Figure 2. Immortalized brown adipocytes from rat were treated with rapamycin (at different doses) for 16h, and then cells were serum-starved for 1 h followed by stimulation with insulin (10 nM) for 10 min. Thereafter, cells were collected in lysis buffer. Phosphorylation of insulin receptor (IR) and PKB/Akt at serine 473 and threonine 308 were analyzed by western blot as well total levels of IR and Akt. Representative western blots of three independent experiments are shown. Abbreviations: Rap (rapamycin), ins (insulin).

Once defined the impact of rapamycin pretreatment in insulin-mediated IRS-1/Akt signaling pathway in brown adipocytes, we analyzed the effect of CsA and FK506. For this goal, differentiated brown adipocytes (day 8) were pretreated with several doses of CsA or FK506 for 16 hours, after that cells were stimulated with 10 nM insulin for a further 10 minutes. CsA pretreatment didn't decreased IR-mediated tyrosine phosphorylation, but decreased total IRS-1 levels (Figure 3A). Consequently, phosphorylation of PKB/Akt at both serine 473 and threonine 308 residues was severely impaired. Similarly, FK506 did not affect IR tyrosine phosphorylation in response to insulin. Regarding total IRS-1, FK506 didn't decrease IRS-1 total protein. However, as occurred with the other IAs, FK506 impaired insulin-stimulated Akt phosphorylation (Figure 3B).

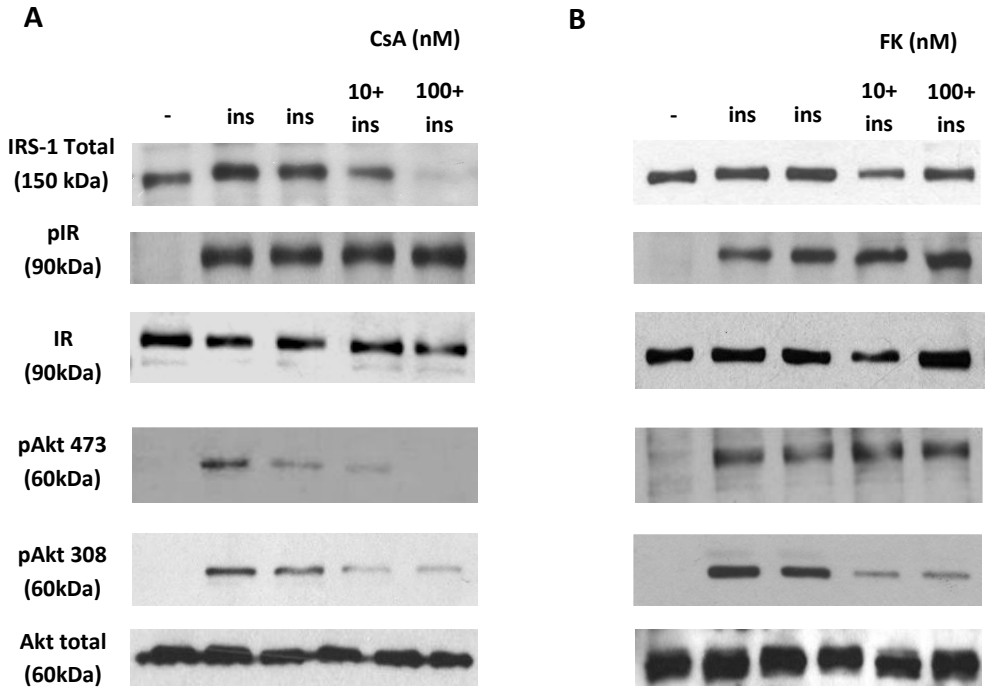

Figure 3. Immortalized brown adipocytes from rat were treated with CsA **(A)** and FK506 **(B)** at different doses for 16h, and then cells were serum-starved for 1 h followed by stimulation with insulin (10 nM) for 10 min. Thereafter, cells were collected in lysis buffer. Expression of insulin receptor substrate 1 (IRS-1), phosphorylation of insulin receptor (IR) and PKB/Akt were analyzed by western blot as well as total IR and total Akt. Representative western blots of three independent experiments are shown. are shown. Abbreviations: CsA (cyclosporine A), FK (tacrolimus), ins (insulin).

To confirm that the effect of IAs on insulin signaling in brown adipocytes was not due to immortalization and/or the differentiation protocol, we prepared primary brown adipocytes from 20 days-old lactating rats as described in Materials and Methods (2.2) and allowed them to differentiate expontaneously during 10 days. As shown in Figure 4, when differentiated primary brown adipocytes were pretreated during 16 hours with 100 nM dose of rapamycin, CsA or FK506 and then stimulated with insulin, the impairment of insulin-mediated PKB/Akt phosphorylation was observed as in the immortalized cell line.

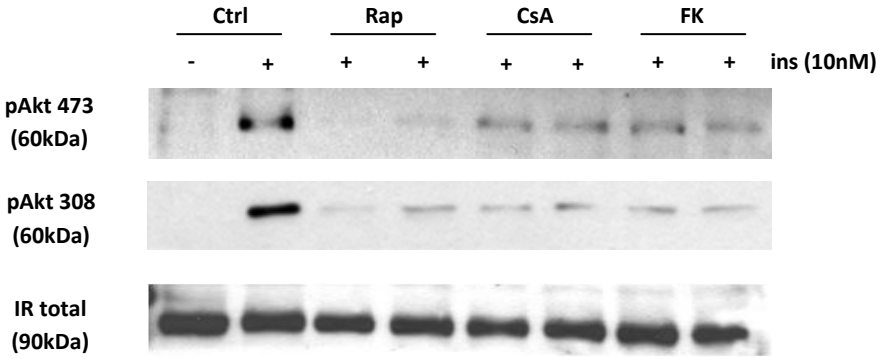

Figure 4. Differenciated brown adipocytes from rat primary culture were treated with IAs (1 μM CsA and FK and 100 nM for Rap) for 16 h, and then cells were serum-starved for 1h followed by stimulation with insulin (10nM) for 10min. Thereafter, cells were collected in lysis buffer. Representative western blots using antibodies against Akt (serine 473 and threonine 308) and total IR as protein loading control are shown. Abbreviations: Ctrl (control), CsA (cyclosporine A), FK (tacrolimus), Rap (rapamycin), ins (insulin).

Rapamycin rapidly induced the phosphorylation of c-Jun NH(2)-terminal kinase (JNK) and IRS-1 at serine 307 (Ser307)

Activation of stress kinases, particularly JNK, has been shown to inhibit insulin-mediated signalling in peripheral tissues by its ability to induce IRS-1 phosphorylation at Ser307, thereby functionally uncoupling IRS-1 from the insulin receptor and promoting IRS-1 degradation (Tanti and Jagger, 2009). On that basis we analyzed JNK phosphorylation in differentiated brown adipocytes treated with the different IAs. In initial experiments we treated differentiated brown adipocytes with 100 nM of rapamycin, CsA or FK506 for 1 hour. At this time-period only rapamycin was able to induce JNK phosphorylation as compared to non-stimulated cells (Figure 5A). However, increased phosphorylation of p38 MAPK, also belonging to the stress kinase family, was not observed in IAs-treated cells. Next, we analyzed the response of brown adipocytes to rapamycin or CsA in a more detailed time-course experiment. Results shown in Figure 5B indicate that the response to rapamycin in inducing JNK phosphorylation was very

rapid, with a maximal effect at 30 minutes. JNK phosphorylation was maintained up to 2 hours of rapamycin stimulation. Importantly, total levels of JNK remained as in control cells.

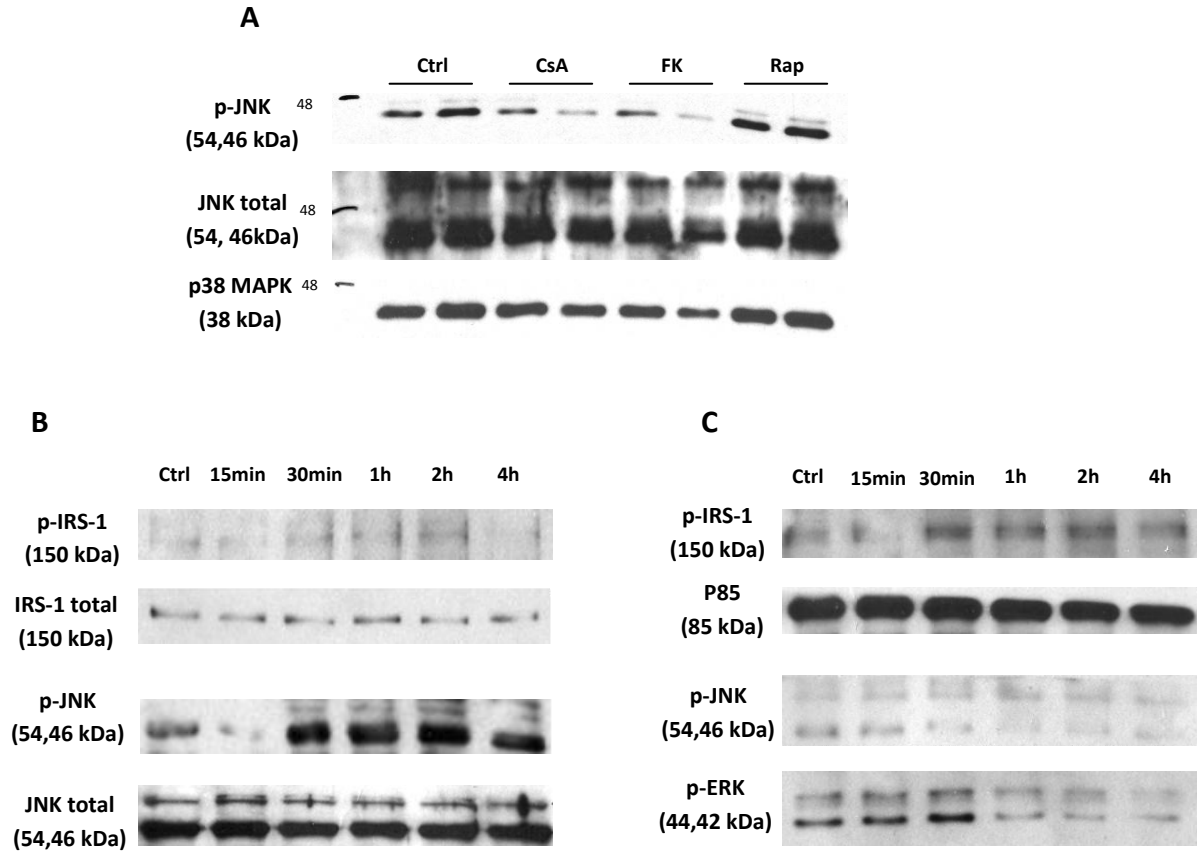

Figure 5. A) Differenciated brown adipocytes from rat (immortalized cells) were treated with IAs (100nM) for 1h. Thereafter, cells were collected in lysis buffer. Representative western blots using antibodies against phospho-JNK, total JNK and phospho-p38 MAPK of three independent experiments are shown. **B)** Differenciated brown adipocytes from rat (immortalized cells) were treated with Rap (100 nM) during different periods of time until 4 h. Representative western blots using phospho-JNK and total JNK are shown. **C)** Cells were stimulated as describe in B and treated with CsA (100 nM). Representative western blots using antibodies against phopho-IRS-1 (Ser307) and total IRS-1 are shown. Abbreviations: Ctrl (control), CsA (cyclosporine A), FK (tacrolimus), Rap (rapamycin).

To inspect if rapamycin-mediated JNK phosphorylation was able to phosphorylate IRS-1 at serine residues, we performed western blot analysis with the phospho-specific anti-IRS-1 Ser307 antibody. In agreement with JNK phosphorylation, treatment of differentiated brown adipocytes with rapamycin induced IRS-1 Ser phosphorylation within similar time-periods (30 minutes-2 hours) (Figure 5B). Besides, total levels of IRS-1 remained unchanged along the experiment.

Since CsA also decreased IRS-1 levels, we performed similar time-course with this compound. As shown in Figure 5C, CsA also increased IRS-1 Ser307 phosphorylation. Although CsA did not increase JNK phosphorylation as shown in Figure 5A, it increased ERK phosphorylation at very early time-periods (15-30 min), time at which IRS-1 Ser307 phosphorylation was detected.

Effect of IAs on insulin-induced glucose uptake and GLUT4 translocation in differentiated brown adipocytes

In primary fetal brown adipocytes, insulin induced glucose uptake in a PI 3-kinase-dependent manner (Valverde AM. et al., 1998). Therefore, we assayed the response to insulin in inducing glucose uptake in our *in vitro* cellular model of immortalized brown adipocytes upon differentiation. For this goal, differentiated brown adipocytes (day 8) were pretreatred with 100 nM rapamycin, CsA or FK506 for 16 h and glucose uptake was analyzed after insulin stimulation for 10 minutes as described in Materials and Methods. Figure 6A shows that differentiated brown adipocytes from rat responded to insulin in inducing glucose uptake with a threefold increase above the controls. Interestingly, this response was significantly decreased in cells that had been pretreated with IAs**.** These results were confirmed by the analysis of GLUT4 translocation by immunofluorescence. As shown is figure 6B, insulin-induced a rapid accumulation of GLUT4 in the plasma membrane in differentiated brown adipocytes whereas GLUT4 was accumulated in internal compartments in non-treated cells. By contrast, in differentiated brown adipocytes treated with IAs GLUT4 immunofluorescence was barely observed in the plasma membrane.

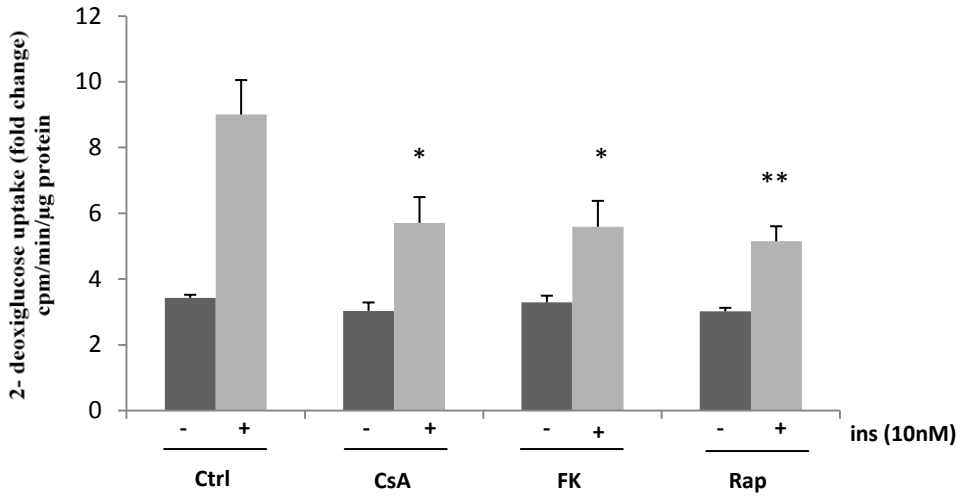

Figure 6. Brown adipocytes were differentiated for 8 days and treated with IAs (100 nM) for 16h. Then cells were serum-starved for 1 h followed by stimulation with insulin (100 nM) for 10min. Thereafter, glucose uptake was analyzed as described in Materials and Methods. Results are expressed in cpm/min/µg protein; *p<0.05, **p<0.01 IAs-treated insulin stimulated cells vs insulin stimulated cells (non-treated with IAs). Abbreviations: Ctrl (control), CsA (cyclosporine A), FK (tacrolimus), Rap (rapamycin), ins (insulin).

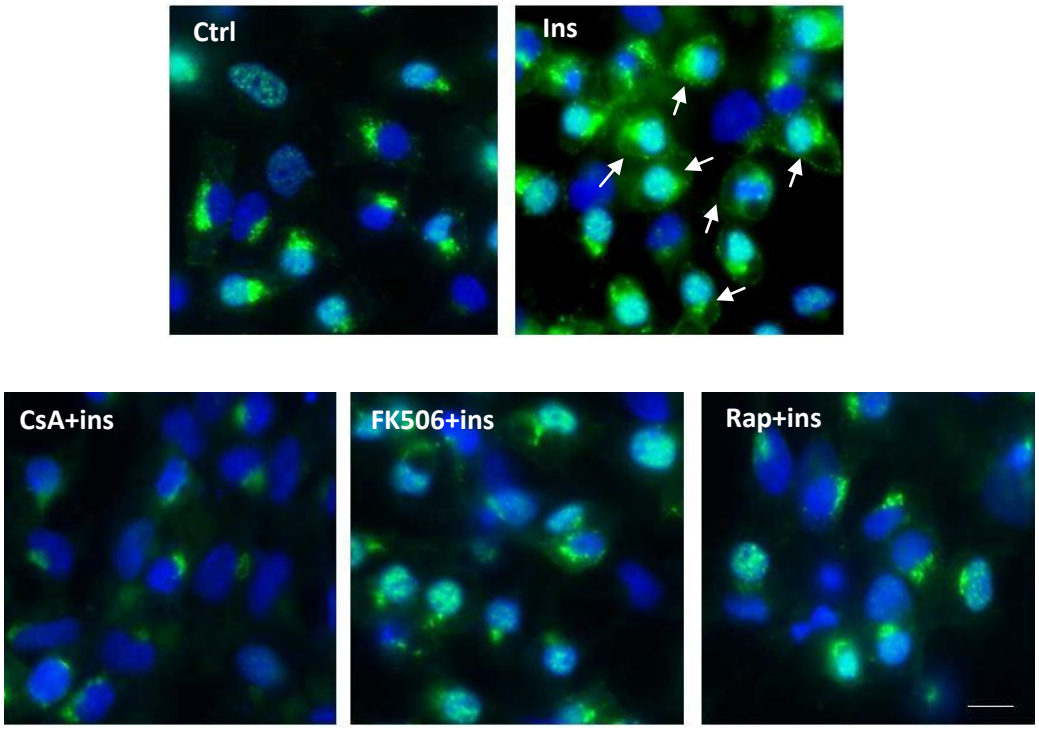

Figure 7. Brown adipocytes were differentiated for 8 days and treated with IAs (100 nM) for 16h. Then cells were serum-starved for 1 h followed by stimulation with insulin (100 nM) for 10min. Thereafter, Immunofluorescence and confocal imaging was analyzed as described in Materials and Methods. Immunofluorescence analysis of GLUT4 translocation were performed using anti-GLUT4 antibody. Nuclei were stained with DAPI. Representative images of two independent experiments are shown. Abbreviations: Ctrl (control), CsA (cyclosporine A), FK (tacrolimus), Rap (rapamycin), ins (insulin). Scale bar shows 60 µm.

Rapamycin decreased the response of differentiated brown adipocytes to norepinephrine (NE) in the induction of *Pgc1a* and *Ucp1* mRNA levels

It has been shown that NE stimulates *Pgc1a* and *Ucp1* mRNA levels in primary rat brown adipocytes (Cao W et al., 2004; Hernandez et al., 2011). Thus, we determined the effect of CsA, FK506 and rapamycin on thermogenesis by measuring *Pgc1a* and *Ucp1* mRNA in our in vitro cellular model of immortalized brown adipocytes upon differentiation. For this goal, brown adipocytes at day 8 of differentiation were cultured in differentiation medium supplemented with CsA or FK506 or rapamycin, each at 100 nM concentration during 24 hours. Then, NE (5 μM) was added directly to the culture plates for a further 16 hours. As shown in the Figures 8A and 8B, treatment with NE induced about 30-fold increases in *Ucp1* mRNA over basal levels and that induction was completely blunted by treatment with rapamycin. On the contrary, treatments with CsA and specially FK506 further induced *Ucp1* mRNA expression (2- and 3.5-fold). CsA and FK506 also increased in similar proportions the basal *Ucp1* mRNA expression, while rapamycin had no effect on basal *Ucp1* mRNA (Figure. 8C).

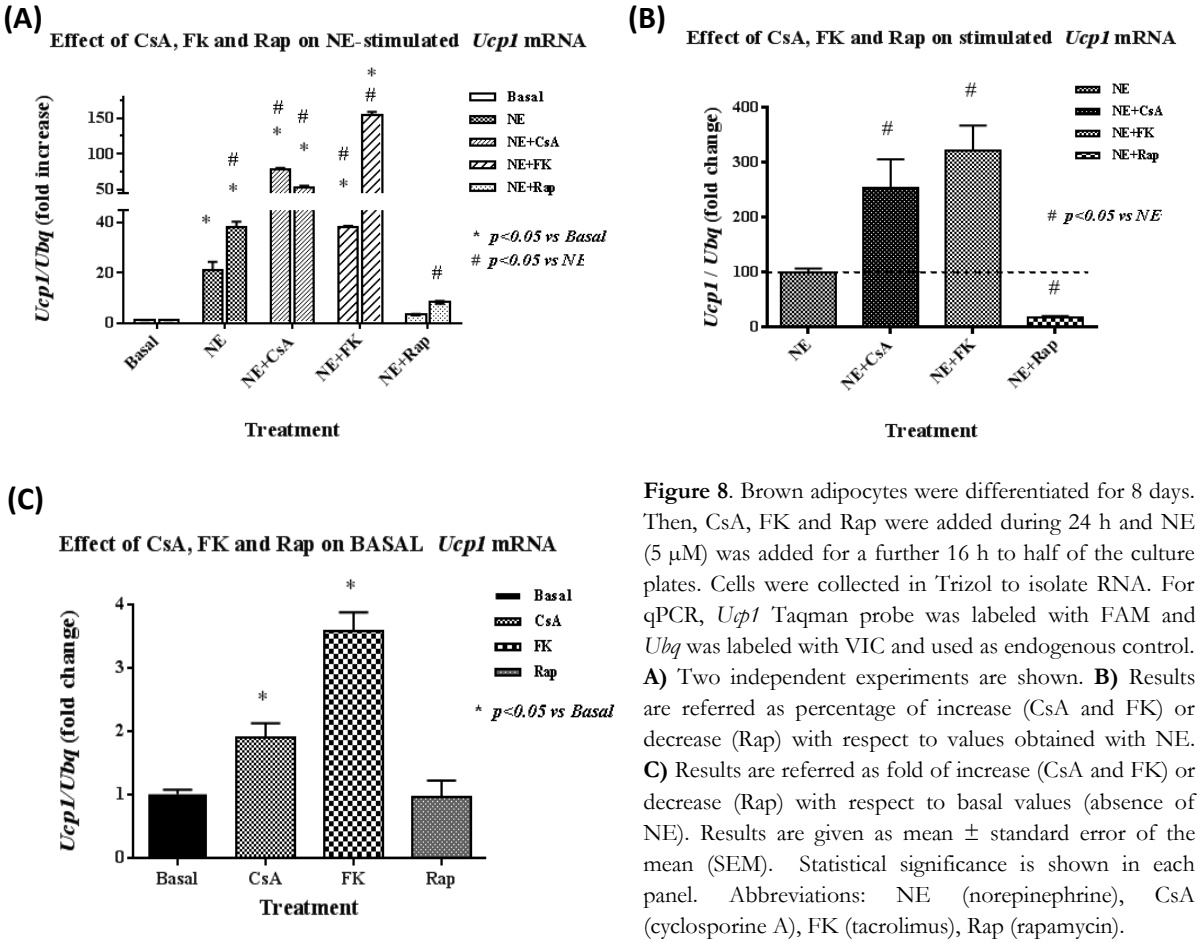

Figure 8. Brown adipocytes were differentiated for 8 days. Then, CsA, FK and Rap were added during 24 h and NE (5 μM) was added for a further 16 h to half of the culture plates. Cells were collected in Trizol to isolate RNA. For qPCR, *Ucp1* Taqman probe was labeled with FAM and *Ubq* was labeled with VIC and used as endogenous control. **A)** Two independent experiments are shown. **B)** Results are referred as percentage of increase (CsA and FK) or decrease (Rap) with respect to values obtained with NE. **C)** Results are referred as fold of increase (CsA and FK) or decrease (Rap) with respect to basal values (absence of NE). Results are given as mean ± standard error of the mean (SEM). Statistical significance is shown in each panel. Abbreviations: NE (norepinephrine), CsA (cyclosporine A), FK (tacrolimus), Rap (rapamycin).

Pgc1a was also determined using the same protocol of treatments. Similarly to the changes in *Ucp1* mRNA, the basal expression of *Pgc1a* mRNA was increased when using CsA and FK506 (2.5 and 3.5-fold, respectively), while no effect was observed with rapamycin (Figures 9A and 9B). As expected, treatment with NE increased *Pgc1a* mRNA by 3.4 fold over basal and such increases were further increased by CsA or FK506 pretreatments and decreased by rapamycin to basal values (Figure 9C). Therefore, the pattern of changes in *Pgc1a* mRNA was similar to that found in *Ucp1* mRNA.

At present, we are setting experiments with the Seahorse technology (see Materials and Methods) in order to get more insights on the effect of IAs on mitochondrial respiration in brown adipocytes.

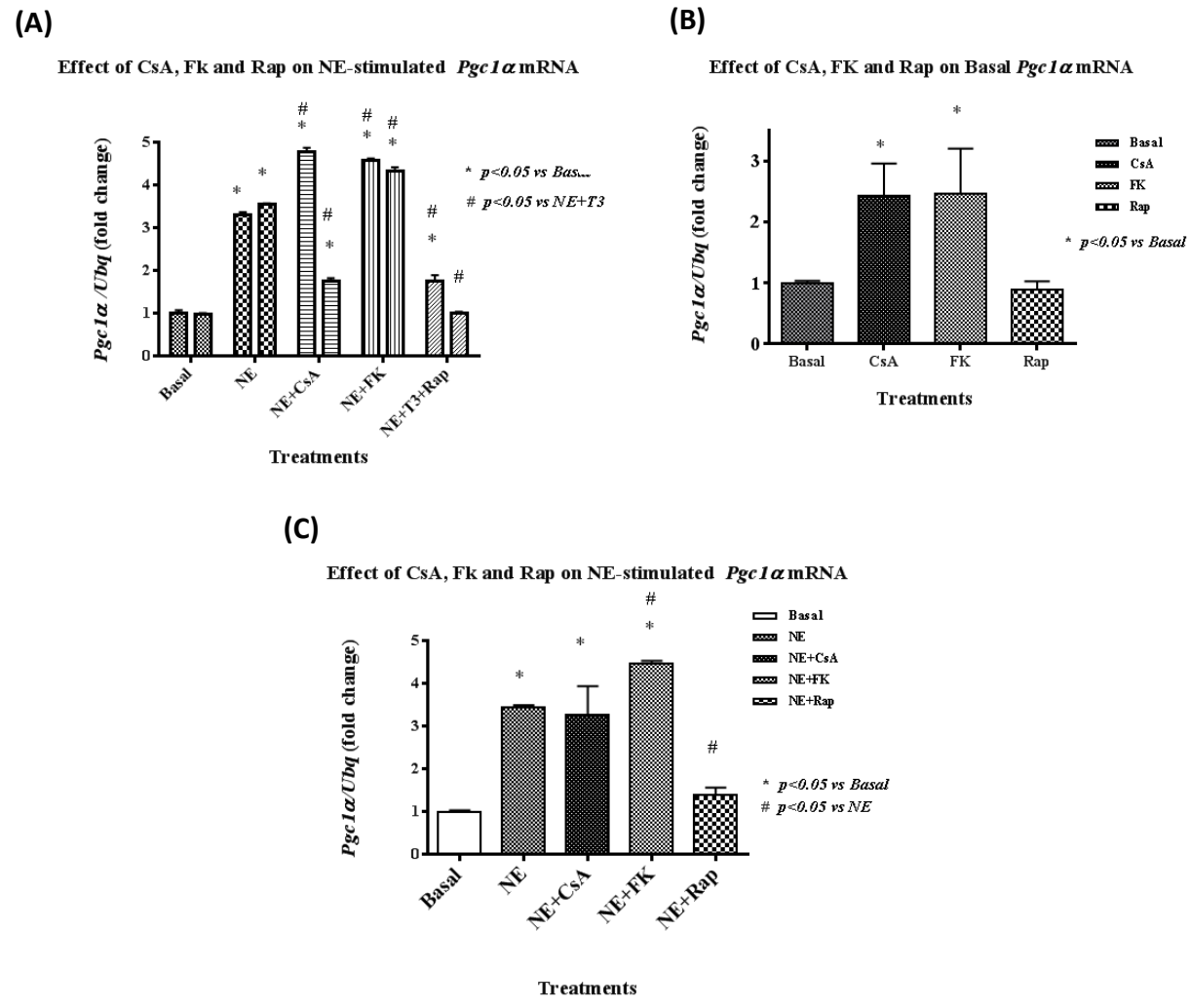

Figure 9 Brown adipocytes were differentiated for 8 days. Then, CsA, FK and Rap were added during 24 h and NE (5 μM) was added for a further 16 h to half of the culture plates. Cells were collected in Trizol to isolate RNA. For qPCR, *Ucp1* Taqman probe was labeled with FAM and *Ubq* was labeled with VIC and used as endogenous control. **A)** Two independent experiments are shown. **B)** Results are referred as percentage of increase (CsA and FK) or decrease (Rap) with respect to values obtained with NE. **C)** Results are referred as fold of increase (CsA and FK) or decrease (Rap) with respect to basal values (absence of NE). Results are given as mean ± standard error of the mean (SEM). Statistical significance is shown in each panel. Abbreviations: NE (norepinephrine), CsA (cyclosporine A), FK (tacrolimus), Rap (rapamycin).

V. Discussion

In rodents, glucose is an important fuel for BAT in vivo. Under physiological insulin stimulation, BAT has an extremely high rate of glucose metabolism (10% of the total glucose turnover rate in the rat), and a number of metabolic genes are induced. Among them, insulin plays an important role in the regulation of UCP1 expression in vivo. Indeed, the maintenance of normal concentrations of UCP1 in BAT has been shown to require normal plasma insulin levels. Conversely, insulin resistance in BAT appears in different physiological situations such as pregnancy, lactation and obesity ((Ferre et al. 1986, Lorenzo et al., 2005). All together these data identify the role of BAT in the overall control of glucose homeostasis. The importance in maintaining functional insulin signaling in BAT for the control of whole body glucose homeostasis was evidenced in mice with specific deficiency of IR in BAT (BATIRKO) that showed glucose intolerance due to a defect in insulin secretion (Guerra et al., 2001). Taking into account these findings, our results have demonstrated for the first time that brown adipocytes are target cells for immunosuppressant action, particularly by their ability to interfere with insulin signaling, suggesting that insulin resistance in BAT might contribute to PTDM.

The first evidence of this negative cross-talk between IAs and insulin signaling in differentiated brown adipocytes was the decrease in total IRS-1 levels upon long-term (16 h) treatment with rapamycin and CsA. During the last years, the IRS proteins have emerged as critical regulators of upstream signaling pathways leading to brown adipocyte differentiation. Particularly, IRS-1 deficiency totally impairs brown adipocyte differentiation (Fasshauer et al., 2001). IRS-1 is also necessary for full UCP1 expression in differentiated brown adipocytes (Tseng et al., 2004) and for its induction in response to insulin in brown preadipocytes (Valverde et al., 2003). Therefore, the fact that IRS-1 levels were decreased by rapamycin or CsA treatment prompted us to hypothesize that insulin signaling downstream IRS-1 that lead to metabolic actions of this hormone could be impaired. Not surprisingly, PKB/Akt phosphorylation at both Ser473 and Thr308 residues was blunted when insulin-stimulated brown adipocytes were pretreated with CsA, rapamycin and also FK506. While this impaired response is directly related with IRS-1 degradation by CsA or rapamycin, the effect of FK506 in blunting PKB/Akt phosphorylation is independent of defects in upstream insulin signaling. Besides, all three compounds significantly reduced insulin-induced GLUT4 translocation and glucose uptake that has been extensively demonstrated to be dependent on PKB/Akt (Huang and Czech, 2007). Our results are in agreement with previous findings in human white adipocytes showing impaired

insulin-induced glucose uptake and GLUT4 translocation upon pretreatment with IAs (Pereira MJ et al., 2012; Lopes P et al., 2013). However, slight differences have been found between the two types of adipocytes regarding insulin signaling. Whereas both calcineurin inhibitors did not affect PKB/Akt phosphorylation in white fat cells (Pereira MJ, unpublish results), both compounds blunted this response in brown adipocytes. These results suggest that activation of Akt/PKB is essential for mediating glucose uptake and GLUT4 translocation exclusively in brown adipocytes and, as suggested by Pereira MJ, CsA and FK506 do not allow translocation of GLUT4 to the cell surface of white adipocytes, as well as in L6 muscle cells, by increasing the rate constant for endocytosis. However, we cannot exclude the possibility of alterations in GLUT4 trafficking in brown fat cells treated with calcineurin inhibitors. On the other hand, rapamycin decreased PKB/Akt phosphorylation in both types of adipocytes (Pereira MJ et al., 2012 and our results) but with different responses in the levels of IRS proteins. Whereas rapamycin decreased IRS2 levels in subcutaneous human adipocytes, it decreased IRS-1 in brown adipocytes. Thus, altogether these results suggest different susceptibility of rapamycin-mediated IRS-1 and IRS2 degradation in different adipose cell types. This interesting issue deserves further investigations.

Phosphorylation of IRS-1 upon serine 307 has been shown to negatively regulate insulin signal transduction. Phosphorylation of serine 307 inhibits insulin-stimulated tyrosine phosphorylation and IRS-1-associated PI-3 kinase activity, potentially through disruption of the protein–protein interaction between IRS-1 and the IR. Serine 307 phosphorylation can be induced by cellular stressors, insulin, TNF-α and lipids, all factors that cause insulin resistance (reviewed by Copps and White MF, 2012). Several lines of evidence support a central role for mTOR in the development of insulin resistance and regulating the phosphorylation of IRS-1 serine 307. In this regard, rapamycin has been shown to prevent the development of insulin resistance and the degradation of IRS-1, likely through the inhibition of serine phosphorylation (Sun XJ et al, 1999; Pederson T et al., 2001; Berg CE et al., 2002). In agreement with these studies, rapamycin prevented IRS-1 serine 307 phosphorylation in human subcutaneous adipocytes (Pereira MJ et al., 2012). As stated above, our data in brown adipocytes clearly demonstrated that long-term (16 h) rapamycin treatment in differentiated brown adipocytes results in IRS-1 degradation that, importantly, is preceded by serine 307 phosphorylation at early time-periods (30 min-2 h). Collectively, our results in brown fat cells suggest that in addition to mTORC1, other/s serine/threonine kinases might be involved in this negative feed-back mechanism.

Anisomycin, a potent cellular stressor, downregulates IRS tyrosine phosphorylation in CHO cells, which is dependent on the presence of both serine 307 and the IRS-1 phosphotyrosine binding domain (Aguirre V et al., 2000). Although several kinases are activated by anisomycin, JNK1 directly binds to IRS-1. Moreover, JNK is activated by similar triggers that activate mTORC1 including hiperinsulinemia, nutrient overloading and oxidative stress (Aguirre et al., 2000; Copps and White, 2012). Importantly, we found that in brown adipocytes rapamycin rapidly induced JNK phosphorylation and this response was paralleled with IRS-1 serine 307 phosphorylation. Therefore in addition to the direct inactivation of the mTORC1 complex, rapamycin might increase oxidative stress leading to an increase in reactive oxygen species (ROS) that activate JNK which, in turn, phosphorylates IRS-1 at serine 307 in an mTORC1-independent manner. Related to this, very recent findings have shown a mechanism of mTOR inactivation by ROS-JNK-p53 pathway that plays an important role in autophagy-dependent senescence in L929 cells (Qi et al., Eur J Pharmacol 2013). Of note, neither p38 MAPK nor ERK were activated by rapamycin at early time-periods.

The direct or indirect activation of JNK by rapamycin seem to be specific of this compound since it was not observed with CsA or FK506. Of note, treatment of brown adipocytes with CsA for 16 hours also led to IRS-1 degradation similar to rapamycin. Similarly, CsA induced IRS-1 Ser307 phosphorylation in an early time-course experiment but in the absence of JNK phosphorylation. Instead, a rapid activation of ERK was observed, suggesting multiple regulatory mechanisms for IAs-mediated insulin resistance in brown adipocytes. Additional investigation is necessary to find out other possible modulators involved on such effects.

The effect of the IAs on UCP1 expression was rather different among them, because rapamycin had no effect on the basal UCP1 expression, but inhibited the NE-mediated increases of UCP1, while CsA and FK506 induced increases in both the basal and NE-stimulated UCP1 expression, with similar fold increases. We can speculate about the mechanisms that lead to such divergent results possibly related to the interference of these drugs on insulin signaling pathways. Indeed, in rat primary cultures of brown adipocytes we have observed that the absence of insulin or serum as well as the inhibition of PKB/Akt or ERK phosphorylation enhance UCP1 expression (unpublished results), and it is possible that the increases observed using CsA and FK506 could be related to this interference in insulin signaling pathways. On the other hand, the inhibitory effect of rapamycin could be related to an effect directly on the cAMP- NE or JNK-mediated pathways (Cao W et al., 2004).

The changes observed in PGC1α are rather parallel to the ones in UCP1, even though at a smaller scale. CsA and FK506 increase the basal PGC1α while rapamycin has no effect. PGC1α increased with NE, and rapamycin inhibited this increase while FK506 induced a small increase.

It has been shown that inhibition of mTOR by rapamycin reduces PGC1α gene expression in cells and mouse skeletal muscle, as well as reduced mitochondrial activity and capacity. The mechanism behind this evidence seems to be mediated through the transcription factor yin and yang (YY1), which is known to be a common target of PGC1α and mTOR (Wang L. et al., 2011, Cunningham JT.et al., 2007). Thus, regarding PGC1α, the reduced levels of its mRNA by rapamycin found in our study in brown adipocytes, could be due to this common feature between PGC1α and mTOR. Moreover, it is also known that mitochondrial targets of PGC1α involved in oxidative phosphorylation, the tricarboxylic acid cycle and uncoupling respiration are also downregulated by rapamycin (Cunningham JT.et al., 2007), corroborating our data that show the inability of NA in inducing UCP1 mRNA in the presence of rapamycin, but more information still is needed regarding brown adipose tissue.

VI. Concluding remarks

Over the last years, studies performed in BAT have led to a paradigm in our understanding of the importance of this unique tissue in adult humans, providing an incremented interest in the potential of activating BAT to enhance energy expenditure and therefore, fight against obesity and diabetes.

IAs has been extensively demonstrated to induce PTDM on subjects treated with these drugs in order to prevent allograft rejection after organ transplantation. Despite the efforts on providing to these patients a good quality of life, further investigation is needed so that, new IAs could be produced or the existing ones may be improved, not only for its proper use but also to decrease the impact of its side effects.

For the first time, this study evidenced the negative cross-talk between signaling pathways triggered by IAs in brown adipocytes and insulin signaling, being IRS-1 a critical modulator. Considering the thermogenic potential of BAT, these data might contribute to understand the physiological importance of brown adipocytes on the energy expenditure and its influence on insulin resistance in humans. Although *in vivo* data will reinforce our *in vitro* studies, insulin resistance in BAT might contribute to PTDM.

VII. Acknowledgements

Without the guidance and support of the following people, this work would not have been concluded.

First of all, I want to express my gratitude to my supervisor Eugénia Carvalho for her supervision, support and guidance, giving to me the opportunity to work on a great laboratory that produces magnificent science and to enrich my knowledge as a researcher.

I would like also to express my gratitude to my co-supervisor Angela Valverde who gave me the golden opportunity to do this wonderful project on the very interesting topic of brown adipocytes, which also helped me doing a lot of research and I came to know about so many new things. Her supervision and advices were of great importance during this project and was always ready to give support on many matters despite the many other ongoing research projects.

Special thank go to Maria Jesus Obregon, Ester Casarrubios and Laura García for their hospitality and friendship. Their coordination and support was of great importance to complete this project as also to learn so many different techniques and experimental tips. From the laboratory of Angela Valderde, I wish to thank my colleagues Maysa Mobasher, Virginia Pardo, Agueda Gonzalez, Pilar Valdecantos, Ana Arroba and Beatriz Santamaria for the help and bright ideas that allowed finishing the high number of experiments performed. From the laboratory of professor Eugénia, I want to thank my colleagues Patricia Lopes, Amelia Fuhrmann, Liane Moura, Marta Santos and Maria João Pereira for their guidance and interest in helping on my first journey as a researcher.

My eternal thankfulness is dedicated to my family, in special to my grandmother and mother, Ester and Maria Helena who have always supported me in my life.

To my "Madrillenos" friends, I want to say many thanks for their fellowship: Ana, Soledad, Sandra, Lara, Léon, Daniela, Petrina and many others.

My very last thank goes to my best friends whose I names don't need to mention.

For this project, I have been financially supported by the European Foundation for the Study of Diabetes (EFSD) which I am very thankful. Other grants: SAF2012-33283 (from MINECO, Spain to AMV), S2010-BMD-2423 (from Comunidad de Madrid, Spain, to AMV and MJO), PTDC/SAU-OSM/104124/2008.

VIII. References

1. Aguirre V, Uchida T, Yenush L, Davis R, White MF. The c-Jun NH2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser307. J Biol Chem 275:9047–9054, 2000
2. Arribas M, Valverde AM, Burks D, Klein J, Farese RV, White MF, Benito M Essential role of protein kinase C zeta in the impairment of insulin-induced glucose transport in IRS-2-deficient brown adipocytes. FEBS Lett. 11;536(1-3):161-6, 2003
3. Berg CE, Lavan BE, Rondinone CM. Rapamycin partially prevents insulin resistance induced by chronic insulin treatment. Biochem Biophys Res Commun. 10;293(3):1021-7. 2002
4. Berger J, Tanen M, Elbrecht A, Hermanowski-Vosatka A, Moller DE, Wright SD & Thieringer R. Peroxisome proliferator-activated receptor-gamma ligands inhibit adipocyte 11 beta-hydroxysteroid dehydrogenase type 1 expression and activity. Journal of Biological Chemistry 276 12629–12635, 2001
5. Böhmer AE, Mendes Ribeiro Corrêa A, de Souza DG, Knorr L, Hansel G, Corbellini LG, Driemeier D, Portela LV, Souza DO. Long-term cyclosporine treatment: evaluation of serum biochemical parameters and histopathological alterations in Wistar rats. Exp Toxicol Pathol. 63(1-2):119-123, 2009
6. Borel JF, Feurer C, Gubler HU, Stähelin H. "Biological effects of Cyclosporine A: a new antilymphocytic agent". Agents. Action. 6:468-475, 1976
7. Bouillaud F, D Ricquier, J Thibault, J Weissenbach: Molecular approach to thermogenesis in brown adipose tissue: cDNA cloning of the mitochondrial uncoupling protein. Proc Natl Acad Sci U S A82(2), 445-448, 1985
8. Bouillaud F, J Weissenbach, D Ricquier: Complete cDNA-derived amino acid sequence of rat brown fat uncoupling protein. J Biol Chem261(4), 1487-1490, 1986
9. Bujalska IJ, Kumar S, Hewison M & Stewart PM. Differentiation of adipose stromal cells: the roles of glucocorticoids and 11 beta-hydroxysteroid dehydrogenase. Endocrinology 140 3188–3196, 1999
10. Burén J, Liu HX, Jensen J, Eriksson JW. Dexamethasone impairs insulin signaling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes. Eur J Endocrinol. 146(3):419-29, 2002
11. Cao W, Daniel KW, Robidoux J, Puigserver P, Medvedev AV, Bai X, Floering LM, Spiegelman BM, Collins S. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. Mol Cell Biol. 24(7):3057-67, 2004
12. Chapman KE, Gilmour JS, Coutinho AE, Savill JS, Seckl JR. 11beta-Hydroxysteroid dehydrogenase type 1—A role in inflammation? Mol Cell Endocrinol. 27;248(1-2):3-8. 2006
13. Charmaine S. Tam, Virgile Lecoultre and Eric Ravussin. "Brown Adipose Tissue : Mechanisms and Potential Therapeutic Targets", Circulation 125:2782-2791, 2012
14. Cohade C, Mourtzikos KA, Wahl RL. "USA-Fat": prevalence is related to ambient outdoor temperature-evaluation with 18F-FDG PET/CT. J Nucl Med. 44:1267–1270, 2003

15. Copps KD, White MF. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS-1 and IRS2. Diabetologia. 55(10): 2565-82, 2012
16. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol Cell Endocrinol. 15; 335(1): 2–13, 2011
17. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature. 2007 Nov 29;450(7170):736-40.
18. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM, Kahn CR. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 9;360(15):1509-17, 2009
19. Deleuze S, Garrigue V, Delmas S, Chong G, Swarcz I, Cristol JP, Mourad G. New-onset dyslipidemia after renal transplantation; is there a diference between tacrolimus and cyclosporine? Transplant Proc. 38(7):2311-3, 2006
20. Di Paolo S, Teutonico A, Leogrande D, Capobianco C, Schena PF. Chronic inhibition of mammalian target of rapamycin signaling downregulates insulin receptor substrates 1 and 2 and Akt activation: A crossroad between cancer and diabetes? J Am Soc Nephrol. 17(8):2236-44, 2006
21. Draper N, Stewart PM. 11beta-hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. J Endocrinol; 186(2):251-71, 2005
22. Düfer M, Krippeit-Drews P, Lembert N, Idahl LA, Drews G. Diabetogenic effect of cyclosporin A is mediated by interference with mitochondrial function of pancreatic B-cells. Mol Pharmacol. 60(4):873-9, 2001
23. Eguchi A, Takei T, Yoshida T, Tsuchiya K, Nitta K. Combined cyclosporine and prednisolone therapy in adult patients with the first relape of minimal-change nephrotic syndrome. Nephrol Dial Transplant. 25(1):124-9, 2010
24. Fasshauer M, Klein J, Ueki K, Kriauciunas KM, Benito M, White MF, Kahn CR Essential role of insulin receptor substrate-2 in insulin stimulation of Glut4 translocation and glucose uptake in brown adipocytes. J Biol Chem. 18;275(33):25494-501, 2000
25. Fasshauer M, Klein J, Kriauciunas KM, Ueki K, Benito M, Kahn CR. Essential role of insulin receptor substrate 1 in differentiation of brown adipocytes. Mol Cell Biol. 21(1):319-29, 2001
26. Foley, J.E. Rationale and application of fatty acid oxi-dation inhibitors in treatment of diabetes mellitus. Diabetes Care 15, 773–784, 1992
27. Fujimoto S, Nabe K, Takehiro M, Shimodahira M, Kajikawa M, Takeda T, Mukai E, Inagaki N, Seino Y. Impaired metabolism-secretion coupling in pancreatic beta-cells: role of determinants of mitochondrial ATP production. Diabetes Res Clin Pract. 77 S2-S10, 2007
28. Gesta S, Tseng YH, Kahn CR. "Developmental origin of fat: tracking obesity to its source". Cell 131 (2): 242–56. 2007
29. Hasan EA, Jessop DS, Power LL, Monk PT, Kirwan JR. Use of dexamethasone-corticotrophin releasing hormone test to access hypothalamic-pituitary-adrenal axis function in rheumatoid arthritis. Int J Endocrinol. 2009:391284, 2009
30. Heit JJ, Apelqvist AA, Gu X, Winslow MM, Neilson JR, Crabtree GR, Kim SK. Calcineurin/NFAT signaling regulates pancreatic beta-cell growth and function. Nature. 21;443(7109):345-9, 2006
31. Heit JJ. Calcineurin/NFAT signaling in the beta-cell: From diabetes to new therapeutics. Bioessays. 29(10):1011-21, 2007

32. Hernandez A, de Mena RM, Martin E, Obregon MJ. Differences in the response of UCP1 mRNA to hormonal stimulation between rat and mouse primary cultures of brown adipocytes. Cell Physiol Biochem. 28(5):969-80, 2011
33. Hjelmesaeth J, Asberg A, Müller F, Hartmann A, Jenssen T. New-onset post transplantation diabetes mellitus: insulin resistance or insulinopenia? Impact of immunosuppressive drugs, cytomegalovirus and hepatitis C virus infection. Curr Diabetes Rev. 1(1):1-10, 2005
34. Houde VP, Brûlé S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, Marette A. Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. Diabetes. 59(6):1338-48, 2010
35. Huang S, Czech MP. The GLUT4 glucose transporter. Cell Metab. 5(4):237-52, 2007
36. Kauh EA, Mixson LA, Shankar S, McCarthy J, Maridakis V, Morrow L, Heinemann L, Ruddy MK, Herman GA, Kelley DE, Hompesch M. Short-term metabolic effects of prednisone administration in healthy subjects. Diabetes Obes Metab. 13(11):1001-7, 2011
37. Kim JW, Sun C, Jeon SY, You YH, Shin JY, Lee SH, Cho JH, Park CG, Yoon KH. Glucocorticoid treatment independently affects expansion and transdifferentiation of porcine neonatal pancreas cell clusters. BMB Rep. 45(1):51-6, 2012
38. Lablanche S, Cottet-Rousselle C, Lamarche F, Benhamou PY, Halimi S, Leverve X, Fontaine E. Protection of pancreatic INS-1 beta-cells from glucose- and fructose-induced cell death by inhibiting mitochondrial permeability transition with cyclosporine A or metformin. Cell Death Dis. 24;2:e134, 2011
39. Lopes P, Fuhrmann A, Sereno J, Pereira MJ, Nunes P, Pedro J, Melão A, Reis F, Carvalho E. Effects of cyclosporine and sirolimus on insulin-stimulated glucose transport and glucose tolerance in a rat model. Transplant Proc. 45(3):1142-8, 2013
40. Maechler P, Wollheim CB. Mitochondrial function in normal and diabetic beta-cells. Nature 13;414(6865):807-12, 2001
41. Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR & Flier JS. A transgenic model of visceral obesity and the metabolic syndrome. Science 294 2166–2170, 2001
42. Miranda S, González-Rodríguez A, Revuelta-Cervantes J, Rondinone CM, Valverde AM. Beneficial effects of PTP1B deficiency on brown adipocyte differentiation and protection against apoptosis induced by pro- and anti-inflammatory stimuli. Cell Signal. 22(4):645-59, 2010
43. Néchad M, Kuusela P, Carneheim C, Björntorp P, Nedergaard J, Cannon B. Development of brown fat cells in monolayer culture. I. Morphological and biochemical distinction from white fat cells in culture. Exp Cell Res. 149(1):105-18, 1983
44. Ohno H, Shinoda K, Spiegelman BM, Kajimura S. PPARγ agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. Cell Metab. 7;15(3):395-404, 2012
45. Ortega-Molina A, Efeyan A, Lopez-Guadamillas E, Muñoz-Martin M, Gómez-López G, Cañamero M, Mulero F, Pastor J, Martinez S, Romanos E, Mar Gonzalez-Barroso M, Rial E, Valverde AM, Bischoff JR, Serrano M. Pten Positively Regulates Brown Adipose Function, energy Expenditure, and longevity. Cell Metab. 7;15(3):382-94, 2012
46. Ost A, Svensson K, Ruishalme I, Brännmark C, Franck N, Krook H, Sandström P, Kjolhede P, Strålfors P. Attenuated mTOR signaling and enhanced autophagy in adipocytes from obese patients with T2D. Mol Med. 16(7-8):235-46, 2010
47. Øzbay LA, Smidt K, Mortensen DM, Carstens J, Jørgensen KA, Rungby J. Cyclosporin and tacrolimus impair insulin secretion and transcriptional regulation in INS-1E beta-cells. Br J Pharmacol. 162(1):136-46, 2011

48. Pederson TM, Kramer DL, Rondinone CM. Serine/threonine phosphorylation of IRS-1 triggers its degradation: possible regulation by tyrosine phosphorylation. Diabetes. 2001 Jan;50(1):24-31.
49. Pereira MJ, Palming J, Rizell M, Aureliano M, Carvalho E, Svensson MK, Eriksson JW. mTOR inhibition with rapamycin causes impaired insulin signaling and glucose uptake in human subcutaneous and omental adipocytes. Mol Cell Endocrinol. 15;355(1):96-105. 2012
50. Pham PT, Pham PM, Pham SV, Pham PA, Pham PC. "New onset diabetes after transplantation (NODAT): an overview" Diabetes Metabolic Syndrome Obesity. 4:175-186, 2011
51. Prasad SS, Prashanth A, Kumar CP, Reddy SJ, Giridharan NV, Vajreswari A. A novel genetically-obese rat model with elevated 11 beta-hydroxysteroid dehydrogenase type 1 activity in subcutaneous adipose tissue. Lipids Health Dis. 17;9:132, 2010
52. Qi D, Rodrigues B. Glucocorticoids produce whole body insulin resistance with changes in cardiac metabolism. Am J Physiol Endocrinol Metab. 292(3):E654-67, 2007
53. Qi M, Zhou H, Fan S, Li Z, Yao G, Tashiro SI, Onodera S, Xia M, Ikejima T. mTOR inactivation by ROS-JNK-p53 pathway plays an essential role in Psedolaric acid B induced autophagy-dependent senescence in murine fibrosarcoma L929 cells. Eur J Pharmacol. pii: S0014-2999(13)00484-6. 2013 [Epub ahead of print]
54. Rafacho A, Marroquí L, Taboga SR, Abrantes JL, Silveira LR, Boschero AC, Carneiro EM, Bosqueiro JR, Nadal A, Quesada I. Glucocorticoids in vivo induce both insulin hypersecretion and enhanced glucose sensitivity of stimulus-secretion coupling in isolated rat islets. Endocrinology. 151(1):85-95, 2010
55. Ranta F, Avram D, Berchtold S, Düfer M, Drews G, Lang F, Ullrich S. Dexamethasone induces cell death in insulin-secreting cells, an effect reversed by exendin-4. Diabetes. 55(5):1380-90, 2006
56. Richard D, Picard F. "Brown fat biology and thermogenesis". Frontiers in Bioscience 16, 1233-1260, January 1, 2011
57. Ricquier D, J Thibault, F Bouillaud, Y Kuster: Molecular approach to thermogenesis in brown adipose tissue. Cell-free translation of mRNA and characterization of the mitochondrial uncoupling protein. J Biol Chem258(11), 6675-6677, 1983
58. Rodriguez-Rodriguez AE, Triñanes J, Velazquez-Garcia S, Porrini E, Vega Prieto MJ, Diez Fuentes ML, Arevalo M, Salido Ruiz E, Torres A. The Higher Diabetogenic Risk of Tacrolimus Depends on Pre-Existing Insulin Resistance. A Study in Obese and Lean Zucker Rats, 2013 [Epub ahead of print]
59. Saiah E. The role of 11beta-hydroxysteroid dehydrogenase in metabolic disease and therapeutic potentialof 11beta-hsd1 inhibitors. Curr Med Chem. 15(7):642-9, 2008
60. Sakoda H, Ogihara T, Anai M, Funaki M, Inukai K, Katagiri H, Fukushima Y, Onishi Y, Ono H, Fujishiro M, Kikuchi M, Oka Y, Asano T. Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. Diabetes. 49(10):1700-8, 2000
61. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell. 21;22(2):159-68, 2006
62. Shaw JE., Sicree, R.A.; Zimmet, P.Z. "Global estimates of the prevalence of diabetes for 2010 and 2030" Diabetes Res. Clin. Pract. 87: 4-14, 2010
63. Shimodahira M, Fujimoto S, Mukai E, Nakamura Y, Nishi Y, Sasaki M, Sato Y, Sato H, Hosokawa M, Nagashima K, Seino Y, Inagaki N. Rapamycin impairs metabolism-secretion coupling in rat pancreatic islets by suppressing carbohydrate metabolism. J Endocrinol. 204(1):37-46, 2010

64. Smorlesi A, Frontini A, Giordano A, Cinti S. The adipose organ: white-brown adipocyte plasticity and metabolic inflammation. Obes Rev: 13 Suppl 2:83-96, 2012
65. Subramanian S, Trence DL. Immunosuppressive Agents: Effects on Glucose and Lipid Metabolism. Endocrinol Metab Clin North Am. 36(4):891-905, 2007
66. Sun XJ, Goldberg JL, Qiao LY, Mitchell JJ Insulin-induced insulin receptor substrate-1 degradation is mediated by the proteasome degradation pathway Diabetes. 48(7):1359-64, 1999
67. Takano A, Usui I, Haruta T, Kawahara J, Uno T, Iwata M, Kobayashi M. Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Mol Cell Biol. 21(15):5050-62, 2001
68. Tanti J. F., Jager J. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. Curr. Opin. Pharmacol. 9, 753–762, 2009
69. Thomson AW, Carroll PB, McCauley J, Woo J, Abu-Elmagd K, Starzl TE, Van Thiel DH. FK 506: a novel immunosupressant for treatment of autoimmune diseases. Rationale and preliminary clinical experience at the University of Pittsburgh. Springer Semin Immunopathol. 14(4):323-44, 1993
70. Tontonoz P, BM Spiegelman: Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem77, 289-312, 2008
71. Tseng YH, Kriauciunas KM, Kokkotou E, Kahn CR. Differential roles of insulin receptor substrates in brown adipocyte differentiation. Mol Cell Biol. 24(5):1918-29, 2004
72. Valverde AM, Kahn CR, Benito M. Insulin signaling in insulin receptor substrate (IRS) -1-deficient brown adipocytes: requirement of IRS-1 for lipid synthesis. Diabetes. 48(11):2122-31, 1999
73. Valverde AM, Benito M, Lorenzo M. The brown adipose cell: a model for understanding the molecular mechanisms of insulin resistance. Acta Physiol Scand. 183(1):59-73, 2005
74. Valverde AM, Teruel T, Navarro P, Benito M, Lorenzo M. Tumor necrosis factor-alpha causes insulin receptor substrate-2-mediated insulin resistance and inhibits insulin-induced adipogenesis in fetal brown adipocytes. Endocrinology. 139(3):1229-38, 1998
75. Valverde AM, Arribas M, Mur C, Navarro P, Pons S, Cassard-Doulcier AM, Kahn CR, Benito M. Insulin-induced up-regulated uncoupling protein-1 expression is mediated by insulin receptor substrate 1 through the phosphatidylinositol 3-kinase/Akt signaling pathway in fetal brown adipocytes. J Biol Chem. 21;278(12):10221-31, 2003
76. Veilleux A, Houde VP, Bellmann K, Marette A. Chronic inhibition of mTOR/S6K1 pathway increases insulin-induced PI3K activity but inhibits Akt2 and glucose transport stimulation in 3T3-L1 adipocytes. Mol Endocrinol. 24(4):766-78, 2010
77. Wang L, Mascher H, Psilander N, Blomstrand E, Sahlin K. Resistance exercise enhances the molecular signaling of mitochondrial biogenesis induced by endurance exercise in human skeletal muscle. J Appl Physiol. 111(5):1335-44, 2011
78. White UA, JM Stephens: Transcriptional factors that promote formation of white adipose tissue. Mol Cell Endocrinol 318(1-2), 10-14, 2010
79. Wolf A, Trendelenburg CF, Diez-Fernandez C, Prieto P, Houy S, Trommer WE, Cordier A. Cyclosporine A-induced oxidative stress in rat hepatocytes. J Pharmacol Exp Ther. 280(3):1328-34, 1997
80. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE. "Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkβ" Science 293(5535): 1673-1677, 2001
81. Zhang P, Zhang X, Brown J, Vistisen D, Sicree R, Shaw J, Nichols G. Global healthcare expenditure on diabetes for 2010 and 2030. Diabetes Res Clin Pract. 87(3): 293-301, 2010

82. Zingaretti MC, Crosta F, Vitali A, Guerrieri M, Frontini A, Cannon B, Nedergaard J, Cinti S. The presence of UCP1 demonstrates that meta-bolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue.FASEB J. 23:3113–3120, 2009