MECHANISMS OF RAPAMYCIN TOXICITY IN PANCREATIC BETA CELLS

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CONTENTS

Chapter 1 – Introduction: islet transplantation and immunosuppression

Chapter 2 – Introduction: actions of rapamycin

Chapter 3 – Materials and methods

Chapter 4 – Experimental results: effect of rapamycin on pancreatic β cells

Chapter 5 – Experimental results: mechanisms of rapamycin toxicity in pancreatic β cells

ABSTRACT

Islet transplantation offers a potential cure for type I diabetes mellitus. The publication of the landmark Edmonton study in 2000, which reported insulin independence in seven consecutive patients, prompted increased interest in this therapy. However, enthusiasm was tempered when the 5 year follow up results of this study were published, with only 10% of recipients maintaining insulin independence. The cause for this graft loss is multifactorial, but there is in vivo and in vitro evidence that suggests immunosuppressive drug toxicity plays an important role. The aim of this thesis was to establish the effects of rapamycin, one of the primary immunosuppressants used in islet transplantation, on murine β cells and islets and elucidate the mechanisms of any toxicity seen.

The intracellular target for rapamycin is mTOR which exists in two complexes, mTORC1 and mTORC2. mTORC1 primarily regulates cell size and proliferation; whereas mTORC2 plays a key role in regulating cell survival via protein kinase B (PKB).

This thesis has demonstrated that rapamycin treatment results in significant reductions in glucose stimulated insulin secretion in the MIN6 mouse insulinoma cell line and isolated rat islets, as well as increased apoptosis in these cell types. Furthermore, it has shown that prolonged rapamycin results in inhibition of mTORC2 assembly, with resultant inhibition of PKB phosphorylation and activity. Overcoming rapamycin induced mTORC2 inhibition with an adenovirus encoding constitutively active PKB ameliorates the detrimental effects of rapamycin on both MIN6 cells and rat islets. This suggests that rapamycin toxicity is mediated predominantly via mTORC2 rather than mTORC1 inhibition.

This work brings into question the use of rapamycin as an immunosuppressant in islet transplantation and also highlights the key role of PKB in β cell survival. Therapies resulting in PKB activation may have the potential to improve outcomes of islet transplantation.

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ABBREVIATIONS

4E-BP	eIF4E binding protein
ADB	assay dilution buffer
AdCaPKB	adenovirus encoding constitutively active PKB
ADP	adenosine diphosphate
AGC	cAMP-dependent, cGMP dependent and protein kinase C
AMP	adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ANOVA	analysis of variance
APS	ammonium persulfate
ASK	apoptosis signal-regulating kinase
ATG	antithymocyte globulin
ATP	adenosine triphosphate
BAD	Bcl_2/$BclX_L$ antagonist causing cell death
bFGF	basic fibroblast growth factor
BMI	body mass index
CDK	cyclin-dependent kinase
CREB	cAMP response element binding
DMEM	Dulbecco modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
eIF	eukaryotic translation initiation factor
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial sodium channel
EPC	endothelial progenitor cell
ERK	extracellular signal-related kinase
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FKBP	FK506 binding protein
FRB	FKBP-rapamycin binding
GAD	glutamic acid decarboxylase
GAP	GTPase activating protein
GLP	glucagon-like peptide
GSIS	glucose stimulated insulin secretion
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
GβL	G protein β-subunit-like protein
HDC	human ductal cell
HIF	hypoxia inducible factor

HLA	human leucocyte antigen
HM	hydrophobic motif
IBMIR	immediate blood-mediated inflammatory response
IGF	insulin-like growth factor
IP	immunoprecipitation
IPGTT	intra-peritoneal glucose tolerance testing
IRS	insulin receptor substrate
JNK	c-jun NH_2-terminal kinase
kdPKB	kinase dead PKB
KGDH	α-ketoglutarate dehydrogenase
KRB	Krebs Ringer buffer
MAPK	mitogen-activated protein kinase
MDM2	murine double minute 2
mLST8	mammalian lethal with Sec13 protein 8
MMF	mycophenolate mofetil
MOI	multiplicity of infection
MPA	mycophenolic acid
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
MTT	3-(4,5-Dimethylthiazol-2-yl)-diphenyltetrazolium bromide
NPI	neonatal porcine islet
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDK	phosphoinositide dependent kinase
PET	positron emission tomography
PGC	peroxisome-proliferator-activated receptor coactivator
PH	pleckstrin homology
PHLPP	PH-domain leucine-rich repeat phosphatase
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIK	phosphatidylinositol kinase
PIP_2	phosphatidylinositol 4,5-bisphosphanate
PIP_3	phosphatidylinositol 3,4,5-bisphosphanate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKI	PKA specific inhibitor peptide
PMSF	phenylmethylsulfonyl fluoride
PP2A	protein phosphatase 2A
PRAS40	proline-rich Akt/PKB substrate 40 kDa
Protor	protein observed with rictor

PVDF	polyvinylidene fluoride
Raptor	regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rictor	rapamycin insensitive companion of mTOR
RPMI	Roswell Park Memorial Institute
rpS6	ribosomal protein S6
S4	syndecan 4
S6K	S6 kinase
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SGK	serum- and glucocorticoid-induced protein kinase
SIN	stress activated protein kinase interacting protein
siRNA	short interfering RNA
STAT	signal transducer and activator of transcription
TEMED	tetramethylethylenediamine
TM	turn motif
TNF	tumour necrosis factor
TOP	5’ terminal oligopyrimidine tract
TOR	target of rapamycin
TRAIL	TNF-related apoptosis-inducing ligand
TSC	tumour suppressor complex
TUNEL	TdT-mediated dUTP nick end labelling
UIC	University of Illinois at Chicago
UKITC	United Kingdom islet transplant consortium
VEGF	vascular endothelial growth factor
WB	Western blotting
WOP	whole organ pancreas
XIAP	X-linked inhibitor of apoptosis
YY1	yin yang 1

CHAPTER 1

Introduction: islet transplantation and immunosuppression

1.1 Type I diabetes mellitus

Type I diabetes mellitus is a metabolic disorder characterized by chronic hyperglycaemia due to insulin deficiency caused by destruction of the insulin secreting beta (β) cells found in the islets of Langerhans in the pancreas. The aetiology of this destruction is yet to be fully elucidated, but autoimmunity plays a strong role, with autoantibodies directed against islet constituents present in up to 90% of newly diagnosed patients (Bilbao, Rica et al. 2000). Chronic hyperglycaemia results in secondary complications of diabetes such as nephropathy, retinopathy, neuropathy and vasculopathy. The Diabetes Control and Complications trial demonstrated that intensive insulin therapy can delay the onset and slow progression of retinopathy, albuminuria and neuropathy. However, this was at the expense of a two to threefold increase in severe hypoglycaemia (The Diabetes Control and Complications Trial Research Group 1993).

1.2 Transplantation for type I diabetes mellitus

The complications and inconvenience of long term insulin therapy for diabetes has led to a search for other therapies. Transplantation of insulin secreting β cells, either as part of a whole organ pancreas (WOP) transplant or as an isolated islet transplant has long been an attractive alternative to long term insulin therapy. Transplantation of the whole pancreas is an established treatment option for some type I diabetics, with over 1,000 WOP transplants performed annually in the United States (U.S. Department of Health and Human Services 2008) and approximately 200 performed annually in the United Kingdom (NHS Blood and Transplant 2009). However, despite advances in technique and post operative management WOP transplantation remains a high risk procedure with a reported morbidity of 25-50% and 1 year mortality of 1-5% (Frank, Deng et al. 2004, Gerber, Pavlicek et al. 2008, Gruessner, Sutherland et al. 2008, Gruessner, Sutherland 2005). Since islet tissue only accounts for 1%

of the total volume of the pancreas, and is all that is required for treatment of type I diabetes, transplantation of the islets alone provides a more elegant solution and one that should, in principle, be associated with less major complications.

1.3 Development of islet transplantation

The first reported islet transplants were carried out in 1893 in Bristol, where extracts of sheep's pancreas were given both orally and subcutaneously to two patients with glycosuria. Unsurprisingly, in the absence of immunosuppression treatment failed in both instances (Watson 1894). The first successful series of islet allografts was reported in 1990 in nine patients undergoing upper abdominal exenteration and liver transplantation, five of whom maintained insulin independence for more than 6 months (Tzakis, Ricordi et al. 1990). Over the course of the 1990s interest in islet transplantation developed and the numbers performed worldwide slowly increased. However, early outcomes were poor with 200 islet allograft transplants performed worldwide between 1990 and 1997 resulting in 96% patient survival, but only 35% graft survival, assessed as basal C-peptide $\geq$ 0.5 ng/ml, and only 10% insulin independence for more than 7 days (Brendel, Hering et al. 1999).

In 2000 the Edmonton group published a landmark report of seven consecutive islet transplant recipients who all attained insulin independence for a median follow-up period of 11.9 months (Shapiro, Lakey et al. 2000). The Edmonton protocol included several changes to previous islet transplant protocols which can be deemed fundamental to the improved outcomes seen. Firstly the immunosuppressive protocol, which is discussed in further detail in section 1.5.1, avoided the diabetogenic effects of glucocorticoids. In addition, further refinements were made to the gold standard islet isolation procedure developed initially by Camillo Ricordi (Ricordi, Lacy et al. 1988), increasing both the yield and purity of the isolate. Thirdly, recipients received multiple islet transplants until insulin independence was

achieved; six patients requiring two metachronous transplants and one requiring a third transplant with islets pooled from two donors. Overall, the total transplanted islet mass contained between 9000 and 14000 islet equivalents per kilogram of recipient's body weight.

The results of the Edmonton trial initiated a surge in enthusiasm for islet transplantation as a potential cure for type 1 diabetes and prompted other units worldwide to set up islet transplant programmes. However, enthusiasm was tempered when the 5 year results of the initial cohort of patients from Edmonton was reported, with only approximately 10% maintaining insulin independence (Ryan, Paty et al. 2005). Although the cause of this decline in islet graft function is multifactorial (see section 1.7), there is some evidence that it is in part related to the toxicity of the immunosuppressive agents used. This has led to a re-evaluation of the most suitable immunosuppressive regimen for islet cell transplantation.

1.4 Principles of islet transplantation

In general, pancreata from cadaveric donors are first offered for whole organ pancreas transplantation. If the organ is not deemed suitable for whole organ transplantation, often because the donor has a high body mass index (BMI), it is offered for islet transplantation. Although raised donor BMI is often seen as a contraindication for WOP transplantation, islet yield tends to be higher from this group of patients as compared to leaner donors (Ricordi 1991, Zeng, Torre et al. 1994), making them more suitable for islet transplantation. Other donor factors shown to significantly influence islet yield include organ cold ischaemic time, donor age, donor blood glucose levels and cause of donor death (Zeng, Torre et al. 1994).

The isolation of islets from the pancreas still follows the general principles introduced by Ricordi in 1988 (Ricordi, Lacy et al. 1988). This involves separation of the islets from the surrounding exocrine tissue in a digestion chamber using a mechanically enhanced continuous enzymatic digestion process. Liberase (Roche Diagnostics GmbH, Mannheim,

Germany) a highly purified blend of collagenase class I and II, combined with the neutral protease thermolysin and with low endotoxins (Brandhorst, Brandhorst et al. 2003, Linetsky, Bottino et al. 1997), is currently the main enzyme utilised. Although the use of Liberase increases the consistency, yield and function of islets, there are significant variations in enzymatic activity between batches and deterioration in enzyme quality over time, which necessitates test isolations of each new batch to maintain quality control.

Following digestion, islets are separated from the acinar tissue using density gradient centrifugation (Ricordi, Lacy et al. 1988). This allows separate analysis of serial fractions to select those with the highest purity for transplantation. The gold standard method is continuous purification using the computerised semi automated COBE-2991 cell processor, as utilized by the majority of clinical islet transplant programmes (Froud, Ricordi et al. 2005, Hering, Kandaswamy et al. 2005, Shapiro, Ricordi et al. 2006).

Once isolated, the purified islet preparation is implanted into the recipient liver via the portal vein. This is accessed percutaneously by a transhepatic route under ultrasonographic and fluoroscopic guidance (Owen, Ryan et al. 2003, Venturini, Angeli et al. 2005). The islets are then infused slowly using a closed bag system, with monitoring of portal venous pressure (Baidal, Froud et al. 2003). The use of a haemostatic substance or coils to seal the entry track has been shown to reduce the risk of post procedural bleeding (Froud, Yrizarry et al. 2004, Villiger, Ryan et al. 2005). Nevertheless, bleeding remains a recognised complication of the procedure, as does portal vein thrombosis and portal hypertension (Venturini, Angeli et al. 2005, Brennan, Shannon et al. 2004, Hafiz, Faradji et al. 2005). Overall, morbidity and mortality from the procedure is low and most patients are discharged from hospital after 1-2 days.

1.5 Immunosuppression for islet transplantation

The transplanted islets obviously represent a foreign antigen to the recipient and immunosuppression is required to prevent rapid rejection. Prior to the development of the Edmonton protocol, the majority of patients undergoing islet transplantation were given an immunosuppressive regimen consisting of antilymphocyte globulin as an induction agent combined with cyclosporine, azathioprine and corticosteroids as maintenance immunosuppression.

Corticosteroids are well known to have significant diabetogenic effects. The mechanisms of this have been extensively investigated in both human and rodent islets and β-cells; these include reduced insulin mediated glucose uptake by skeletal muscle via reduced glycogen synthase activity (Ekstrand, Schalin-Jantti et al. 1996), impaired insulin mediated suppression of endogenous glucose production by the liver (Rizza, Mandarino et al. 1982), inhibition of insulin secretion by β-cells (Ullrich, Berchtold et al. 2005) and increased β-cell death (Ranta, Avram et al. 2006).

The calcineurin inhibitors cyclosporine and tacrolimus have both been shown to have detrimental effects on β-cell function. Cyclosporine decreases insulin mRNA levels in human islets (Polastri, Galbiati et al. 2002) and reduces both human (Oetjen, Baun et al. 2003) and murine insulin gene transcription (Tamura, Fujimura et al. 1995). Tacrolimus has also been shown to inhibit human insulin gene transcription (Oetjen, Baun et al. 2003), lower the insulin stimulation index (Polastri, Galbiati et al. 2002) and decrease insulin secretion in human islets (Nielsen, Mandrup-Poulsen et al. 1986). Both calcineurin inhibitors have been shown to cause direct morphological changes to human islets in pancreatic allograft biopsies (Drachenberg, Klassen et al. 1999). These changes include cytoplasmic swelling,

vacuolization, apoptosis and decreases in dense-core secretory granules. The findings appeared to be more frequent and severe in tacrolimus treated patients.

The Edmonton protocol was designed to avoid these diabetogenic effects of corticosteroids and to minimise the effects of tacrolimus. This was achieved by substituting corticosteroids for daclizumab, and using tacrolimus at low doses. However, the main immunosuppressive agent utilized by the protocol was rapamycin, produced commercially as sirolimus, an immunosuppressant which was thought to have little or no detrimental effects on islet survival or function.

1.5.1 The Edmonton protocol

Induction therapy consisted of daclizumab, a humanized monoclonal interleukin-2 receptor antibody, given over a 10-week period. Maintenance therapy comprised sirolimus (target levels 12-15 ng/ml for the first three months, 7-10 ng/ml thereafter) and low dose tacrolimus (target levels 3-6 ng/ml). As mentioned previously early outcomes were encouraging with all seven patients included in the initial study remaining insulin independent at a median follow-up of 11.9 months (Shapiro, Lakey et al. 2000). However, longer term outcomes were less promising. Of a further 65 patients to receive the Edmonton protocol 80% had functioning grafts at 5 years, as assessed by C-peptide secretion, but only about 10% of these were insulin independent(Ryan, Paty et al. 2005). The Edmonton protocol has also been adopted as part of an international trial, with 9 centres recruiting 36 patients (Shapiro, Ricordi et al. 2006). Unfortunately, results were inferior to the in house studies performed in Edmonton. C-peptide secretion was detectable in 70% of patients at 2 years, with only 14% remaining insulin independent at 2 years. In addition, a quarter of patients were switched from sirolimus to either mycophenolate mofetil or azathioprine because of side-effects.

Following the initial promise of the Edmonton protocol, a number of other centres adopted the regimen for use in their islet transplant programmes. The Philadelphia group initially reported seven patients who completed an islet transplant protocol encompassing the Edmonton immunosuppressive regimen (Markmann, Deng et al. 2003). All seven patients achieved insulin independence, five requiring only a single islet infusion. A later report from the same group included 12 patients receiving an islet transplant under the Edmonton protocol (Frank, Deng et al. 2004). All patients had detectable C-peptide, and eleven patients achieved insulin independence. However, only about 55% of patients had C-peptide detectable at 2 years post transplant and only 20% were insulin independent at 2 years. The Zurich group performed 13 simultaneous islet and kidney transplants under the Edmonton protocol for patients with end stage diabetic nephropathy (Gerber, Pavlicek et al. 2008). Only 31% of patients were insulin independent at 1 year, and only 15% at 38 months post transplant.

In summary all the studies of islet transplantation utilising the Edmonton protocol show similar results with good initial graft function and insulin independence in the majority, but a rapid decline in the rate of insulin independence from 1 year post transplant.

1.5.2 Modifications to the Edmonton protocol

Given the limitations of the Edmonton protocol outlined above, a number of studies have investigated modifications to the protocol aimed at improving the long term results of islet transplantation. The majority of these have investigated different induction strategies. One of the potential mechanisms of islet cell loss following islet transplantation is recurrent autoimmunity, mediated by autoreactive CD8+ T-cells. Induction with T-cell antibodies, such as antithymocyte globulin (ATG) may be protective against this autoimmune response.

The Vancouver group reported 10 patients who received islet transplants using ATG and methylprednisolone as induction, with sirolimus (target levels 5-10 ng/ml) and tacrolimus (target levels 5-10 ng/ml) as maintenance therapy in 8 patients and mycophenolate mofetil (MMF) and tacrolimus in 2 patients(Warnock, Meloche et al. 2005). All patients achieved insulin independence, with 50% remaining insulin independent at the end of a follow up period ranging from 6 to 21 months. These results were, if anything, inferior to those achieved using the Edmonton protocol. The likely detrimental effects of corticosteroid induction may well have counteracted any beneficial effects of ATG.

Gillard et al investigated whether ATG induction provided adequate immunosuppression to allow avoidance of calcineurin inhibitors. They compared 5 patients undergoing islet transplantation under ATG induction with sirolimus and tacrolimus maintenance, with 5 patients treated with ATG induction and sirolimus monotherapy as maintenance (Gillard, Ling et al. 2008). They concluded that sirolimus monotherapy is insufficient in islet transplantation, with no patients in this group achieving insulin independence over 6 months post transplantation, as compared to 3 out of 5 patients in the sirolimus/tacrolimus arm.

Other anti-T cell antibodies have also been utilized in islet transplantation. The Minnesota group performed single-infusion islet transplantation on 6 patients using hOKT3γ1 for induction with tacrolimus and sirolimus as maintenance therapy (Hering, Kandaswamy et al. 2004). hOKT3γ1 is an anti-CD3 monoclonal antibody with dual activity against autoreactive and alloreactive T cells. Four out of six patients remained insulin independent at 1 year post transplant. Given only a single infusion of islets was administered these results suggest hOKT3γ1 may confer some benefit over daclizumab induction.

A number of groups have investigated the addition of the anti-TNFα receptor antibody etanercept, or the anti-TNFα antibody infliximab, to the Edmonton protocol. Cure et al from

Miami performed seven islet transplants using the Edmonton regimen and etanercept in patients who had already received a kidney transplant (Cure, Pileggi et al. 2008). Six patients (86%) achieved insulin independence after 1or 2 islet infusions, but 1 year insulin independence was only 30%, with detectable C-peptide production in 86%. The University of Illinois at Chicago (UIC) protocol adds etanercept and exenatide, a glucagon-like peptide-1 analogue, to the Edmonton protocol. The UIC group have reported a comparison of 6 patients transplanted using their protocol (group 1) with 4 patients transplanted using the Edmonton protocol (group 2) (Gangemi, Salehi et al. 2008). All group 1 patients became insulin independent after their initial islet transplant, whilst all group 2 patients became insulin independent following 2 or 3 islet infusions. However, at 15 months follow up post transplant whilst all group 2 patients remained insulin independent, only 4 out of the 6 group 1 patients remained so. Therefore, in this series etanercept did seem to lower the number of islets required initially to achieve insulin independence, perhaps via a combined mechanism of reducing inflammation and improving islet engraftment. However, it did not seem to confer long term protection.

The Miami group performed 16 islet transplantations using the Edmonton protocol, with patients randomised to receive infliximab or not prior to islet infusion. All patients achieved insulin independence, with the majority requiring 2 islet infusions. 79% of the study group remained insulin independent at 1 year and 43% at 18 months post transplantation. However, the authors identified no clinical benefit from the addition of infliximab to the regimen (Froud, Ricordi et al. 2005).

1.5.3 Departures from the Edmonton protocol

In addition to evaluating the use of hOKT3γ1 for induction, the Minnesota group have also investigated an immunosuppressive regimen that differs substantially from those described above; the main aim being to limit calcineurin inhibitor exposure (Hering, Kandaswamy et al. 2005). They performed 8 islet transplants using a multi-agent induction protocol comprising ATG, methylprednisolone, daclizumab and etanercept. Maintenance therapy was with sirolimus and tacrolimus initially, with tacrolimus gradually replaced with MMF at 1 month post transplantation. Of note all patients received a single islet infusion prepared from a single donor pancreas. Insulin independence was achieved in all 8 patients, with 5 patients remaining insulin independent for longer than 1 year post transplantation. Given that these were single donor, marginal dose islet transplants (mean 7271 islet equivalents/kg), these results show some promise.

The Minnesota group have also published results of 6 islet transplants performed using a refined version of the above protocol (Bellin, Kandaswamy et al. 2008). Induction therapy was with ATG and etanercept. Maintenance immunosuppression comprised cyclosporine and everolimus, another rapamycin derivative, for the first year post transplant. Everolimus was then substituted for MMF or mycophenolic acid (MPA). Five patients achieved insulin independence, one with a single islet infusion and the remainder with 2 infusions. Four patients remain insulin independent at over 3 years post transplant. This protocol has resulted in arguably the most promising long term survival data reported for islet transplantation to date.

The Vancouver group have also utilised MMF as a replacement for sirolimus rather than tacrolimus. They have reported the results of 21 patients undergoing islet transplantation with maintenance immunosuppression comprising only tacrolimus and MMF in 17 patients, tacrolimus and sirolimus converted to tacrolimus and MMF in 2 patients, and tacrolimus and

sirolimus in 2 patients(Fung, Warnock et al. 2007). Only 17 patients achieved insulin independence, and only 7 remained insulin independent at the end of a median follow-up period of 29 months. This regimen therefore seems to have no advantage, in terms of graft function and survival, over those using sirolimus. Indeed the results are inferior to those from a previous study from the same centre outlined above. Unfortunately, it is not possible to determine why the results of the Vancouver group do not match the findings of the Minnesota group when substituting MMF or MPA for sirolimus or everolimus, given the other substantial differences between the two protocols.

Another regimen aimed at minimizing calcineurin inhibitor exposure has recently been published. Froud et al report three patients who received islet transplants using induction with the CD-52 monoclonal antibody, alemtuzumab (Campath)(Froud, Baidal et al. 2008). Maintenance was with sirolimus and tacrolimus for the initial 3 months and sirolimus and MPA thereafter. Two of the three patients achieved insulin independence, both remaining insulin independent at 2 years post transplantation. In addition, islet function as measured by the mixed meal stimulation index, mixed meal peak C-peptide and C-peptide production was significantly better in this group than in a historical control treated under the Miami version of the Edmonton protocol. However, evaluation of the results should be guarded given the small number of patients involved. Nevertheless, further investigation of the role of alemtuzumab in islet transplantation is warranted.

1.6 Goals of islet transplantation

The long term goal of all those involved in both the clinical and scientific aspects of islet transplantation has been to provide a cure for type I diabetes. However, as discussed above, despite many years of endeavour prolonged insulin independence has proved elusive. Nevertheless, islet transplantation does result in significant improvement in other outcome measures.

The main indications for islet transplantation are currently severe hypoglycaemia, reduced awareness of hypoglycaemia or metabolic lability with frequent, wide, swings in blood glucose levels. The long term follow-up data from the Edmonton group show that both severe hypoglycaemia, as quantified by the HYPO score, and metabolic lability, as measured by the lability index, improved significantly following islet transplantation and that this effect was sustained for 4 years post transplant (Ryan, Paty et al. 2005). Gangemi et al also reported complete resolution of severe hypoglycaemia in all islet recipients studied, with significant reductions in HYPO score sustained at 12 months post transplant (Gangemi, Salehi et al. 2008). These observations have led some to argue that the aim of islet transplantation should not be insulin independence, but avoidance of severe hypoglycaemia and improvement of metabolic lability (Lehmann, Spinas et al. 2008).

1.7 Islet loss following transplantation

1.7.1 Monitoring islet grafts following transplantation

Regardless of the goals of islet transplantation, one of the keys to improving outcomes is to reduce islet loss following transplantation. A number of insults contribute to islet loss at different stages following transplantation. However, the exact contribution that each of these mechanisms makes to graft loss remains unclear. This arises in part due to difficulties in monitoring islets post transplantation. For solid organ transplants such as kidney or liver, tissue biopsies are used to monitor grafts and detect causes of graft dysfunction. However, this approach is much more difficult in islet transplantation, since islet grafts comprise 5 to 10 g of tissue distributed within a 1 to 2 kg organ. Therefore sampling of transplanted islets is a real challenge. This inability to effectively monitor and assess islet grafts makes evaluation of the effectiveness of immunosuppressive regimens difficult and prevents further optimisation of islet transplant protocols.

Recently the use of percutaneous needle core biopsies of the liver for histological assessment of islet grafts has been reported. However, although this study showed that this is feasible, only 31% of the biopsies contained islet tissue, limiting its practical use (Toso, Isse et al. 2009).

Non-invasive techniques to monitor transplanted islets are also under development. These include positron-emission tomography (PET) (Eich, Eriksson et al. 2007, Kim, Doudet et al. 2006, Toso, Zaidi et al. 2005)and magnetic resonance imaging (MRI) (Biancone, Crich et al. 2007, Evgenov, Medarova et al. 2006, Koblas, Girman et al. 2005, Tai, Foster et al. 2006). However, both techniques are still at the experimental phase and require labelling of islets prior to transplantation. It is therefore likely to be some years before they enter clinical use.

Hence, investigation into the causes of islet graft loss has so far been limited. Perhaps the best evidence comes from post mortem histological analysis of islet transplant recipients (Davalli, Maffi et al. 2000, Sever, Demetris et al. 1992, Smith, Kent et al. 2008, Westermark, Westermark et al. 2008). Overall, these studies suggest a predominantly non immunological cause for islet graft loss.

The potential causes of islet graft loss will now be explored in more detail.

1.7.2 Early inflammatory responses

It has been demonstrated experimentally that up to 70% of the transplanted islets may be destroyed in the early period post transplantation (Davalli, Ogawa et al. 1995, Biarnes, Montolio et al. 2002). Since this loss is seen in both allogeneic and syngeneic transplantation, the primary cause is likely to be non immunological.

Indeed, incompatibility between islets and blood has been shown to trigger detrimental thrombotic reactions, resulting in disruption of islet morphology and subsequent islet loss, an effect termed the instant blood-mediated inflammatory reaction (IBMIR). This is triggered by activation of the intrinsic coagulation pathway by collagen expressed on the islet surface (Bennet, Groth et al. 2000) and activation of the extrinsic coagulation pathway by tissue factor secreted by islets (Moberg, Johansson et al. 2002). This results in coagulation, platelet activation and complement activation (Bennet, Sundberg et al. 1999) followed by infiltration of the islets by neutrophilic granulocytes and macrophages (Moberg, Korsgren et al. 2005).

Another factor responsible for early islet graft loss is a non specific local cytokine inflammatory response (London, Robertson et al. 1994), potentially mediated by tumour necrosis factor-α, interleukin-1β and nitric oxide production by resident hepatic macrophages (Kupffer cells) (Bottino, Fernandez et al. 1998)and resulting in islet cell death.

1.7.3 Hypoxia

Alongside these effects, the islets are subjected to prolonged hypoxia during the revascularization process until engraftment has taken place. This process is likely to occur via a chimeric vascular tree with islets revascularized by endothelial cells from both donor and recipient (Nyqvist, Kohler et al. 2005). The revascularization of islets is not immediate with the first signs of angiogenesis appearing from 2 days post transplant and complete vascularization not occurring for 10 to 14 days (Jansson, Carlsson 2002). During this period islets rely on diffusion of oxygen and nutrients for survival. Even once vascularized, the vascular density of transplanted islets is approximately 25% of that seen in native islets (Lau, Carlsson 2009). This poor vascularity is accompanied by pO_2 levels within transplanted islets of approximately 10% of those seen within native islets (Carlsson, Palm et al. 2001). It is likely that this chronic hypoxia is another factor responsible for the loss of islets over time.

1.7.4 Immunological reactions

Both alloimmunity and autoimmunity have been shown to play a role in islet graft failure. Autoantibodies directed against islet cells, glutamic acid decarboxylase (GAD 65) and protein tyrosine phosphatase-like protein IA-2 (IA-2) have been demonstrated in 23%, 14-52% and 6% of islet transplant recipients respectively (Bosi, Braghi et al. 2001, Jaeger, Hering et al. 1999). This included subjects who had been both autoantibody positive and negative prior to transplantation. Furthermore, the presence of autoantibodies appears to affect overall outcome of islet transplantation. Islet graft failure has been shown to occur earlier in those recipients who are autoantibody positive prior to transplantation (Jaeger, Brendel et al. 1997). Also, pre transplant cellular autoreactivity has been shown to have a significant effect on both insulin independence and C-peptide production (Roelen, Huurman et al. 2009).

Evidence for alloimmunity in islet graft failure includes an increase in graft specific alloreactive T cells demonstrated in peripheral blood of patients with failing islet grafts (Roep, Stobbe et al. 1999). In one of these patients alloreactivity was preceded by a sharp increase in a number of islet autoantigens suggesting a possible reciprocal relationship between allo- and autoimmunity. Further evidence for the role of alloimmunity comes from a study demonstrating the importance of histocompatibility in the outcome of islet transplantation (Mohanakumar, Narayanan et al. 2006). In this series 3 out of 7 patients undergoing islet transplantation were sensitized to donor HLA pre-transplant. Early graft failure was seen in all 3 of these patients. Increased alloreactivity following transplantation, as measured by cytotoxic T lymphocyte precursor assay, has also been shown to be associated with lower insulin independence and C-peptide production in islet transplant recipients treated with tacrolimus and rapamycin or rapamycin alone (Roelen, Huurman et al. 2009).

Despite the above evidence, pathological examination of one patient who died with a failed islet transplant performed under the Edmonton protocol and one who died with a functioning islet transplant showed no evidence of allo or autoimmune damage to the transplanted islets (Davalli, Maffi et al. 2000, Smith, Kent et al. 2008). This provides evidence for a predominantly non immunological cause for the chronic loss of intrahepatic islets and subsequent graft failure.

1.7.5 Immunosuppressive drug toxicity

As mentioned above, chronic hypoxia may well contribute to non immunological islet loss. However, one of the primary causes may well be drug toxicity. It is important to recognise that serum levels of the immunosuppressant drugs are higher in the portal circulation than in the systemic circulation as they are yet to undergo first pass metabolism in the liver. Tacrolimus and rapamycin levels have been shown to be up to three times higher in the portal circulation as compared to the systemic circulation in islet transplant recipients (Desai, Goss et al. 2003). As mentioned above the Edmonton protocol was designed to minimise the toxic effects of the immunosuppressive agents used. Although rapamycin was thought to have minimal toxic effects on islets there is increasing evidence in the literature that it has significant effects on both islet survival and function; this is discussed in detail in sections 4.1.1 and 4.1.2. Therefore, the immunosuppressive storm that islets are subjected to in the portal circulation may well play a key role in the loss of islet mass over time.

1.8 Conclusion

Despite marked improvements in the short term success of islet transplantation as a treatment for type I diabetes since the introduction of the Edmonton protocol, long term outcomes remain poor. The reasons for this decline are multifactorial. However, there is human autopsy evidence which suggests a predominantly non immunological cause. Transplanted islets are subjected to high concentrations of immunosuppressive drugs following transplantation and although the Edmonton protocol was designed to minimize drug toxicity, there is increasing evidence that rapamycin in particular has toxic effects on pancreatic β cells. As such, the aim of this thesis is to investigate the effects of rapamycin on pancreatic β cell survival and function, and to elucidate the mechanisms of any toxicity seen. The following chapter

discusses the mechanism of action of rapamycin and its effects on cell signalling pathways, with particular reference to pancreatic β cell homeostasis.

CHAPTER 2

Introduction: actions of rapamycin

Rapamycin is a lipophilic macrocyclic lactone, which was first discovered in soil samples from Rapa Nui, otherwise known as Easter Island. It is a fermentation product of the actinomycete *Streptomyces hygroscopicus*. It was initially investigated for its antifungal properties, but was soon found to be more potent as an immunosuppressive agent (Martel, Klicius et al. 1977). However, it was not developed for clinical use until after the successful introduction of its fellow immunosuppressant and structural analogue, tacrolimus.

2.1 Mechanism of action

The intracellular target for rapamycin is the immunophilin, FK506 binding protein 12 (FKBP12). Immunophilins are binding proteins for immunosuppressive agents and are all members of the cyclophilin or FKBP families (Sigal, Dumont 1992, Walsh, Zydowsky et al. 1992). The cyclophilin family binds cyclosporin A, whereas the FKBP family bind FK506 and rapamycin.

FKBP12 was first discovered as the binding protein of tacrolimus, also known as FK506, and was later found to also bind rapamycin (Heitman, Movva et al. 1991b, Fruman, Burakoff et al. 1994). In order to exert its immunosuppressive effects, rapamycin must first bind to FKBP12 (Bierer, Somers et al. 1990) and strains deficient in FKBP are resistant to rapamycin (Koltin, Faucette et al. 1991).

The search for the intracellular target of the rapamycin:FKBP complex resulted in the discovery in yeast of a central regulator of cell proliferation and growth, named the target of rapamycin (TOR) (Heitman, Movva et al. 1991a) and subsequently the discovery of the homologous mammalian target of rapamycin (mTOR) (Sabatini, Erdjument-Bromage et al. 1994).

mTOR is a 289 kDa serine/threonine kinase, which is a member of the PI kinase (PIK)-related kinases (see figure 2.1). This family of kinases are characterized by a common C-terminal phosphatidylinositol (PI) kinase homology domain, termed a FAT (*F*RAP, *A*TM and *T*RRAP) domain. Structurally, mTOR also possesses up to 20 tandem HEAT repeats at the amino-terminal region, each comprising a protein-protein interaction structure of two tandem anti parallel α helices found in *h*untington, *e*longation factor 3, *A* subunit of protein phosphatase 2A and *T*OR (HEAT). This is followed by a further FAT domain. The rapamycin:FKBP complex interacts with the FKBP-rapamycin binding (FRB) domain of mTOR, adjacent to the catalytic kinase domain (Choi, Chen et al. 1996, Chen, Zheng et al. 1995). This binding interferes with mTOR function and inhibits the signal transduction pathways outlined below in section 2.2.3, resulting in the arrest of the cell cycle in mid to late G1 phase.

It is this cell cycle arrest and subsequent inhibition of cell proliferation in T and B lymphocytes that results in the immunosuppressive effects. Rapamycin inhibits human, porcine and murine T-lymphocyte proliferation induced by various interleukins, alloantigens and co-stimulation with anti-CD28, as well as inhibiting the activation of B lymphocytes by bacterial lipopolysaccharide (Dumont, Staruch et al. 1990, Kay, Kromwel et al. 1991, Bertagnolli, Yang et al. 1994).

The mTOR kinase is known to be present in two distinct complexes, known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Although they have the same central mTOR component these complexes have differing associated proteins, and distinct upstream and downstream signalling pathways. However, both complexes potentially play a role in the immunosuppressive and toxic effects of rapamycin.

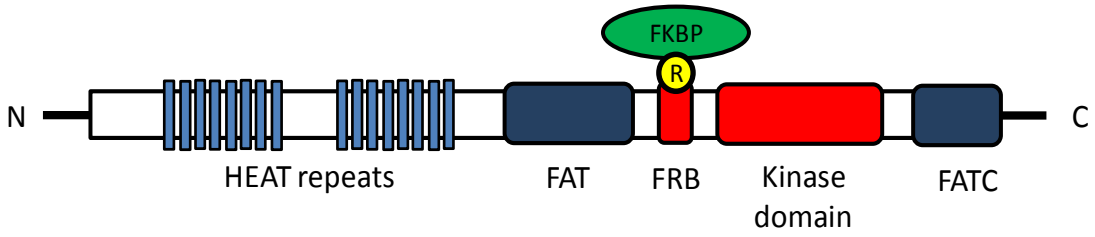

Figure 2.1 Schematic structure of mTOR. HEAT – protein-protein interaction structure of two tandem anti parallel α helices found in *h*untington, *e*longation factor 3, *A* subunit of protein phophatase 2A and *T*OR; FAT – domain structure shared by *F*RAP, *A*TM and *T*RRAP, which are all PIKK family members; FRB – *F*KBP12/*r*apamycin (R) *b*inding domain; FATC – *FAT C*-terminus.

2.2 mTOR complex 1

2.2.1 Structure of mTOR complex 1

mTORC1 comprises of mTOR, Raptor, mLST8 and Deptor (see figure 2.2). Raptor (regulatory associated protein of mTOR), an evolutionarily conserved 149 kDa protein, binds both to mTOR and independently to the downstream effectors S6K1 and 4E-BP1 (Hara, Maruki et al. 2002, Kim, Sarbassov et al. 2002). As such, it appears to serve as a scaffold for the mTORC1 signalling pathway. The mammalian lethal with Sec13 protein 8 (mLST8), previously identified as G protein β-subunit-like protein (GβL), is a 36 kDa protein that, like Raptor, is conserved amongst eukaryotes. It interacts, independently of Raptor, with the kinase domain of mTOR and stabilises the mTOR-raptor association (Kim, Sarbassov et al. 2003). mLST8 and Deptor are the only known proteins common to both mTORC1 and mTORC2. Deptor is a 48 kDa protein which contains n-terminal DEP (dishevelled, egl-10, pleckstrin) domains and also binds mTOR near the kinase domain. Experiments overexpressing or depleting Deptor suggest that it negatively regulates the kinase activity of both mTORC1 and mTORC2 (Peterson, Laplante et al. 2009). Interestingly Deptor is found only in vertebrates, suggesting that its regulatory function is a recent evolutionary adaptation.

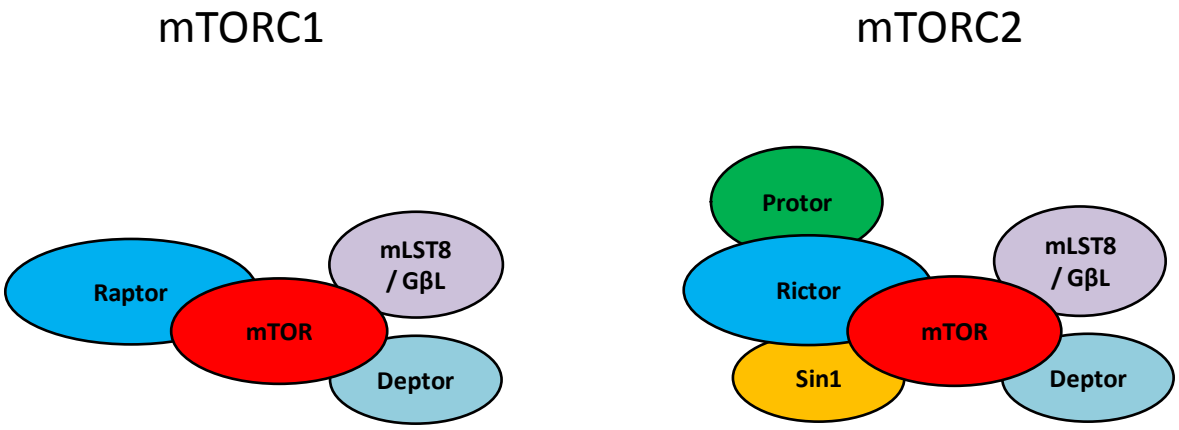

Figure 2.2 Components of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTOR – *m*ammalian *t*arget *o*f *r*apamycin, Raptor - *r*egulatory *a*ssociated *p*rotein of m*TOR*, mLST8 - *m*ammalian *l*ethal with *S*ec13 pro*t*ein *8*, GβL- *G* protein *β*-subunit-*l*ike protein, Rictor – *r*apamycin *i*nsensitive *c*ompanion of m*TOR*, Sin1 - *s*tress activated protein kinase *in*teracting protein 1, Protor - *pro*tein observed with ric*tor*.

2.2.2 Upstream signalling of mTORC1

Consistent with its role as a key regulator of cell proliferation and growth, mTORC1 activity is regulated by nutrients, growth factors and by cellular energy levels (figure 2.3).

The control of mTORC1 by insulin and other growth factors is mediated by a functional connection between the mTORC1 signalling pathway and the insulin/IGF-PI3K signalling pathway. Growth factors such as insulin and IGF activate phosphatidylinositol 3-kinase (PI3K), this enzyme phosphorylates phosphatidylinositol 4,5-bisphosphanate (PIP_2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Whitman, Downes et al. 1988). PIP_3 binding to the PH domain of PKB recruits it to the plasma membrane along with phosphoinositide-dependent kinase 1 (PDK1) (Burgering, Coffer 1995, Franke, Yang et al. 1995). PKB activation occurs by phosphorylation at Ser473 by mTORC2 (Sarbassov, Guertin et al. 2005) and at Thr308 by PDK1 (Alessi, James et al. 1997). Activated PKB phosphorylates and subsequently inhibits the tumour suppressor complex TSC1/2 (Inoki, Li et al. 2002, Potter, Pedraza et al. 2002, Manning, Tee et al. 2002), providing the functional link between the mTORC1 and insulin/IGF-PI3K signalling pathways. A further regulator of the PKB and mTORC1 pathway is the mTOR substrate PRAS40 (Proline-rich Akt/PKB substrate 40 kDA) which binds to and inhibits mTOR. PRAS40 is phosphorylated on Thr246 by PKB, which results in its dissociation from mTOR and removes its inhibitory effect (Vander Haar, Lee et al. 2007, Kovacina, Park et al. 2003).

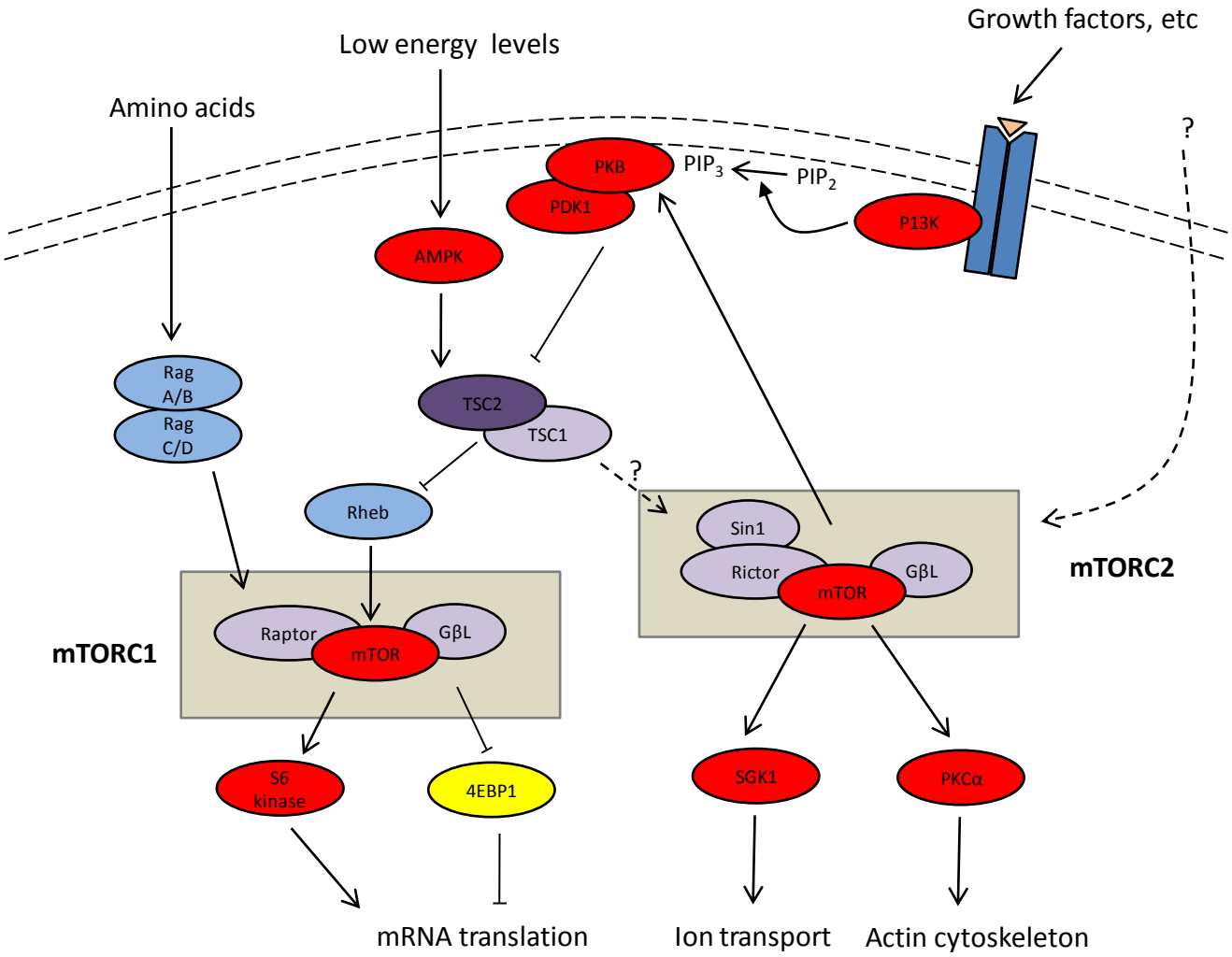

Figure 2.3 mTOR signalling pathways. Following stimulation by insulin and other growth factors, PI3K converts PIP_2 into PIP_3 which localizes PKB to the membrane where it is activated by PDK1 and mTORC2. Activated PKB phosphorylates and inhibits TSC1/2. Rheb, a small GTPase that is inhibited by TSC2, positively modulates mTORC1 activity. mTORC1 phosphorylates S6 kinase 1/2 and 4EBP1 resulting in increased mRNA translation. Amino acid sufficiency activates mTORC1 via Rag A/B and C/D. Under low energy conditions the AMP/ATP ratio rises and activates AMPK, which phosphorylates and activates the TSC1/2 complex resulting in mTORC1 inhibition. mTORC2 activity is mediated via predominantly unknown pathways. mTORC2 phosphorylates and activates PKB, SGK1 and PKC. *Arrows denote stimulatory effects, block ends denote inhibitory effects; solid lines denote direct effects, dashed lines denote indirect effects.*

TSC1 and TSC2, also known respectively as hamartin and tuberin, have tumour suppressor activity and mutations within them lead to the development of benign tumours known as hamartomas and the syndrome of tuberous sclerosis. The two complexes interact through their N termini, forming a heterodimer and acting as an upstream negative regulator of mTOR (Inoki, Li et al. 2002, Gao, Zhang et al. 2002, Tee, Fingar et al. 2002). The direct target of TSC1/2 is the small GTPase Rheb (Ras homolog enriched in brain). TSC2 acts as a GTPase activating protein (GAP) for Rheb, catalysing the conversion from its GTP-loaded to its GDP-loaded form and thereby negatively regulating its activity(Zhang, Gao et al. 2003, Garami, Zwartkruis et al. 2003, Castro, Rebhun et al. 2003, Inoki, Li et al. 2003). Rheb itself positively modulates mTORC1 function through an as yet unknown mechanism (Inoki, Li et al. 2003, Stocker, Radimerski et al. 2003, Saucedo, Gao et al. 2003).

Until recently the mechanisms by which nutrients, in particular amino acids, activated mTORC1 were largely unknown. However, the Rag subfamily of Ras small GTPases have recently been identified as key mediators of amino acid signals to mTORC1 (Sancak, Peterson et al. 2008, Kim, Goraksha-Hicks et al. 2008). It has been proposed that amino acids signal RagA or RagB GTP binding, which in turn stimulates their binding to Raptor. The Rags then mediate mTORC1 localization to vesicles that contain Rheb resulting in increased mTORC1 activity.

Given the high levels of cellular energy required for cell growth and cell cycle progression, both processes regulated by mTORC1, it is logical that mTORC1 activity is linked to cellular energy status. Under low energy conditions the AMP/ATP ratio rises. This rise results in the activation of the 5'AMP-activated protein kinase (AMPK) (Hardie, Carling 1997). AMPK subsequently phosphorylates TSC2 (Inoki, Zhu et al. 2003), up regulating its activity and subsequently down regulating mTORC1.

2.2.3 Downstream targets of mTORC1

The primary targets of mTORC1 are the eIF4E-binding proteins (4E-BPs) and the S6 kinase proteins (S6K), both of which are key components of mRNA translation and, in particular, recruitment of ribosomes to mRNA.

2.2.3.1 4E-BPs

The 4E-BPs are a family of translational repressor proteins, the mammalian family consisting of three proteins, 4E-BP1, 4E-BP2 and 4E-BP3 (also known as PHAS-I, II and III). The 4E-BPs repress translation by binding to eukaryotic translation initiation factor 4E (eIF4E) and preventing its binding to eIF4G (Inoki, Zhu et al. 2003). For translation to commence, eIF4E must bind to the 5' cap of mRNA as part of a ribosome initiation complex including eIF4G. This results in the unwinding of the mRNA proximal secondary structure and allows binding of the 40s ribosomal subunit with other initiation factors.

The binding of 4E-BPs to eIF4E is dependent on their phosphorylation state. Hypophosphorylated 4E-BPs bind with high affinity, whereas hyperphosphorylation promotes dissociation from eIF4E. mTORC1 directly phosphorylates 4E-BP1 at Thr37 and Thr46, this phosphorylation is required for subsequent phosphorylation at Thr70 and then Ser65, followed by release of 4E-BP1 from eIF4E (Brunn, Hudson et al. 1997, Gingras, Raught et al. 2001, Heesom, Denton 1999). Therefore, mTORC1 activity results in the phosphorylation and subsequent release of 4E-BP1 from eIF4E. This allows formation of the eIF4E/eIF4G complex and initiation of translation.

2.2.3.2 S6 kinase

Two similar S6 kinase proteins, S6K1 and S6K2, are present in mammalian cells and are key regulators of cell growth, via increased mRNA translation. S6K mediated activation of ribosomal protein S6 (rpS6) is thought to promote translation of 5' terminal oligopyrimidine tract (TOP) mRNAs. These contain a short polypyrimidine sequence immediately next to the 5' cap and exclusively encode for essential translational components such as ribosomal proteins and elongation factors (Jefferies, Fumagalli et al. 1997).

The direct downstream targets of S6K have yet to be fully elucidated. Ribosomal protein S6 (rpS6) is the most extensively studied S6K substrate. However, there is evidence that a S6K target other than rpS6 is responsible for the regulation of 5'TOP mRNA translation (Pende, Um et al. 2004). One proposed alternative is eIF4B, which stimulates the RNA helicase eIF4A in unwinding RNA secondary structure, and is specifically phosphorylated at Ser422 by S6K1/S6K2 (Raught, Peiretti et al. 2004). There is also evidence that mTOR itself may mediate TOP mRNA translation. Interestingly, this appears to be via a novel pathway independent of Raptor or Rictor (Patursky-Polischuk, Stolovich-Rain et al. 2009).

2.2.3.3 Other mTORC1 targets

The activity of mTORC1 has been shown to directly correlate with mitochondrial metabolism (Schieke, Phillips et al. 2006). mTORC1 has been shown to directly regulate Yin Yang 1 (YY1), a transcriptional activator which along with its co-activator PGC-1α (peroxisome-proliferator-activated receptor coactivator), regulates the expression of many mitochondrial genes (Cunningham, Rodgers et al. 2007).

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor which is partially activated by cytokine stimulation of receptor tyrosine kinases resulting in phosphorylation at Tyr705 (Wen, Zhong et al. 1995). However, full activation requires

further phosphorylation at Ser727. mTOR has been shown to phosphorylate STAT3 at this residue, an event that was inhibited by rapamycin (Yokogami, Wakisaka et al. 2000).

The serum- and glucocorticoid-inducible kinase 1 (SGK1) has also been identified as a potential mTORC1 substrate. mTOR induced SGK1 phosphorylation at Ser422 led to the phosphorylation and upregulation of the cyclin-dependent kinase inhibitor $p27^{KIP}$ (Hong, Larrea et al. 2008). As described in section 2.3.3 mTORC2 also appears to phosphorylate SGK1 (Garcia-Martinez, Alessi 2008). Further research in this area should clarify whether SGK1 is a substrate of mTORC1 or mTORC2, or whether both complexes play a role.

mTORC1 also regulates hypoxia inducible factor 1α (HIF1α), although it is not clear whether this regulation is direct or indirect. HIF1α is stabilised under hypoxic conditions, allowing it to form a dimer with HIF1β. This dimer translocates to the nucleus and induces expression of genes involved in angiogenesis, erythropoiesis and glucose metabolism (Wang, Jiang et al. 1995). Under hypoxic conditions, Rheb induced activation of mTOR potently enhances the transcriptional activity of HIF1α (Land, Tee 2007).

Other reported targets of mTORC1 involved in translation include eIF4G, which is a key scaffolding protein in the ribosome initiation complex (Raught, Gingras et al. 2000) and the translation elongation factor eEF2 kinase (Browne, Proud 2004).

Finally, mTORC1 also plays a role in stimulating transcription from rRNA genes by RNA polymerase I (Pol I), ribosomal protein genes by RNA Pol II and tRNA/5S genes by RNA Pol III (Hay, Sonenberg 2004).

2.2.4 The role of mTORC1 in β cell function

A vital aspect in the preservation of glucose homeostasis is the maintenance of pancreatic β cell mass and also the ability for β cell mass to increase in response to insulin resistant states such as obesity. This increase in β cell mass results from increases in neogenesis and replication, hypertrophy and reductions in apoptosis. β cell proliferation has been shown to have a dominant role in both maintaining and increasing β cell mass in adult life (Dor, Brown et al. 2004). Although the regulation of islet mass is complex, it is clear that mTORC1 plays a key role.

A number of studies using genetically modified mice have provided evidence for the importance of mTORC1 signalling in the regulation of β cell mass and proliferation. Mice with a specific knockdown of PDK1 in pancreatic β cells show reductions in islet density, number and size with resultant hyperglycaemia (Hashimoto, Kido et al. 2006). Although this reveals a role for PKB signalling in regulation of β cell mass, it does not specifically demonstrate the role of mTORC1, as PKB has a number of downstream targets other than the mTORC1 signalling pathway. However, other studies have shown that upregulation of mTORC1 by overexpression of Rheb (Hamada, Hara et al. 2009) or deletion of TSC1 (Mori, Inoki et al. 2009) or TSC2 (Rachdi, Balcazar et al. 2008, Shigeyama, Kobayashi et al. 2008) leads to increases in β cell size and therefore mass, with resultant improvements in glucose tolerance. These effects were reversed by rapamycin, providing further evidence that the changes seen were mediated via mTORC1. In addition, knock-in of a non-phosphorylating rpS6 lead to a mouse phenotype of decreased β cell size, hypoinsulinaemia and impaired insulin tolerance (Ruvinsky, Sharon et al. 2005). Furthermore, mice deficient in S6K1 are also hypoinsulinaemic and glucose intolerant with diminished β cell size (Pende, Kozma et al. 2000). Taken together, these findings demonstrate the critical role of mTORC1 in the regulation of β cell size and mass and subsequent glucose tolerance.

There is less knowledge on the exact mechanisms by which mTORC1 signalling specifically regulates cell cycle progression. However, recent work has demonstrated the role of mTORC1 in modulating the synthesis and stability of cyclin D2 and D3 (Balcazar, Sathyamurthy et al. 2009). These cyclins form a complex with cyclin-dependent kinase 4 (cdk4), the activity of which is known to control cell cycle progression in β cells.

mTORC1 also appears to play a role in insulin secretion in pancreatic β cells. Knockdown of TSC1 in mice results in significant increases in insulin production, independent of β cell number (Mori, Inoki et al. 2009). In addition, treatment of rats with leucine and subsequent activation of mTORC1 resulted in increased glucose-stimulated insulin secretion (da Silva, Zoppi et al. 2010, Filiputti, Rafacho et al. 2010). As explored in detail in section 4.1.1, the functional effects of rapamycin on insulin secretion from β cells have also been extensively studied.

The control of insulin secretion in pancreatic β cells involves a number of complex signalling pathways, and as such the mechanisms by which mTORC1 regulates insulin secretion remain under investigation. One proposed mechanism is that inhibition of mTORC1 decreases mitochondrial function, specifically the activity of α-ketoglutarate dehydrogenase (KGDH). This results in reduced carbohydrate metabolism and therefore reduced mitochondrial ATP production (Shimodahira, Fujimoto et al. 2010). Intracellular ATP levels are known to be one of the key regulators of insulin secretion in β cells (Maechler, Wollheim 2001).

2.2.5 The role of mTORC1 in the development of insulin resistance

As well as the specific role of mTORC1 in β cell homeostasis described above, it has also been suggested that mTORC1 is involved in the development of insulin resistance in peripheral tissues, a key factor in the development of type II diabetes. However, there is some contradiction in the published literature.

Studies of S6K1 knockout mice have shown enhanced insulin sensitivity mediated via loss of a negative feedback loop between S6K1 and the insulin receptor substrate 1 (IRS1) (Um, Frigerio et al. 2004). This negative feedback loop reduces phosphorylation of IRS1 at Ser307 and Ser636/639, which have been implicated in the development of insulin resistance. Also, activation of S6K1 by knockdown of TSC1/2 or overexpression of Rheb has been shown to induce insulin resistance (Harrington, Findlay et al. 2004, Shah, Wang et al. 2004). The mechanisms appear to include inhibition of murine IRS1 function via phosphorylation at Ser302 (corresponding to Ser307 in the human sequence), which prevents binding of IRS1 to the insulin receptor, and accelerated degradation of IRS1 and IRS2. In humans, mTORC1 activation by infusion of amino acids increased IRS1 serine phosphorylation and was accompanied by increased insulin resistance (Tremblay, Krebs et al. 2005). Overall, these *in vivo* findings suggest that activation of mTORC1 and subsequent downregulation of IRS1/2 plays a key role in the development of insulin resistance. It would therefore be expected that inhibition of mTORC1 by rapamycin would increase insulin sensitivity.

However, two studies in humans have demonstrated the converse. Rapamycin has been shown to inhibit phosphorylation of IRS1 at Ser307 in human adipocytes, mimicking type II diabetes (Danielsson, Ost et al. 2005). In addition, an *in vivo* study using peripheral blood monocytes from patients undergoing long term rapamycin treatment demonstrated downregulation of IRS1 and IRS2 associated with insulin resistance (Di Paolo, Teutonico et

al. 2006). Rapamycin has also been shown to increase insulin resistance in the *Psammomys obesus* rat model of type II diabetes (Fraenkel, Ketzinel-Gilad et al. 2008).

The above discrepancies may be explained by differences in the effects of rapamycin between species, disparity between *in vitro* and *in vivo* studies and by differing effects of acute and chronic rapamycin exposure. It is noteworthy that the in vivo and human studies correlate in implicating rapamycin in the development of insulin resistance.

2.2.6 The role of mTORC1 in angiogenesis

To survive and function effectively transplanted islets need rapid and adequate revascularization after engraftment. As described above the complete revascularization of islets does not occur for 10 to 14 days following engraftment (Jansson, Carlsson 2002). Even once vascularized, the vascular density of transplanted islets is approximately 25% of that seen in native islets (Lau, Carlsson 2009) and is accompanied by pO_2 levels within transplanted islets of approximately 10% of those seen within native islets (Carlsson, Palm et al. 2001).

Vascular endothelial growth factor (VEGF) is a 35-45 kDa cytokine expressed by endothelial and other cell types. It plays a major role in regulating the process of angiogenesis. mTORC1 activity has been shown to play a role in the regulation of VEGF production via HIF-1. Activation of mTORC1 by Rheb overexpression results in enhanced transcriptional activity of HIF-1α, an effect which requires the direct interaction between Raptor and HIF-1α. The increased HIF-1α activity was mirrored by increases in VEGF secretion, and both effects were reversed by rapamycin (Land, Tee 2007). Rapamycin treatment has been shown to reduce *in vitro* expression of VEGF by both mouse and human islets (Cantaluppi, Biancone et al. 2006, Cross, Richards et al. 2007).

In addition, rapamycin has direct effects on the endothelial cells necessary for angiogenesis. Rapamycin treatment at clinically relevant concentrations inhibited the outgrowth of endothelial cells from freshly purified human islets and reduced revascularization of human islets transplanted subcutaneously into SCID mice (Cantaluppi, Biancone et al. 2006). Furthermore, at sub-therapeutic concentrations rapamycin induced rapid apoptosis of endothelial progenitor cells (EPCs) (Miriuka, Rao et al. 2006). These EPCs are bone marrow derived mononuclear cells which are an important component of the angiogenic mechanism.

Therefore inhibition of mTORC1 by rapamycin treatment has the potential to affect the outcome of islet transplantation not only via direct effects on pancreatic β cell function but also via indirect effects on islet revascularization and peripheral insulin resistance.

2.3 mTOR complex 2

2.3.1 Structure of mTOR complex 2

mTORC2 consists of mTOR, Deptor and mLST8 in common with mTORC1, but also Sin1, Protor and Rictor (see figure 2.2). Rictor, or rapamycin-insensitive companion of mTOR, is a 200 kDa protein that is structurally distinct from Raptor and is essential for both mTORC2 complex formation and its kinase activity (Sarbassov, Ali et al. 2004, Jacinto, Loewith et al. 2004). Sin1, or stress activated protein kinase interacting protein 1, has been shown to be essential for Rictor-mTOR interaction and may serve as a scaffold between mTORC2 and its downstream target PKB (Jacinto, Facchinetti et al. 2006, Yang, Inoki et al. 2006). Protor (protein observed with Rictor) binds to Rictor, which regulates its expression (Pearce, Huang et al. 2007). However, Protor does not appear to be required for the assembly of the other components of mTORC2 nor its kinase function and its protein structure lacks any obvious domains to provide clues to its exact role. As with mTORC1, Deptor binds near to the kinase

domain of mTOR and negatively regulates the kinase activity of mTORC2 (Peterson, Laplante et al. 2009).

As suggested by the naming of Rictor (*rapamycin insensitive* companion of mTOR), mTORC2 was originally thought not to be inhibited by rapamycin (Sarbassov, Ali et al. 2004, Jacinto, Loewith et al. 2004). However, later work has shown that in certain cell types prolonged rapamycin treatment does indeed inhibit mTORC2 assembly and kinase activity (Sarbassov, Ali et al. 2006, Zeng, Sarbassov dos et al. 2007). This has not yet been demonstrated in pancreatic β cells.

The mechanism of inhibition of mTORC2 by rapamycin has not yet been elucidated. Possible explanations included the binding of the FKBP12-rapamycin complex to free mTOR, preventing subsequent binding of Rictor (Sarbassov, Ali et al. 2006). Rapamycin has also been shown to regulate the phosphorylation of Rictor (Akcakanat, Singh et al. 2007). It is possible that rapamycin mediated dephosphorylation of Rictor inhibits mTORC2 assembly by cytoplasmic translocation of nuclear Rictor (Rosner, Hengstschlager 2008).

2.3.2 Upstream signalling of mTORC2

Unlike mTORC1, the upstream targets of mTORC2 have yet to be fully elucidated. The activity of mTORC2 appears to be controlled by phosphatidylinositol 3-kinase (PI3K) (Sarbassov, Guertin et al. 2005, Huang, Dibble et al. 2008) and, unlike mTORC1, is relatively insensitive to nutrients or energy conditions (Alessi, Pearce et al. 2009).

The TSC1-TSC2 complex also appears to activate mTORC2, with its disruption resulting in decreased mTORC2 kinase activity (Huang, Dibble et al. 2008). This seems to be independent of the GAP activity of the TSC1-TSC2 complex against Rheb and subsequent

inhibition of mTORC1. This study also demonstrated a physical association between the TSC1-TSC2 complex and mTORC2, suggesting a likely mode for this regulation.

In addition, it has been suggested that S6K1 may play a role in the regulation of mTORC2 via phosphorylation of Rictor at threonine 1135. Expression of a phosphorylation site mutant of Rictor (T1135A) has been shown to increase phosphorylation of PKB, one of the downstream targets of mTORC2 (Dibble, Asara et al. 2009, Julien, Carriere et al. 2010, Treins, Warne et al. 2010). However, it does not affect other downstream targets and this phosphorylation event itself does not appear to relate to mTORC2 integrity or *in vitro* kinase activity. Therefore the true significance of the role of S6K1 in the regulation of mTORC2 has yet to be determined.

2.3.3 Downstream targets of mTORC2

The first downstream target of mTORC2 to be described was protein kinase C α (PKCα). PKCα is a serine/threonine kinase which is ubiquitously expressed in mammalian cells and plays a role in the regulation of a number of key cellular processes including apoptosis, proliferation, motility and differentiation (Nakashima 2002). mTORC2 activity results in phosphorylation of PKCα at serine 657, which is necessary for its kinase activity, and plays an important role in the organization of the actin cytoskeleton (Sarbassov, Ali et al. 2004, Jacinto, Loewith et al. 2004).

Interestingly, PKCα has also been shown to regulate mTORC2 activation itself (Partovian, Ju et al. 2008). PKCα is targeted to the plasma membrane by the single-pass transmembrane protein syndecan-4 (S4). Here it is required for recruitment of the mTORC2 components to membrane lipid raft domains where PKB activation takes place. In S4 deficient cells

decreased levels of mTORC2 components are seen in the rafts with subsequent reductions in mTORC2 and PKB activation.

After a long search mTORC2 was found in 2005 to be the elusive kinase responsible for phosphorylation of protein kinase B (PKB), also known as Akt, at serine 473 (Sarbassov, Guertin et al. 2005). PKB is a serine/threonine kinase that regulates a wide range of signalling pathways that include proliferation, cell growth, differentiation, glucose transport and protein and glycogen synthesis. However, perhaps its key role is in controlling cell survival. The activation, downstream targets and biological functions of PKB, particularly related to pancreatic β cells, will be discussed in detail in chapter 4.

mTORC2 has also been shown to be responsible for the activation of SGK1 (serum- and glucocorticoid-induced protein kinase 1) by phosphorylation at serine 422 (Garcia-Martinez, Alessi 2008). SGKs are stimulated by insulin and other growth factors and one of their roles is control of ion transport. In particular, they stimulate sodium transport into epithelial cells by enhancing the stability and expression of the ENaC (epithelial sodium channel) (Diakov, Korbmacher 2004).

PKCα, PKB and S6K all belong to the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family. This kinase family is characterized by the presence of a unique interaction between the C-tail, partly comprised of a conserved hydrophobic motif (HM), and the catalytic domain. This interaction regulates kinase activity (Kannan, Haste et al. 2007). Optimal activation of AGC kinases occurs by phosphorylation of the activation loop (A-loop) in the catalytic domain by PDK1 and at the HM site of the carboxyl terminus (Newton 2003). A further highly conserved region in the carboxyl terminus of AGC kinases is the turn motif (TM), phosphorylation of which stabilizes the kinase core (Newton 2003). mTORC2 has been shown to control phosphorylation of both the TM and HM sites of PKB

and PKC, thereby regulating both their kinase activity and stability (Facchinetti, Ouyang et al. 2008, Ikenoue, Inoki et al. 2008).

Surprisingly, given the broad knowledge of the role of mTORC1 in pancreatic β cell function, no work has been done to investigate the specific role of mTORC2. However, there is a large body of research on the importance of PKB in β cell function, which will be explored in depth in section 4.1.4.

2.4 Summary and aims

Rapamycin has been the primary immunosuppressant used in a number of islet transplant programmes for type I diabetes. This choice was based on the assumption that it was less toxic to pancreatic β cells than other immunosuppressants such as corticosteroids and tacrolimus. However, there is evidence that drug toxicity plays a key role in the poor long term outcomes of islet transplantation.

The immunosuppressive effects of rapamycin are mediated via inhibition of mTORC1. However, mTORC1 is known to play a key role in pancreatic β cell homeostasis. One would therefore expect mTORC1 inhibition to have detrimental effects on β cells. In addition, rapamycin has been shown to inhibit mTORC2 in certain cell types. If this is the case in β cells, and rapamycin results in reduced PKB activation, this may have further adverse effects. Therefore the aims of my work were firstly to confirm the toxic effects of rapamycin on pancreatic β cells and then to investigate the mechanism of any effects seen, with an emphasis on the role of mTORC2 signalling.

CHAPTER 3

Materials and methods

3.1 General reagents and materials

Unless stated, all chemicals were of analytical grade and were routinely purchased from Sigma-Aldrich (Gillingham, UK), Fisher Scientific (Loughborough, UK) or Melford (Ipswich, UK). Bacterial cell culture reagents were from Melford (Ipswich, UK). Disposable plastics (1.5ml microfuge tubes, non-filtered pipette tips, tubes) were obtained from Sarstedt (Leicester, UK) or VWR (Lutterworth, UK). Filtered pipette tips were purchased from Axygen (Thistle Scientific, Glasgow, UK). Rapamycin was purchased from Calbiochem (Nottingham, UK). [γ^{32}P] ATP was purchased from GE Health Care (Amersham, UK). Recombinant adenovirus encoding constitutively active PKB was purchased from Vector Biolabs (Philadelphia, PA, USA). 3-(4,5-Dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Gillingham, UK).

3.2 Mammalian cell culture

Cell lines used in this study were Human Embryonic Kidney 293 (HEK-293) and Mouse Insulinoma 6 cells (MIN6). MIN6 cells, which respond to physiological changes in glucose concentrations (Miyazaki, Araki et al. 1990, Ishihara, Asano et al. 1993), were kindly provided by Prof. Jun-Ichi Miyazaki, Osaka University Medical School, Japan.

Tissue culture plates and flasks were from NUNC (Fisher Scientific, Loughborough, UK) or TPP (LSL, Rochdale, UK). Tissue culture pipettes were from Greiner Bio-One (Stonehouse, UK) or Corning Life Sciences (Fisher Scientific, Loughborough, UK).

3.2.1 Maintenance of cell lines

HEK-293 cells were used at approximately 80% confluence and were grown in full Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Gillingham, UK) containing 25mM glucose supplemented with 10% heat-inactivated foetal calf serum (FCS) (Gibco BRL; Invitrogen, Paisley, UK), 100μg/ml streptomycin and 100units/ml penicillin sulphate (Sigma-Aldrich, Gillingham, UK), equilibrated with 5% CO_2, 95% air at 37°C. Cells were split every 2-3 days.

MIN6 cells were used at approximately 80% confluence between passages 16 to 51. For experiments on glucose stimulated insulin secretion, low passages were used. MIN6 cells were grown in DMEM containing 25mM glucose supplemented with 15% heat-inactivated FCS, 100μg/ml streptomycin, 100units/ml penicillin sulphate, 200μg/ml neomycin, 40mM $NaHCO_3$ and 75μM β-mercaptoethanol, equilibrated with 5% CO_2, 95% air at 37°C. The medium was changed every 2-3 days and cells split every 7 days.

3.2.2 Cell splitting

When cells reached approximately 80% confluence, the media was removed, cells were washed once in 1X phosphate buffered saline (PBS) (Sigma-Aldrich, Gillingham, UK) and then incubated with 1ml 1X trypsin/EDTA (0.5%) (Gibco BRL; Invitrogen, Paisley, UK) for 2-5 minutes at 37°C. Once detached from the plate, cells were immediately resuspended in DMEM. Resuspended MIN6 cells were split 1:3 to 1:4 for maintenance, or as required for experiments; 293 cells were split 1:5 to 1:10 for maintenance, or as required for experiments.

3.2.3 Islet isolation and culture

Pancreatic islets were isolated from 200-250g male Wistar rats by collagenase digestion and Histopaque density gradient centrifugation by a modification of the method of Guest and Rhodes *et al* (Guest, Rhodes et al. 1989), itself a modification of the method of Lacy and Kostianovsky (Lacy, Kostianovsky 1967). Briefly, six male Wistar rats were anaesthetized and the pancreas was inflated by injecting 6ml of Roswell Park Memorial Institute (RPMI) 1640 medium containing 11mM glucose (Sigma-Aldrich, Gillingham, UK) and additional 1mg/ml collagenase (Serva, Heidelberg, Germany) through the common pancreatic duct. After the animals were killed by exsanguination from an incision in the heart, the pancreas was removed. The excised pancreata were then incubated at 37°C for 17 minutes. Following incubation the pancreata were then individually hand shaken for 1 minute. The partially disaggregated tissue was then centrifuged for 3 minutes at 200 x *g* at 4˚C. The pelleted material was then resuspended in RPMI 1640 medium and subjected to another cycle of resuspension and centrifugation. The pelleted material was then resuspended in RPMI 1640 medium containing 5% fetal calf serum and then filtered through a 6.5cm-diameter plastic tea strainer. Each filtrate was centrifuged for 3 minutes at 200 x *g* at 4˚C and the pellets were resuspended in 10ml of Histopaque 1077 (Sigma-Aldrich, Gillingham, UK) and over-layered with 10ml of RPM1 1640. The tubes were centrifuged for 20min at 1600 x *g* at 4˚C and islets were recovered from the RPMI/Histopaque-1077 interface and washed once in RPMI 1640 medium containing 5% fetal calf serum. The islets were then hand-picked under a stereomicroscope to ensure high purity of the preparation. The islets were then cultured at 37°C 5% CO_2, 95% air in CMRL 1066 (Gibco BRL; Invitrogen, Paisley, UK) medium containing 5.6mM glucose and 1% bovine serum albumin (BSA) (Sigma-Aldrich, Gillingham, UK) instead of 10% fetal calf serum, to prevent flattening of the islets on the

bottom of the dish. Unless otherwise specified, the islets were cultured overnight prior to treatments.

3.2.4 Infection of cell lines with recombinant adenoviruses

Adenoviral mediated transduction of MIN6 cells was performed as follows. Growth medium was aspirated from cells and replaced with 400µl of DMEM (for a 4cm diameter plate) supplemented with antibiotics, minus FCS. High titre viral stock was added to the cells (for details see figure legends) and incubated at 37°C/5% CO_2. After one hour, 1.2ml of complete DMEM (containing FCS) was added to the cells, and the incubation continued for a further 24-48h prior to experimentation.

3.2.5 Infection of isolated islets with recombinant adenoviruses

Adenoviral mediated transduction of islets was carried out in microfuge tubes. Growth medium was removed and 50µl of RPMI 1640 medium supplemented with 5% FCS added. 1µl of high titre adenoviral stock (diluted 1:10) was added to the islets and incubation continued at 37°C/5% CO_2. After one hour, the islets were transferred to a dish containing 1.5ml RPMI 1640 medium with added 5% FCS.

3.3 Experimental techniques

3.3.1 Buffers and reagents

Krebs Ringer Bicarbonate buffer (KRB)

115mM NaCl

5mM KCl

10mM $NaHCO_3$

2.5mM $MgCl_2$

2.5mM $CaCl_2$

Supplemented with 20mM HEPES pH 7.4

MIN6 lysis buffer

1% v/v Triton X-100

10mM β-glycerophosphate pH 7.4

50mM Tris-HCl pH 7.5

1mM EDTA pH 8

1mM EGTA

1mM sodium orthovanadate

50mM sodium fluoride

1mM benzamidine

0.2mM PMSF

1μg/ml leupeptin

1μg/ml pepstatin A

0.1% v/v β-mercaptoethanol

CHAPS lysis buffer for immunoprecipitation

0.3% v/v CHAPS

40mM HEPES pH 7.5

120mM NaCl

1mM EDTA pH 8

10mM sodium pyrophosphate

10mM β-glycerophosphate pH 7.4

50mM sodium fluoride

0.5mM sodium orthovanadate

1mM benzamidine

0.2mM PMSF

1μg/ml leupeptin

1μg/ml pepstatin A

3.3.2 Treatment of cell lines

Rapamycin at the concentrations stated in the figure legends was added directly to the cell culture medium. For all experiments the control comprised cells cultured in the absence of rapamycin for a period of time equivalent to the longest rapamycin treatment. At the end of the treatment period the media was removed and discarded. All treatments were stopped by the addition of ice-cold MIN6 lysis buffer (see section 3.3.1), or CHAPS lysis buffer (see section 3.3.1) for mTOR/Rictor immunoprecipitation. The lysates were then centrifuged for 10 min at 16,000 × *g*.

3.3.3 Treatment of isolated islets

For treatments, islets were counted into microfuge tubes containing KRB. Islets were then washed in the pre-incubation medium and were collected by centrifugation at 800rpm for 1 minute. The supernatant was removed and the wash step repeated and islets were then transferred into 4cm diameter Sterilin plates (Fisher Scientific, Loughborough, UK). Treatments were performed as described in the figure legends. Controls were as described above for the cell lines. After treatment, islets were collected and transferred back into microfuge tubes and collected by centrifugation 16,000 × *g* for 30 seconds at 4˚C. The supernatant was discarded and the experiment continued as described in section 3.6.1 (glucose-stimulated insulin secretion) and section 3.7.3 (cell death detection).

3.4 Recombinant adenoviral techniques

3.4.1 Amplification of adenoviral stocks

The commercial adenovirus encoding constitutively active PKB (AdCaPKB) was used to infect confluent T-75 flasks of HEK-293 cells as follows. The media was removed and 4mls fresh DMEM media without FCS added, followed by 20µL AdCaPKB. After 1 hour a further 2.4ml of DMEM supplemented with 10% FCS and 5.6ml of DMEM without serum were added. Approximately 3-5 days post-infection, when 70-90% of the cells were floating, the cells were harvested as follows. Cells were transferred to 50ml conical tubes and pelleted at 1800 x *g* for 5 minutes at 4˚C. The supernatant was removed and the cell pellet resuspended in 500µL PBS, frozen in a dry ice/ethanol bath then thawed in a 37°C water bath and vortexed. This cycle was repeated 3 more times to ensure complete cell lysis. The samples were then centrifuged at 3200 x *g* for 10 minutes at 4˚C to pellet cell debris. The pellet was discarded and the resulting viral supernatant was stored at -80°C.

3.5 Protein techniques

3.5.1 Buffers and reagents

10X Tris-Glycine buffer

30g Tris base

144g Glycine

1L ddH_20 qsp

SDS-PAGE running buffer

1X Tris-Glycine buffer

0.1% w/v SDS

Semi-dry transfer buffer

1X Tris-Glycine buffer

0.01% w/v SDS

20% v/v Methanol

10X PBS (Phosphate buffered saline)

3g KCl

100g NaCl

14g Na_2HPO_4

3g KH_2PO_4

1L ddH_20 qsp

pH to 7.4

PBS-Tween (PBST)

1X PBS

0.1% v/v Tween-20 (Sigma)

Laemmli sample buffer (4X)

0.25M Tris pH 6.8

4% w/v SDS

40% v/v Glycerol

10% v/v β-mercaptoethanol

20μg/ml Bromophenol blue

Destain/fixing solution

50% v/v Methanol

10% v/v Acetic acid

3.5.2 Antibodies

Antibodies used for Western blotting and immunoprecipitation are listed in table 3.1 **(**New England Biolabs, Hitchin, UK) (University of Dundee, Dundee, UK) (Transduction Laboratories; BD Biosciences, Oxford, UK).

Antibody	Obtained from	Primary Dilution	Secondary antibody	Application
Anti-mTOR	New England Biolabs	1/1000	Rabbit	WB
Anti-raptor	New England Biolabs	1/1000	Rabbit	WB
Anti-Rictor	New England Biolabs	1/500	Rabbit	WB
Anti-mTOR	University of Dundee	-	Protein A	IP
Anti-rictor	University of Dundee	-	Protein A	IP
Anti-phospho ribosomal protein S6 (Ser^{240} Ser^{244})	New England Biolabs	1/1000	Rabbit	WB
Anti-ribosomal protein S6	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho Akt/PKB (Ser473)	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho Akt/PKB (Thr308)	New England Biolabs	1/500	Rabbit	WB
Anti-Akt/PKB	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho GSK-3α/β (Ser21/9) Rabbit mAb	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho MDM2 (Ser166)	New England Biolabs	1/500	Rabbit	WB
Anti-phospho PKCα/βII (Thr638/641)	New England Biolabs	1/1000	Rabbit	WB
Anti-PKCα	Transduction Laboratories	1/1000	Rabbit	WB
Anti-GAPDH	New England Biolabs	1/10000	-	WB
HRP-coupled anti-Rabbit	New England Biolabs	1/3000	-	WB
HRP-coupled anti-Mouse	New England Biolabs	1/3000	-	WB

WB = Western Blotting IP = Immunoprecipitation

Table 3.1 Antibodies used for Western blotting and immunoprecipitation

3.5.3 Sample preparation

Following experimentation, cells were scraped off 4cm diameter plates in 100µl of ice cold MIN6 lysis buffer and transferred to a clean microfuge tube. The lysate was vortexed for 10 seconds, and centrifuged at 16000 x *g* at 4°C for 10 minutes. The post nuclear supernatant was removed and transferred to a fresh microfuge tube. Cell lysates were then stored at -80°C until required.

3.5.4 Bradford assay

The Bradford protein assay was used to determine protein content of cell lysates. Bradford reagent (Bio-Rad Laboratories, Hemel Hempstead, UK) was diluted with distilled water 1/5 for use. 2µl of samples of unknown protein content was mixed with 8µl of ddH_2O and 1ml of diluted Bradford reagent and incubated for 5 minutes at room temperature. The A_{595} of the reaction mixture was measured on a WPA spectrophotometer, and the unknown protein content of samples was determined by linear regression against a standard curve of BSA (Sigma-Aldrich, Gillingham, UK) protein standards (0 to 20µg). Protein contents of individual lysates were normalised to the sample with the lowest protein content using the appropriate lysis buffer used in the experiment.

3.5.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

3.5.5.1 Polyacrylamide gel casting

Following assembly of the glass plates from either Bio-Rad (Hemel Hempstead, UK) or ATTO (GRI, Braintree, UK) gel systems, the running gel was poured between the plates and then immediately overlaid with dH_2O. Once the running gel had set (approximately 30 minutes) the dH_2O was removed and the stacking gel poured on top. The comb was inserted immediately after and the gel left to set for a further 30 minutes. The polyacrylamide gel recipes used are shown in table 4.2 (Acrylamide: Bis acrylamide ratio of 37.5:1). 12.5% gels were used for resolution of all proteins other than mTOR, Rictor and Raptor for which 7.5% gels were used. (BDH; VWR, Lutterworth, UK).

Solution	Atto gel (x2)		Biorad gel (x2)		Stacking gel (x2)
	7.5%	**12.5%**	**7.5%**	**12.5%**	
40% Acrylamide (BDH)	2.925ml	4.725ml	1.95ml	3.15ml	1.24ml
2% Bis-Acrylamide[1]	1.56ml	2.52ml	1.04ml	1.68ml	0.65ml
1.5M Tris-HCl, pH 8.8	3.75ml	3.75ml	2.5ml	2.5ml	-
1M Tris-HCl, pH 6.8	-	-	-	-	1.25ml
H_2O	6.54ml	3.78ml	4.36ml	2.52ml	6.7ml
10% SDS	0.15ml	0.15ml	0.1ml	0.1ml	0.1ml
TEMED[2]	7.5µl	7.5µl	5µl	5µl	10µl
10%APS[3] (add just before pouring)	75µl	75µl	50µl	50µl	75µl

[1]N,N'-methylene bis-acrylamide (BDH/Bio-Rad)

[2]NNN'N' tetramethylethylenediamine

[3]Ammonium persulfate

Table 4.2 Polyacrylamide gel recipes used for SDS-PAGE

3.5.5.2 Running of gels

The gels were immersed in SDS-PAGE running buffer in the appropriate Bio-Rad or ATTO system gel tanks. Laemmli sample buffer (4X) was added to protein samples prepared as in section 3.5.3 and 3.5.4 in a 1:3 ratio to give a 1X final concentration. Samples were then vortexed and centrifuged at 16000 x *g* at 4˚C for 1 minute. Protein samples were boiled for 3 minutes, then centrifuged briefly at full-speed and loaded alongside a protein marker (Prestained Protein Marker, Broad Range (7-175kDA); New England Biolabs, Hitchin, UK). SDS-PAGE gels were run vertically at 180 volts for approximately 90 minutes until the bromophenol blue had just run off the gel. Gels for mTOR, Rictor and Raptor were run for 120 minutes due to the large molecular weight of these proteins

3.5.5.3 Western blotting

Once the gels had been run, proteins were transferred on to Immobilon-P PVDF filters (Millipore, Watford, UK) using a Semi-Dry Transfer Cell (Bio-Rad, Hemel Hempstead, UK). Briefly, membranes were pre-soaked in 100% methanol for 1 minute. Six Whatman 3mm papers per gel, along with the membrane and gel were then equilibrated in semi-dry transfer buffer for 5 minutes. Three Whatman papers were placed on the cell and air bubbles removed. The membrane was then placed onto the papers and the gel placed on top of the membrane. Finally, a further 3 Whatman papers were placed on top. Any air bubbles and then excess transfer buffer were removed. Transfer was performed at 15 volts for 38 minutes. Transfer efficiency was assessed by transfer of the prestained protein markers.

Following transfer, membranes were blocked in PBST with 5% milk for one hour at room temperature with shaking. Where antibodies were diluted in PBST with 5% milk the blocking step was followed by an overnight incubation at 4°C in the specific antibody. If primary antibodies were diluted in PBST with 5% BSA, the membrane was washed 3 times for 10

minutes in PBST prior to incubation. Antibody concentrations were adjusted according to manufacturer's instructions. Following overnight incubation, the membrane was washed for 10 minutes in PBST 3 times. The membrane was then incubated in the appropriate secondary antibody (diluted 1:3000 in 5% milk PBST) for 1 hour at room temperature. The membrane was then washed 3 times for 10 minutes with PBST. Detection of proteins was performed by enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, UK). The membrane was then exposed to X-ray film (Kodak; GRI, Braintree, UK) for 1 minute to 1 hour, depending on protein signal. Proteins of interest were identified by comparison of their size to the broad range protein markers.

3.5.6 Immunoprecipitation

For immunoprecipitation, MIN6 cells were grown on 10cm plates. Following treatment, cell lysis was performed in 1mL of CHAPS lysis buffer (section 3.3.1) on ice for 20mins. Lysate and antibody (4µg antibody/1.2µg protein) were incubated overnight at 4°C with rotation. 25µl of Protein-G Sepharose beads (Sigma-Aldrich, Gillingham, UK) per sample (bed volume) were washed twice in 500µl of CHAPS lysis buffer, centrifuged at 800 x *g* for 1 minute and the supernatant discarded. The lysate/antibody solution was added to the Protein-G Sepharose beads and incubated for two hours at 4°C with rotation. After incubation, the Protein-G Sepharose beads were centrifuged at 2000rpm for two minutes. The supernatants were removed and stored at -80°C. The Protein-G Sepharose beads were then washed 4 times in 1ml of CHAPS lysis buffer, centrifuged at 800 x *g* for one minute and resuspended in 30µl each of 2X Laemmli sample buffer. Samples were boiled for 3 minutes, then loaded straight on to SDS-PAGE gels, or were stored at -80°C until required. Protein A secondary antibody (1:10000 dilution) was used.

3.5.7 PKB kinase assay

MIN6 cells were infected with recombinant adenovirus expressing constitutively active PKB as described in section 3.2.4. 24 hours post infection MIN6 cells were treated as described in figure 5.9. Protein lysates were prepared as described in section 3.5.3. PKB protein was immunoprecipitated for 3 hours at 4 °C with rotation with 25µl immobilized PKB antibody (Roche, Burgess Hill, UK). The beads were then centrifuged at 14000 x *g* for 30 seconds at 4 °C and the supernatant was removed. The immunocomplexes were washed three times with 500µl of MIN6 lysis buffer then two times with 1ml of Buffer B (50mM Tris-HCl, pH 7.5, 0.03% (w/v) Brij-35, 0.1mM EGTA and 0.1% 2-mercaptoethanol) and once with 1ml of ADB (assay dilution buffer) (20mM MOPS, pH7.2, 25mM β-glycerophosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol). The immunocomplexes were resuspended and incubated in a total volume of 75µl containing 10µl of ice cold ADB, 10µM PKI (PKA-specific inhibitor peptide) (Promega, Southampton, UK) to inhibit any cAMP-dependent protein kinases that may non-specifically bind to the complex, 10µl (approximately 30mM) of Crosstide substrate peptide (GRPRTSSFAEG) (Invitrogen, Paisley, UK). The reaction was started by adding 10µl of diluted [γ-32P] ATP stock solution (500µM ATP and 75mM magnesium chloride in ADB with γ-32P to a final concentration of 1µCi/µl). The reactions were incubated for 10 minutes at 30°C with continuous shaking on an Eppendorf Thermomixer at 1000rpm. Then the immunocomplexes were centrifuged at 2000rpm for 30 seconds and 25µl of the supernatant was spotted onto the center of a 2cm x 2cm square of Whatman P81 phosphocellulose paper (Fisher Scientific, Loughborough, UK) to terminate the reaction. The assay squares were washed three times with 0.75% phosphoric acid for 5 minutes per wash at room temperature followed by one wash in acetone. The squares were then transferred into vials and 3ml of scintillation cocktail added and counted by liquid scintillation spectrometry.

3.6 Functional assays

3.6.1 Glucose-stimulated insulin secretion assay

Following treatment, islets or MIN6 cells were incubated in 1ml KRB supplemented with 1mM glucose (basal) for 60 minutes at 37°C. The supernatant was collected and centrifuged at 2300 x *g* for 10 min at 4ºC. It was then stored on ice for imminent use or at -80°C for future use. Incubation continued in a further 1ml KRB containing 20mM glucose (stimulated) for a further 60 minutes at 37°C. The supernatant was collected and processed as above. For MIN6 cells the cell pellet was lysed in ice cold acid/ethanol solution (HCl 1.5% v/v, ethanol 75% v/v, H_20 23.5% v/v) prior to measurement of cellular insulin content. Insulin concentration of the supernatants and content of the lysate was assayed using an anti-mouse (for MIN6 cells) or anti-rat (for islets) insulin ELISA kit (DRG Instruments GmbH, Marburg, Germany) with mouse or rat insulin as a standard in accordance with the manufacturer's instructions. Briefly, 50µl of either the standards or samples were dispensed into the wells of a 96 well plate. 50µl of anti-insulin-HRP conjugate was then added. The plate was incubated for 2 hours at 30°C on a horizontal shaker set at 700 ± 100rpm. Each well was then washed 6 times with wash buffer, then 200µl of the freshly prepared revelation solution added into each well and the plate incubated for 15 min at room temperature on a horizontal shaker set at 700 ± 100 rpm. The reaction was terminated by dispensing 50µl of stopping reagent into each well. The absorbance was read at 450nM on a Novostar plate reader (BMG Labtech, Aylesbury, UK).

3.7 Cell viability assays

3.7.1 MTT assay

MIN6 cells were split 1:6 into 96 well plates and treated with rapamycin as indicated in figure legends. Following treatment 20µL of 5mg/ml MTT solution was added to each well and incubated at 37°C for 2 hours. The media was aspirated and the reaction terminated with 200µl dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Gillingham, UK) using gentle pipetting to dissolve all crystals. Absorbance was read at 450nm using a Novostar plate reader (BMG Labtech, Aylesbury, UK).

3.7.2 Annexin V / propidium iodide Assay

This assays works on the following principles. Annexin V binds to phosphatidylserine residues which become expressed on the cell surface during apoptosis. Propidium iodide (PI) is a membrane impermeant fluorescent DNA stain that is excluded from viable cells. However, during necrosis the cell membrane becomes permeable, allowing PI staining of nuclear DNA (see figure 3.1). Flow cytometry allows the evaluation of the fluorescence of individual cells. As such the percentage of a cell population stained with annexin V and or PI can be determined.

Following treatment, the media was removed and reserved in a universal container. Then 100µl of 1X trypsin/EDTA (0.5%) was added and the cells incubated for 4 minutes at 37°C. 1ml of DMEM was added and the cells dispersed by gentle pipetting. This media was removed, added to the reserved media and centrifuged at 200 x g for 5 minutes at room temperature. The media was discarded and the cell pellet gently resuspended in 2mls fresh DMEM supplemented with FCS and antibiotics. The cells were equilibrated by incubation at 37°C for 30 minutes. 1ml of cell suspension was transferred to a 5ml round bottom tube (BD Falcon; BD Biosciences, Oxford, UK) for use in the flow cytometer. Tubes were centrifuged

at 200 x g for 10 minutes at room temperature and the media was swiftly poured off. Cell staining was performed using the Annexin-V-Fluos staining kit (Roche, Burgess Hill, UK) as per manufacturer's instructions. Briefly, per sample, 1µl of Annexin-V-Fluos labelling reagent was prediluted in 100 µl incubation buffer and 2 µl of propidium iodide solution added. The cell pellet was resuspended in 100 µl labelling solution and vortexed. The solution was incubated for 15 minutes at room temperature. Prior to analysis by flow cytometry 0.5ml of incubation buffer was added per sample. Quantification of staining was performed using either a FACScan or FACSCalibur flow cytometer and Cellquest software (all BD Biosciences, Oxford, UK). Initially dot plots of SSC-H *versus* FSC-H were used to gate out debris and cell clusters, then Annexin-V staining was analysed on the FL1-H channel and propidium iodide on FL2-H channel for the FACScan and FL3-H channel for the FACSCalibur.

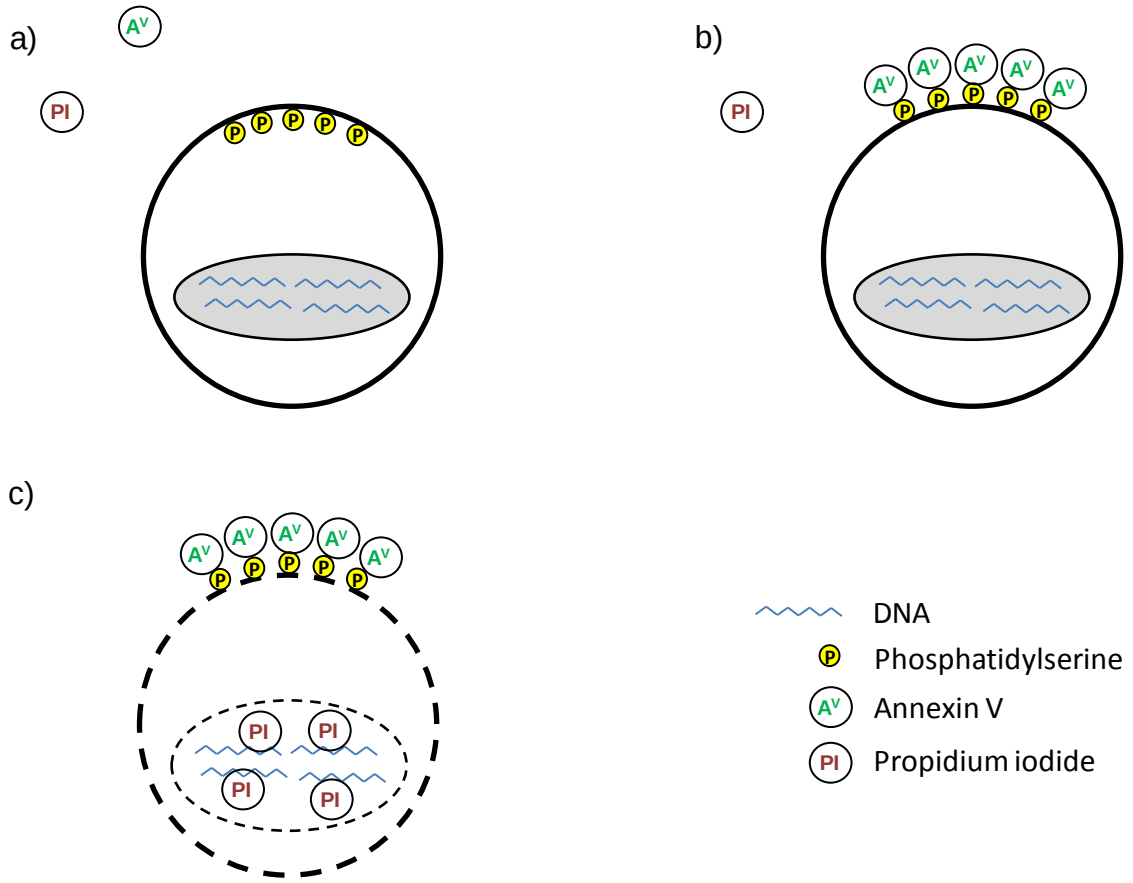

Figure 3.1 Principles of Annexin V / Propidium iodide staining. (a) In live cells phosphatidylserine residues are intracellular and do not bind annexin V, cell membrane integrity is maintained and the DNA stain propidium iodide cannot enter the cell. On flow cytometry there will be low annexin V and propidium iodide staining. (b) In apoptosis, phosphatidylserine residues are present on the cell surface and annexin V is bound; cell membrane integrity is maintained and propidium iodide cannot enter the cell. On flow cytometry there will be high annexin V binding, but low propidium iodide staining. (c) In necrotic cells phosphatidylserine residues are present on the cell surface and annexin V is bound; cell membrane and nuclear integrity is lost and propidium iodide binds to DNA. On flow cytometry both annexin V and propidium iodide staining are high.

3.7.3 Cell death detection assay

Evaluation of cell death in islets was performed using the Cell Death Detection ELISA Plus (Roche, Burgess Hill, UK), as per manufacturer's instructions. Briefly, following islet treatment and culture the medium was removed. The islets were resuspended in 200µl of the provided lysis buffer for 30 minutes at room temperature and then centrifuged at 200 x g for 10 minutes. 20µl of supernatant was transferred into the streptavidin coated microplate along with the provided positive control and background control (incubation buffer). 80µl of immunoreagent was then added to each well and the microplate incubated with gentle shaking at 300rpm for 2hrs at room temperature. Each well was washed 3 times with 250-300µl Incubation Buffer. 100µL ABTS solution was then added to each well and incubation continued on a plate shaker at 250rpm for 10-20mins prior to adding 100µl ABTS Stop Solution. Absorbance was measured at 405nm against ABTS Solution and ABTS Stop Solution as a blank using a Novostar plate reader (BMG Labtech, Aylesbury, UK).

3.8 Quantification and statistical analysis

Immunoblot band intensities were quantified using the Bio-Rad GS-800 Densitometer and Quantity One software (Bio-Rad Laboratories, Hemel Hempstead, UK). All data are given as mean ± SEM, and statistical analyses were performed using a one-way or two-way ANOVA plus Bonferroni's post hoc test and regarded as significant if $P < 0.05$. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

CHAPTER 4

Experimental results: effects of rapamycin on β cells

4.1 Introduction

There have been a number of studies investigating the effects of rapamycin treatment on both pancreatic β cell function and survival, which are summarised in tables 4.1 and 4.2. As described below the research is heterogeneous with a wide variety of cell types and rapamycin concentrations used. This has resulted in varied conclusions as to whether rapamycin has detrimental effects on pancreatic β cells or not. The key question for clinical islet transplantation is whether rapamycin has adverse effects on transplanted human islets *in vivo*. Unfortunately for both ethical and scientific reasons this question has not yet been fully answered.

4.1.1 Effects of rapamycin on pancreatic β cell function

Detrimental effects of rapamycin have been shown in rat (RIN-5F) and hamster (HIT-T15) insulinoma cell lines. Pre-treatment with 100 nM rapamycin, but not 10 nM, significantly reduced glucose stimulated insulin secretion (GSIS) in both RIN-5F and HIT-T15 cell lines (Fuhrer, Kobayashi et al. 2001). This study also demonstrated an acute insulin release stimulated by direct rapamycin treatment in a concentration range of 100-300 nM, which was blocked by rapamycin pre-treatment. Another group have also shown rapamycin at concentrations between 1 and 100 ng/ml to have significant effects on GSIS in HIT-T15 cells (Paty, Harmon et al. 2002).

Treatment of the mouse insulinoma cell line, MIN6, with a cocktail of immunosuppressive drugs containing rapamycin 25 ng/ml, mycophenolate 17.5 µg/ml and tacrolimus 75 ng/ml, resulted in significant decreases in GSIS and total cellular insulin content (D'Amico, Hui et al. 2005).

A number of groups have investigated the effects of rapamycin on murine islet function. Fabian et al showed decreased insulin secretion from murine islets after 72 hours *in vitro* incubation with 100 ng/ml rapamycin (Fabian, Lakey et al. 1993). Further studies have corroborated this with rapamycin 1 nmol/l, 10 nmol/l and 100nmol/L reducing glucose-stimulated insulin secretion in cultured rat islets, but this was only significant at the highest concentration (Bell, Cao et al. 2003). Rapamycin 30 ng/ml has been also shown to significantly impair GSIS in cultured mouse islets (Zhang, Su et al. 2006).

Additional *in vivo* work has shown detrimental effects of rapamycin treatment on β cell function. Rats treated with rapamycin 1.5mg/kg/day had significantly increased plasma and urinary glucose levels, although post-mortem histological appearances of the pancreata were normal (Whiting, Woo et al. 1991). These findings are corroborated by Song et al who showed significantly increased blood glucose concentration following intraperitoneal glucose tolerance testing (IPGTT) in rats treated with 0.3 and 0.6 mg/kg rapamycin for 4 weeks versus control (Song, Han et al. 2009). Furthermore, murine islet allograft recipients treated with rapamycin 5 mg/kg/day had significantly higher blood glucose levels than control (Fabian, Lakey et al. 1993). Zhang et al used a mouse syngeneic islet transplant model and demonstrated significantly higher blood glucose in mice treated with 1 or 1.5mg/kg rapamycin. Additionally, glucose tolerance was significantly impaired with 0.5, 1 and 1.5 mg/kg rapamycin, and GSIS significantly reduced with 1 or 1.5 mg/kg rapamycin (Zhang, Su et al. 2006).

However, work by other groups has been in conflict with the above results, either showing no effect or even beneficial effects with rapamycin treatment. Bussiere et al investigated the effects of rapamycin on syngeneic murine islet grafts, neonatal porcine islets (NPIs) and human islets. The results for syngeneic murine islet grafts showed that 20 ng/ml rapamycin treatment did not affect mean daily blood glucose. However, rapamycin treated animals had

significantly elevated blood glucose levels 60 minutes following a dextrose bolus and insulin content was reduced in islet grafts harvested from these animals. Nevertheless, no significant decrease in GSIS was seen in either human islets or NPIs cultured with 20 ng/ml rapamycin (Bussiere, Lakey et al. 2006).

Further work on the effects of rapamycin on cultured human islets has shown significant increases in stimulated insulin secretion following 24hrs culture in 8, 16 and 32 ng/ml rapamycin, but significant decreases in basal insulin secretion following 120 hours culture in 16 ng/ml rapamycin (Marcelli-Tourvieille, Hubert et al. 2007). Of note, concerns regarding the statistical analyses used in this study should be raised, with differences in insulin secretion between groups compared individually rather than by analysis of variance.

A study in dogs which had undergone total pancreatectomy and islet autotransplant showed beneficial effects of rapamycin treatment at 1mg/kg for 30 days. Rapamycin treatment significantly increased glucose clearance without a change in fasting plasma glucose levels. In addition, total and stimulated insulin release following glucose infusion was significantly higher with rapamycin treatment, as were fasting plasma insulin levels (Kneteman, Lakey et al. 1996).

Hyder et al cultured day-7 post-natal (P7) rat islets, adult rat islets and adult pig islets for 3 weeks in medium containing Edmonton protocol immunosuppressants (tacrolimus 5 ng/ml, rapamycin 15 ng/ml and daclizumab 10 ng/ml). In the adult rat islets they found no change in GSIS at 1 and 3 weeks, but significantly lower GSIS after 3 weeks. In addition the GSIS of both porcine islets and P7 rat islets was significantly lower at both 1 and 3 weeks (Hyder, Laue et al. 2005).

As can be seen from the above, different studies report conflicting results, even for the same cell type. This reflects differing rapamycin doses and treatment periods. However, it may also

be a reflection of islet purity. A study comparing the effects of rapamycin on pure (>90%) and impure (40-60%) islets found GSIS to be significantly higher in impure islets treated with rapamycin, but saw no difference in pure islets. Macrophage content, and subsequent cytokine release was reduced in impure islet preparations treated with rapamycin (Mita, Ricordi et al. 2008). This may explain the differences seen in GSIS, as cytokines are known to have detrimental effects on β cell function.

Authors	Experimental model	Rapamycin dose	Treatment duration	Significant findings
Fuhrer et al	RIN-5F cell line	100nM	30 mins	↓ GSIS
	HIT-T15 cell line	100nM	30 mins	↓ GSIS
Paty et al	HIT-T15 cell line	1-100ng/mL	48 hours	↓ GSIS
D'Amico et al	MIN6 cell line	25ng/mL	48 hours	↓ GSIS, ↓insulin content
Fabian et al	Murine islets	100ng/mL	72 hours	↓ Insulin secretion
	Murine allograft	5mg/kg/day	7 days	↑ Blood glucose
Bell et al	Rat islets	100nmol/L	4 days	↓ GSIS
Zhang et al	Mouse islets	30ng/mL	24 hours	↓ GSIS
	Murine syngeneic graft	0.5-1.5mg/kg	14 days	↓ GSIS
Whiting et al	Rat in-vivo	1.5mg/kg/day	13 days	↑ Plasma & urinary glucose
Song et al	Rat in-vivo	0.3-0.6 mg/kg	4 weeks	↑ Blood glucose
Bussiere et al	Murine syngeneic graft	0.2mg/kg	26 days	Impaired glucose tolerance
Marcelli-Tourvieille et al	Human islets	16ng/mL	120 hours	↓ Basal insulin secretion
Kneteman et al	Canine autotransplant	1mg/kg	30 days	↑ Glucose clearance, ↑ insulin release
Hyder et al	Post-natal rat islets	15ng/mL	3 weeks	↓ GSIS 1 & 3 weeks
	Adult rat islets	15ng/mL	3 weeks	↓ GSIS >3 weeks
	Adult porcine islets	15ng/mL	3 weeks	↓ GSIS 1 & 3 weeks

Table 4.1 Summary of studies investigating the effects of rapamycin on pancreatic β cell function (GSIS, glucose stimulated insulin secretion)

4.1.2 Effects of rapamycin on pancreatic β cell survival

As with the functional effects of rapamycin on β cells, the reported effects on β cell survival are diverse.

Bell et al used MIN6 cells, human islets and rat islets. They showed that 100 nmol/l rapamycin had a significant impact on the viability of both rat and human islets, whilst 1 and 10 nmol/l led to small insignificant decreases in viability. MIN6 cells treated with 10 nmol/l and 100 nmol/l rapamycin showed significantly increased apoptosis (Bell, Cao et al. 2003).

In contrast, another study showed apoptosis, as determined by DNA fragmentation and apoptotic nuclei counting, to be reduced in human islets treated with 8, 16 and 32 ng/ml rapamycin (Marcelli-Tourvieille, Hubert et al. 2007).

A further study investigated the effects of rapamycin on neonatal porcine islets (NPIs) and human ductal cells (HDCs), which are possible β cell progenitors. Rapamycin 10ng/ml inhibited HDC expansion by 53% and 20ng/ml by 50% following 6 days treatment. This reduction in cell number was not due to increased apoptosis. Furthermore, rapamycin 20ng/ml significantly reduced NPI cell number to 28% of control (Bussiere, Lakey et al. 2006).

Zahr et al investigated the effects of rapamycin on β cell proliferation using the murine pregnancy model, where there is a high background rate of β cell proliferation. They found that the proportion of β cells was significantly reduced by rapamycin 0.2 mg/kg/day for 5-8 days, islet yield was lower from treated animals, and the total insulin content of pancreata was reduced with rapamycin treatment. However, there was no difference in GSIS from isolated islets and no difference in the intraperitoneal glucose tolerance test between rapamycin treated and untreated animals (Zahr, Molano et al. 2007).

Authors	Experimental model	Rapamycin dose	Treatment duration	Significant findings
Bell et al	MIN6 cell line	10-100nmol/L	1-4 days	↑Apoptosis
	Human islets	100nmol/L	4 days	↓ Viability
	Rat islets	100nmol/L	4 days	↓Viability
Marcelli-Tourvieille et al	Human islets	8-32ng/mL	120 hours	↓ Apoptosis
Johnson et al	Human islets	10-30µg/L	24 hours	↑ Cleaved caspase-3
Bussiere et al	Neonatal porcine islets	10ng/mL	6 days	↑ Apoptosis

Table 4.2 Summary of studies investigating the effects of rapamycin on pancreatic β cell survival

Finally, in isolated human islets treatment with 10-30µg/L rapamycin for 24 hours has been shown to significantly increase levels of cleaved caspase-3 (Johnson, Ao et al. 2009), one of the key initiators of the apoptotic pathway. This study is of particular relevance as the concentrations of rapamycin used are similar to those seen in the portal vein of individuals undergoing islet transplantation.

In summary, the reported effects of rapamycin on pancreatic β cell function and survival are contradictory. However, the majority of studies in murine β cells show detrimental effects on function and survival. In contrast, the effects seen in human islets vary considerably and no clear conclusion can be made. It is possible that human islets are more resistant to the detrimental effects of rapamycin than murine islets, but no study has been conducted investigating the effects of rapamycin treatment longer than 120 hours on human islet function and survival. This is due, in part, to the technical difficulties with maintaining human islets in culture for this time period. Furthermore, it is not feasible to carry out an *in vivo* study of the effects of rapamycin on pancreatic β cells in humans.

Given the discrepancies in the literature outlined above the first aim of this thesis was to clarify the effects of rapamycin on the function and survival of the β cell types used in this research, namely the mouse insulinoma cell line, MIN6, and isolated rat islets.

4.2 Results

4.2.1 Effects of rapamycin on MIN6 function

Following treatment with 200nM rapamycin for 24 or 48hrs, MIN6 cells were incubated in low concentration glucose (1mM) followed by high concentration glucose (20mM). Insulin concentration of supernatants was determined by ELISA. As seen in figure 4.1 200nM rapamycin resulted in significant ($P<0.001$) reductions in both basal insulin secretion (0 *versus* 24 *versus* 48hrs = 10.64(±0.53) *versus* 7.48(±0.19) *versus* 3.82(±0.02) pmol/mg respectively) and stimulated insulin secretion (0 *versus* 24 *versus* 48hrs = 26.65(±0.10) *versus* 13.66(±0.11) *versus* 8.76(±0.07) pmol/mg respectively).

This reduction in GSIS could result from either a defect in insulin synthesis, or a defect in insulin secretion. To clarify this further, cell lysates were assayed for total insulin content. If total insulin content is unaffected it can be presumed that rapamycin does not affect insulin synthesis and the defect is in insulin secretion.

Total cellular insulin content was not affected by rapamycin treatment (figure 4.2) (0 *versus* 24 *versus* 48hrs = 3561.15(±34.19) *versus* 3716.30(±126.84) *versus* 3823.28(±10.77) pmol/mg respectively, $P=0.125$), suggesting that the effects of rapamycin are a result of a secretory defect. This is consistent with the known role of mTORC1 in insulin synthesis described in section 2.2.4.

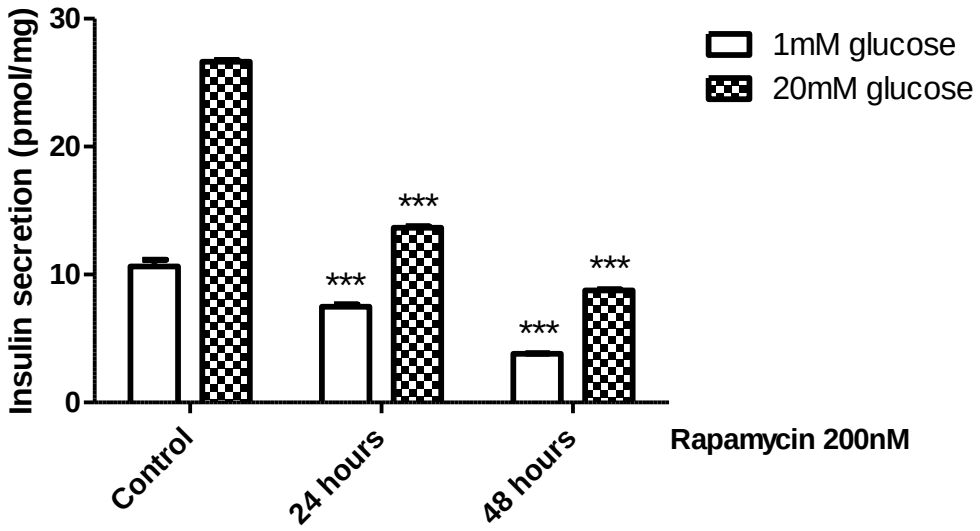

Figure 4.1 Effects of rapamycin on insulin secretion in MIN6 cells. MIN6 cells were treated with 200nM rapamycin as indicated. Following treatment cells were incubated in KRB containing 1mM glucose for 1hr followed by KRB containing 20 mM glucose for a further 1hr. Supernatants were collected and assayed for insulin concentration using ELISA. Data is displayed as mean ± SEM, *** P<0.001 *versus* relevant control using one-way ANOVA and Bonferroni's post hoc test. Results represent 3 independent experiments.

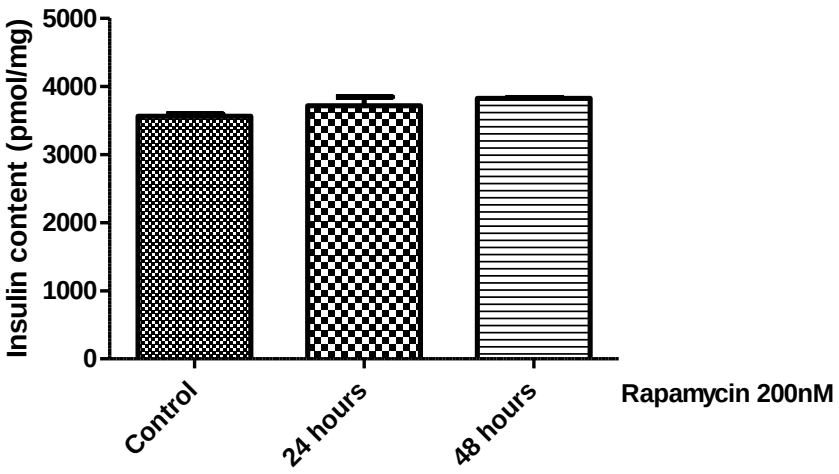

Figure 4.2 Effects of rapamycin on insulin content in MIN6 cells. Cells from figure 4.1 were lysed and insulin content assayed by ELISA. Results represent 3 independent experiments and data is displayed as mean ± SEM. No significant differences seen between groups using one-way ANOVA.

4.2.2 Effects of rapamycin on rat islet function

Having demonstrated detrimental effects of rapamycin on MIN6 function, similar experiments were conducted on isolated rat islets. Following treatment with 200nM rapamycin for 48 or 72 hours, rat islets were incubated in low concentration glucose (1mM) followed by high concentration glucose (20mM). Insulin concentration of supernatants was again determined by ELISA. Figure 4.3 demonstrates that rapamycin also has detrimental effects on rat islet function. Significant (P=0.0007) reductions were seen in basal insulin secretion (0 *versus* 48 *versus* 72hrs = 301.66(±12.04) *versus* 160.88(±15.63) *versus* 156.23(±21.12) pmol/mg respectively) and in stimulated insulin secretion (0 *versus* 48 *versus* 72hrs = 543.29(±62.29) *versus* 416.10(±1.45) *versus* 360.99(±28.90) pmol/mg respectively) following 48 and 72hrs rapamycin treatment.

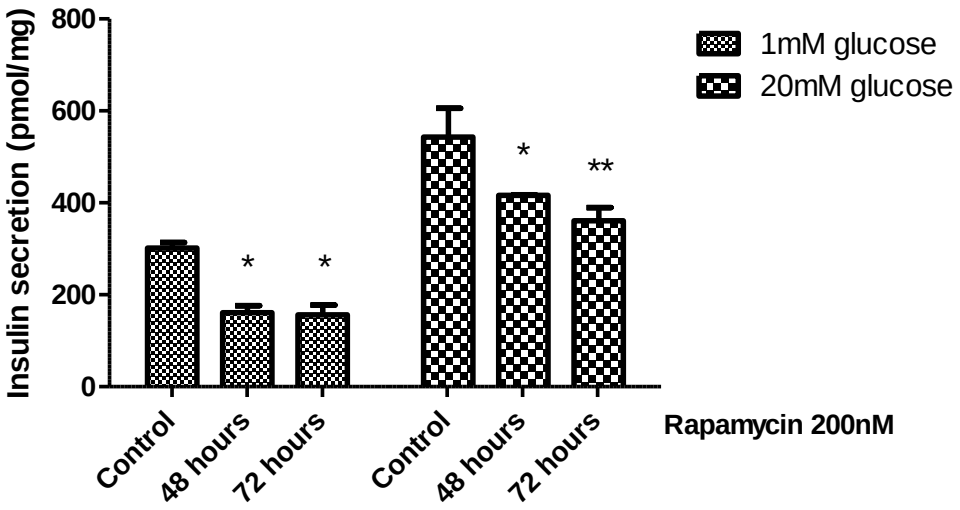

Figure 4.3 Effects of rapamycin on insulin secretion in rat islets. Isolated rat islets were treated with 200nM rapamycin as indicated. Following treatment islets were incubated in KRB containing 1mM glucose for 1hr followed by KRB containing 20 mM glucose for a further 1hr. Supernatants were collected and assayed for insulin concentration using ELISA. Data is displayed as mean ± SEM, *P=0.05-0.01, **=P0.001-0.01 *versus* relevant control using two-way ANOVA plus Bonferroni's post hoc test. Results shown are of 3 independent experiments.

4.2.3 Effects of rapamycin on MIN6 cell size

Given the well defined role of mTORC1 in the regulation of cell size, one would expect rapamycin treatment to result in diminished size of MIN6 cells. This was investigated using flow cytometry, with forward scatter used as an estimate of cell size. Importantly, cell staining with annexin V and propidium iodide was used to allow apoptotic and dead cells to be excluded from analysis (see section 3.7.2) as part of the apoptotic process is cell shrinkage. As expected, 24 hours rapamycin treatment resulted in a significant ($P<0.0001$) reduction in MIN6 cell size, which was independent of increased apoptosis or necrosis (figure 4.4).

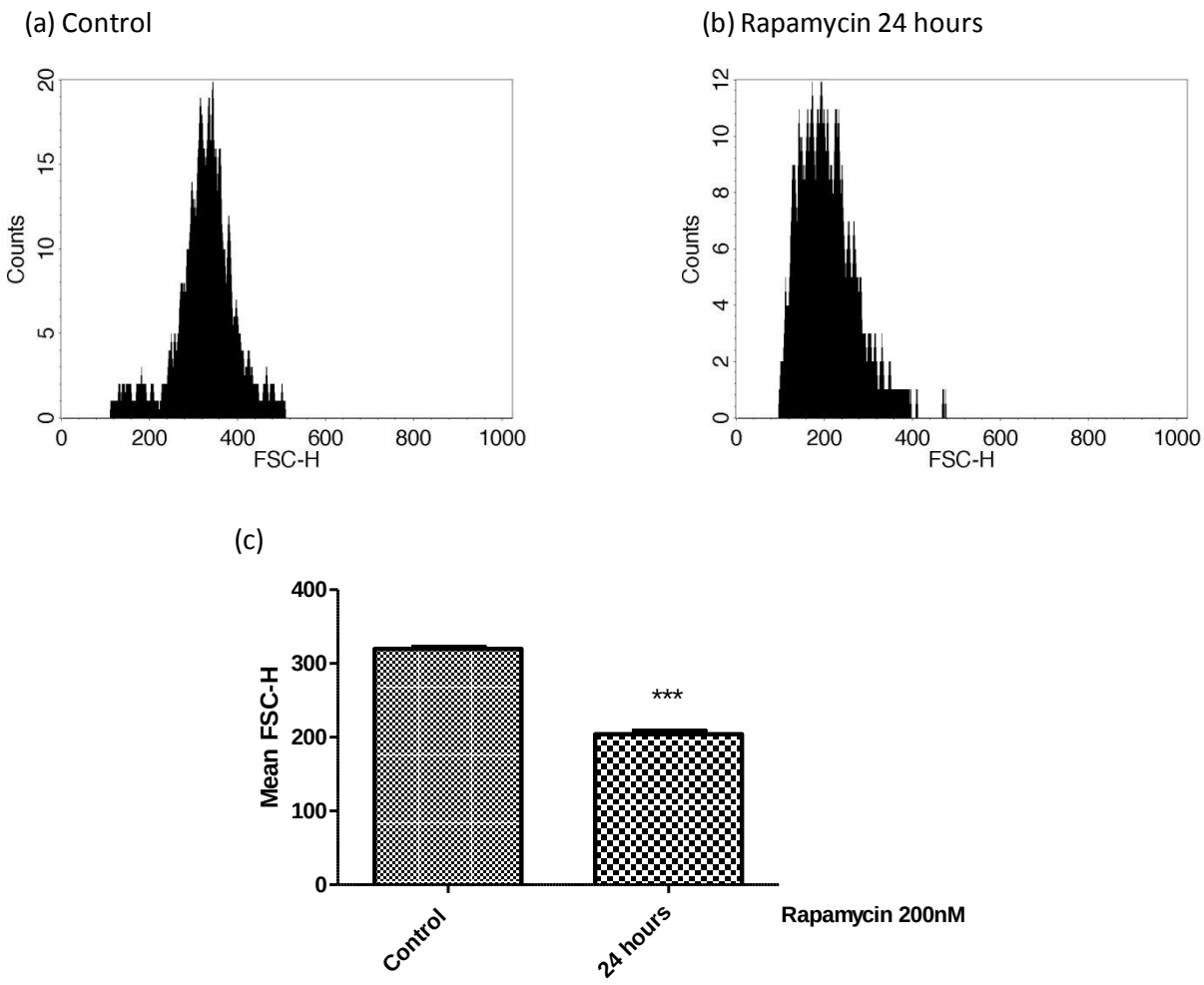

Figure 4.4 Effects of rapamycin on MIN6 cell size. MIN6 cells were incubated for 24hrs with (b) or without (a) 200nM rapamycin. Following treatment cells were stained with annexin V and propidium iodide and size of live cells measured by forward scatter (FSC-H) on flow cytometry. (c) cumulative results from 3 independent experiments. Data is displayed as mean ± SEM, *** P<0.001 *versus* control using Student's t-test.

4.2.4 Effects of rapamycin on MIN6 cell viability

The effects of treatment with 200nM rapamycin for 24, 48 and 72hrs on MIN6 cell viability were first assessed using the MTT colorimetric assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazole that is converted to purple formazan by reductases in the mitochondria of living cells (Mosmann 1983). Therefore, the more living cells the more purple formazan is produced.

Rapamycin treatment resulted in significant ($P<0.0001$) decreases in absorbance at 72 and 144 hours (figure 4.5). This suggests a significant reduction in the number of live cells. However, these findings may also be partly attributable to rapamycin-induced decreases in mitochondrial metabolism in the surviving MIN6 cells.

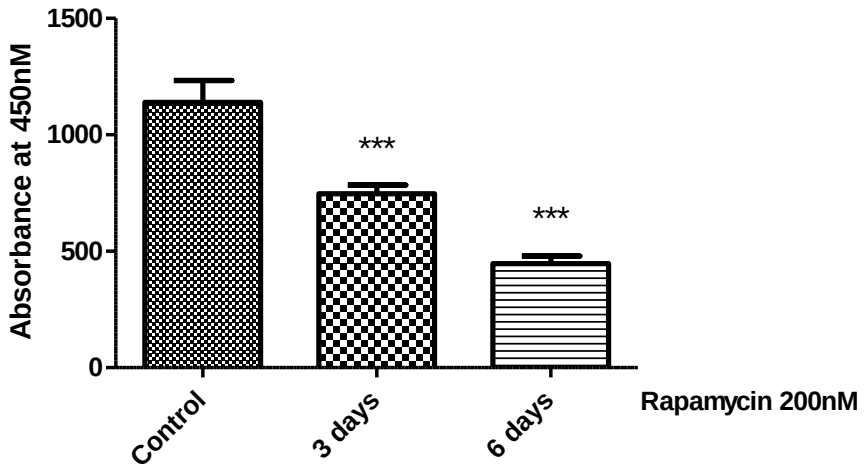

Figure 4.5 Effects of rapamycin on MIN6 cell viability. MIN6 cells were treated with 200nM rapamycin as indicated. Cell viability was assessed by MTT assay. Results shown are of 3 independent experiments. Data is displayed as mean ± SEM, ***P<0.001 *versus* control using one-way ANOVA plus Bonferroni's post hoc test.

Therefore, to provide a more specific measure of cell viability, the effects of rapamycin on MIN6 cell viability was assessed by staining with propidium iodide (PI) and annexin V and analysing fluorescence using flow cytometry (see section 3.7.2).

Figure 4.6 shows the results of the annexin V / PI staining in MIN6 cells. For the dot plots, the X axis (FL2-Height) represents PI staining and the Y axis (FL1-Height) represents annexin-V staining, with each dot representing a single cell. The lower left quadrant (low PI, low annexin-V staining) includes all live cells, the upper left quadrant (low PI, high annexin-V staining) includes all apoptotic cells, the upper right quadrant (high PI, high annexin-V staining) includes all necrotic cells and the lower right quadrant (high PI, low annexin-V staining) represents cellular debris.

As is clearly demonstrated from both the dot plots and from the pooled data shown in figure 4.6c, 200nM rapamycin results in a significant increase in MIN6 cell apoptosis at 24,48 and 72 hours (% apoptotic cells 0 *versus* 24 *versus* 48 *versus* 72hs = 6.8±0.8 *versus* 44.9±6.8 *versus* 68.7±5.0 *versus* 51.5±9.8 respectively; P=0.0002).

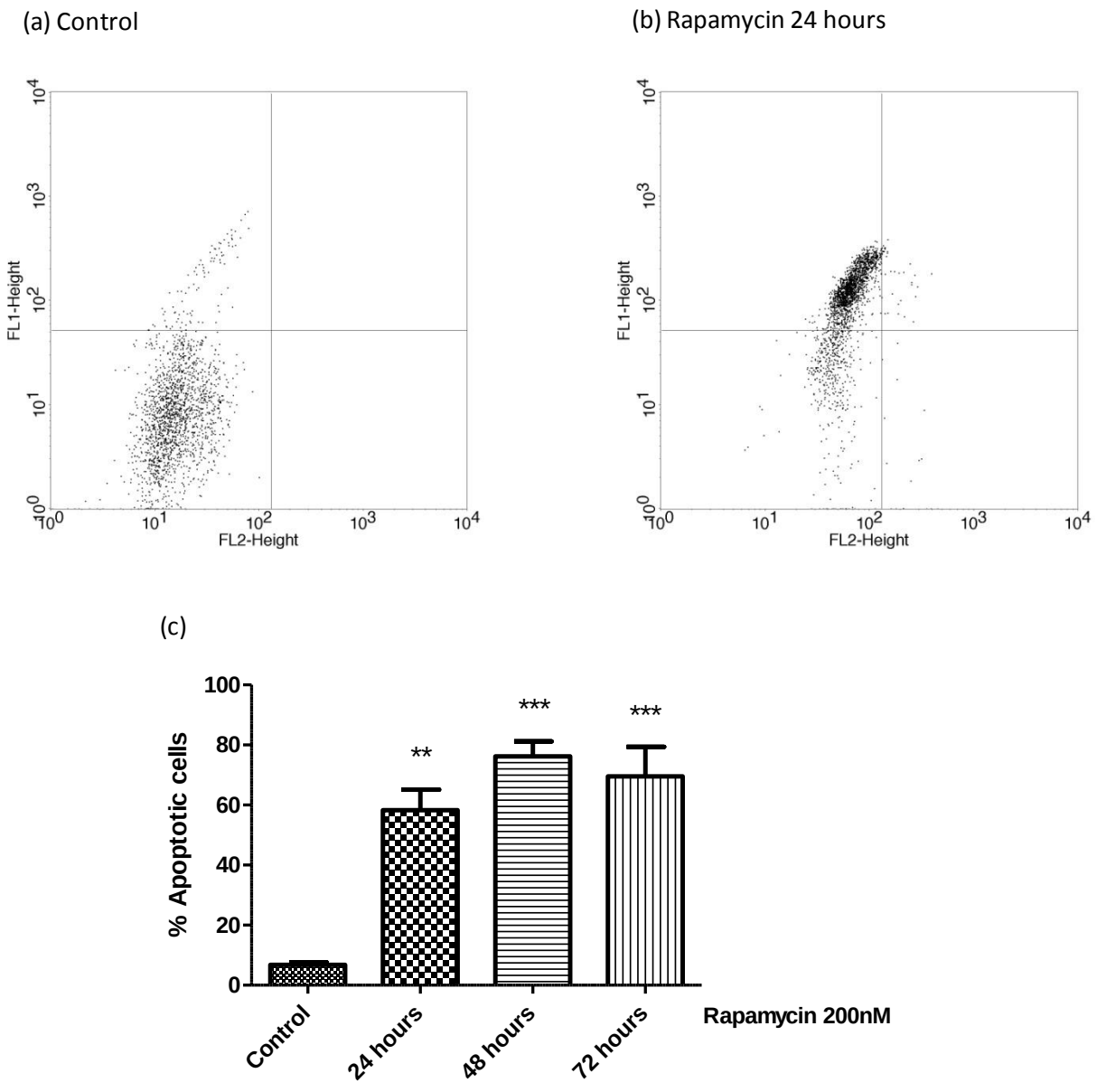

Figure 4.6 Effects of rapamycin on MIN6 cell apoptosis. MIN6 cells were treated with 200nM rapamycin as indicated. Following treatment cells were dispersed, stained with annexin V and propidium iodide and analysed by flow cytometry. FL1-Height = propidium iodide, FL2-Height = annexin V. (a) Control (b) Rapamycin 24hrs. (c) pooled % of apoptotic cells on flow cytometry from 3 independent experiments. Data is displayed as mean ± SEM, **=P0.001-0.01,***P<0.001 *versus* control using one-way ANOVA plus Bonferroni's post hoc test.

4.2.5 Effects of rapamycin on rat islet viability

Since dispersion of islets into a single cell suspension results in cell death (Aikin, Hanley et al. 2006), it is not feasible to use flow cytometry to assess islet viability. An *in-situ* cell death detection kit based on the TUNEL assay (*T*dT-mediated d*U*TP *n*ick *e*nd *l*abelling) was therefore utilised. DNA fragmentation is one of the hallmarks of apoptosis. As well as producing double stranded, low molecular weight DNA fragments, DNAse activity introduces strand breaks or 'nicks' into high-molecular-weight DNA. These processes can be identified by labelling the free 3'-OH termini with fluorescein dUTP, catalysed by terminal transferase (TdT). The fluorescein dUTP labelling is then quantified using a sandwich ELISA technique.

Following treatment with 200nM rapamycin, isolated rat islets were subjected to the above assay. Both 48 and 72 hours treatment resulted in significant increases in rat islet cell death ($P<0.001$) (figure 4.7), corroborating with the findings in MIN6 cells.

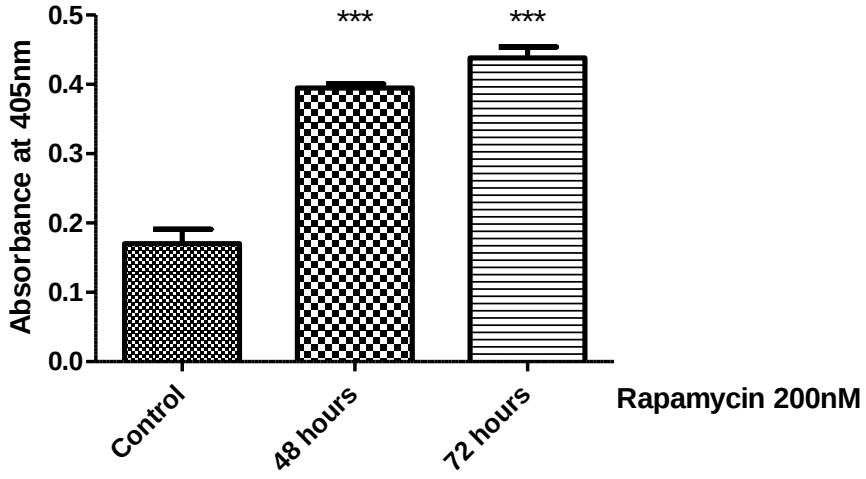

Figure 4.7 Effect of rapamycin on rat islet cell death. Following isolation, rat islets were treated with 200nM rapamycin as indicated. Cell death was determined using the In-Situ Cell Death Detection ELISA. Data is displayed as mean ± SEM and is obtained from 3 independent experiments. ***P<0.001 *versus* control using one-way ANOVA plus Bonferroni's post hoc test.

4.3 Discussion

My findings clearly demonstrate toxic effects in pancreatic β cells following prolonged rapamycin exposure. Treatment with 200nM rapamycin resulted in significant decreases in basal insulin secretion, stimulated insulin secretion and cell viability in both MIN6 cells and isolated rat islets. This corroborates with much of the published research précised earlier in this chapter.

It can be argued that the 200nM concentration of rapamycin used in these experiments is a supra therapeutic dose compared to the levels used in clinical islet transplantation. This is true, but the main aim of this study was to elucidate the mechanisms of rapamycin toxicity in pancreatic β cells. It is therefore important that there are significant differences in the function and viability between treated and untreated cell populations to allow alterations in cell signalling pathways to be detected.

The next stage of this thesis was to establish the mechanisms of the rapamycin mediated pancreatic β cell toxicity seen, focusing on the particular contributions of mTORC1 and mTORC2. The main downstream target of mTORC2 is PKB, which plays a central role in β cell homeostasis. This is now discussed in further detail.

CHAPTER 5

Experimental chapter: mechanisms of rapamycin toxicity in pancreatic β cells

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5.1 Introduction

As discussed above, the main downstream target of mTORC2 is protein kinase B (PKB), which is known to regulate a number of key cellular processes, including cell survival mechanisms.

5.2 Protein kinase B

Protein kinase B (PKB), also known as Akt or RAC, is a 57 kDa serine/threonine kinase containing an N-terminal pleckstrin homology (PH) domain first characterized in 1991 (Bellacosa, Testa et al. 1991, Coffer, Woodgett 1991, Jones, Jakubowicz et al. 1991b). As mentioned previously, it is a member of the AGC kinase family (section 2.3.3). In mammals, three distinct isoforms of PKB have been identified (PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3), which although structurally similar, differ in their expression profile (Jones, Jakubowicz et al. 1991a, Konishi, Kuroda et al. 1995). All three isoforms have been shown to be expressed in pancreatic β cells (Holst, Mulder et al. 1998).

5.2.1 Activation of PKB

As described in section 2.2.2, PKB activation occurs by phosphorylation of Thr308 by PDK1 and Ser473 by mTORC2. Phosphorylation of Thr308 is not dependent on phosphorylation of Ser473 and vice versa, and phosphorylation of either residue leads to partial activation of PKB. However, phosphorylation of both residues results in a synergistic high level activation of the enzyme (Alessi, Andjelkovic et al. 1996).

In pancreatic β cells PKB is activated by a number of upstream effectors. These include insulin, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF1) (Burgering, Coffer 1995, Aikin, Hanley et al. 2006, Holst, Mulder et al. 1998, Alessi, Andjelkovic et al. 1996).

The mechanism of insulin mediated PKB activation is via the insulin receptor which phosphorylates tyrosine residues on the insulin receptor substrates (IRS) 1 and IRS2. Tyrosine phosphorylation of IRS1/2 leads to subsequent binding and activation of PI3K (Myers, Backer et al. 1992, Yonezawa, Ueda et al. 1992). IGF1 binding to its receptor activates the intrinsic tyrosine kinase activity of the IGF1 receptor β subunit, which in turn phosphorylates IRS-2, leading to recruitment of PI3K (Sun, Wang et al. 1995). Other growth factors such as EGF, PDGF and FGF rely on the intrinsic tyrosine kinase activity of their receptors to recruit PI3K directly into a receptor signalling complex (Hawkins, Anderson et al. 2006, Kazlauskas, Cooper 1990, Valius, Bazenet et al. 1993). These complexes can be large and contain a number of scaffolding or adaptor molecules. As such the exact mechanisms by which PI3K is activated following recruitment by receptor kinase activity remain poorly understood.

Chronic (>40min) exposure to glucose has also been shown to directly activate PKB (Srinivasan, Bernal-Mizrachi et al. 2002), although the exact mechanism is not clear. It does not appear to be due to glucose induced insulin secretion, but may be mediated via increased IRS-2 expression caused by glucose-induced rises in intracellular calcium and or cAMP concentrations. Alternatively, PKB may be activated via a cAMP-dependent activation of cAMP-nucleotide guanine exchange factor (GEF) and PKA (Dickson, Rhodes 2004).

The gluco-incretin hormone glucagon-like peptide 1 (GLP1) also activates PKB in pancreatic β cells (Buteau, Roduit et al. 1999, Cornu, Yang et al. 2009, Wang, Li et al. 2004). Again, the mechanism of this activation has not be been clearly elucidated, although it is likely to be via similar cAMP dependent mechanisms as for glucose induced PKB activation. In addition, GLP1 has been shown to activate IGF1 receptor expression and signalling, which in turn required secretion of IGF2, suggesting an IGF2/IGF1 receptor autocrine loop (Cornu, Yang et al. 2009).

PKB is inhibited by protein phosphatase 2A (PP2A), which results in dephosphorylation of Thr308 (Resjo, Goransson et al. 2002) and by PH-domain leucine-rich repeat phosphatase (PHLPP) which dephosphorylates PKB at Ser473 (Gao, Furnari et al. 2005).

5.2.2 Downstream targets of PKB

PKB plays a key role in the regulation of a number of cellular processes including mitogenesis, survival, protein synthesis and differentiation and, as such, has an array of downstream targets (see figure 5.1).

5.2.2.1 Mitogenesis

5.2.2.1.1 GSK-3

Glycogen synthase kinase-3 (GSK-3) is one of the first and best characterised substrates of PKB (Cross, Alessi et al. 1995). GSK-3 phosphorylates cyclin D1 on Thr286, promoting its translocation from the nucleus to the cytoplasm where it undergoes rapid degradation (Diehl, Cheng et al. 1998). Increased cyclin D1 expression is key to initiating a cell's entrance into the cell cycle and subsequent increased mitogenesis. PKB rapidly phosphorylates GSK-3α at Ser21 and GSK-3β at Ser9, resulting in their inhibition (Sutherland, Leighton et al. 1993). Thus PKB mediated GSK3 inhibition depresses cyclin D phosphorylation and degradation, promoting mitogenesis.

5.2.2.1.2 $p27^{KIP}$

$p27^{KIP}$ is a cell cycle regulator which forms part of the cyclin D/Cdk4/$p21^{CIP}$/$p27^{KIP}$ complex that plays a central role in mediating cell growth and, conversely, also potently inhibits cyclin-dependent kinase 1(Cdk1) and Cdk2 (Dickson, Rhodes 2004). PKB phosphorylates $p27^{KIP}$ at Thr157, which promotes its cytosolic retention and degradation, removing the inhibition of Cdk1 and 2 (Liang, Zubovitz et al. 2002, Shin, Yakes et al. 2002). In addition, PKB phosphorylates and inhibits the forkhead transcription factor, which is required for transcription of $p27^{KIP}$ (Medema, Kops et al. 2000).

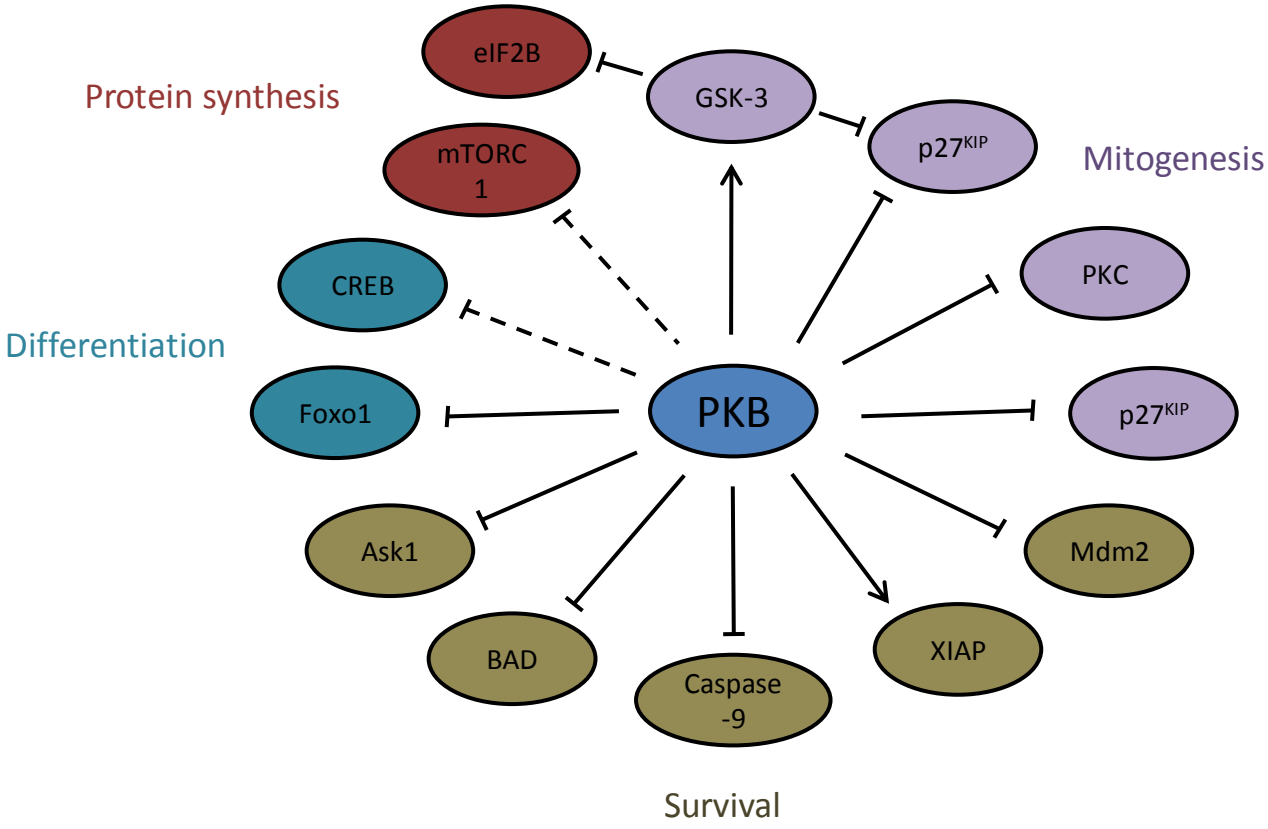


Figure 5.1 Downstream targets of protein kinase B (PKB). Arrows denote stimulatory effects, block ends denote inhibitory effects; solid lines denote direct effects, dashed lines denote indirect effects. Ask – apoptosis signal-regulating kinase, BAD – Bcl2/BclX_L antagonist causing cell death, CREB – cAMP response element binding, GSK – glycogen synthase kinase, Mdm – murine double minute, PKC – protein kinase C, XIAP – X-linked inhibitor of apoptosis.

5.2.2.1.3 $p21^{CIP}$

$p21^{CIP}$ plays a similar role in cell cycle regulation to $p27^{KIP}$. PKB phosphorylates $p21^{CIP}$ at Thr145, which then interacts with 14-3-3 proteins that sequester it in the cytoplasm and prevent it from entering the nucleus where it must be located to inhibit Cdks (Zhou, Liao et al. 2001). Complicating matters, PKB mediated inactivation of GSK3 results in reduced GSK3 induced phosphorylation of $p21^{CIP}$, preventing its degradation and resulting in activation of the cyclin D/Cdk4/$p21^{CIP}$/$p27^{KIP}$ complex (Rossig, Badorff et al. 2002).

5.2.2.1.4 PKC

Protein kinase C ζ (PKCζ) has also been shown to be phosphorylated by PKB, resulting in its translocation from the cytoplasm to the nucleus and subsequent activation (Dickson, Rhodes 2004, Buteau, Foisy et al. 2001). This activation of PKCζ results in increased cell proliferation. The mechanism by which cell growth is increased is unclear as little is known about the targets of PKCζ. Possible candidates include NFκB and PDX-1 (Buteau, Foisy et al. 2001).

5.2.2.2 Survival

5.2.2.2.1 Mdm2

Murine double minute 2 (Mdm2) is a ubiquitin ligase which interacts with the p53 tumour suppressor protein (Momand, Zambetti et al. 1992). PKB phosphorylates Mdm2 at Ser166 and Ser186, promoting its entry to the nucleus where it binds to p53, blocking its transcriptional activity and targeting it for degradation (Gottlieb, Leal et al. 2002, Mayo, Donner 2001). p53 activity in pancreatic β cells has been shown to be linked to apoptosis, and increased PKB activity therefore reduces apoptosis via this pathway (Wrede, Dickson et al. 2002).

5.2.2.2.2 XIAP

X-linked inhibitor of apoptosis protein (XIAP) is one of a family of proteins that inhibit apoptosis by directly binding and inhibiting caspase activity (Liston, Roy et al. 1996). PKB phosphorylates XIAP at Ser87, protecting it from ubiquitination and degradation, thereby promoting cell survival (Dan, Sun et al. 2004).

5.2.2.2.3 Caspase-9

The caspase proteases are key executioners of apoptosis, and caspase-9 initiates the pathway. PKB has been shown to phosphorylate caspase-9 at Ser196 in humans, inhibiting its protease activity (Cardone, Roy et al. 1998). However, this residue is not conserved in monkey or rodent homologues, and this is therefore unlikely to represent a key regulator of apoptosis.

5.2.2.2.4 BAD

Bcl2/BclX_L antagonist causing cell death (BAD) is a pro-apoptotic protein that associates with anti apoptotic proteins on cell membranes such as BclX_L, inhibiting their cell survival action (Yang, Zha et al. 1995). PKB phosphorylates BAD at Ser136 causing its sequestration in the cytosol and preventing its association with anti apoptotic proteins (Datta, Dudek et al. 1997, del Peso, Gonzalez-Garcia et al. 1997), thus promoting cell survival.

5.2.2.2.5 Ask1

Apoptosis signal-regulating kinase 1 (Ask1) activates mitogen-activated protein kinases (MAPK) kinases which in turn activate MAPKs such as the c-jun NH_2-terminal kinases (JNK) (Ichijo, Nishida et al. 1997). JNK activation is involved in apoptosis. PKB directly phosphorylates Ask1 at Ser, resulting in inhibition of Ask1 and reduced JNK activity with anti apoptotic effects in human islets (Aikin, Maysinger et al. 2004).

5.1.3.3 Protein Synthesis

5.2.2.3.1 mTORC1

As described in section 2.2.2 PKB activation leads indirectly to mTORC1 activation via TSC1/2 and Rheb. mTORC1 in turn phosphorylates 4E-BP1 and p70S6K, both of which regulate protein synthesis via increases in mRNA translation.

5.2.2.3.2 eIF2B

GSK3 inhibition by PKB prevents it from phosphorylating and inhibiting the eukaryotic initiation factor 2B (eIF2B) at Ser535 (Wang, Paulin et al. 2001), and consequently protein synthesis is increased.

5.2.2.4 Pancreatic β cell differentiation

5.2.2.4.1 CREB

PKB has been shown to potently induce PKA mediated phosphorylation of cAMP response element-binding (CREB) protein at Ser133 (Du, Montminy 1998). Phosphorylation of CREB is associated with the regulation of insulin and IRS-2 gene expression required for pancreatic β cell differentiation and survival (Jhala, Canettieri et al. 2003).

5.2.2.4.2 Foxo1

Foxo1 is a member of the forkhead transcription factor family and is phosphorylated in the nucleus at Thr24, Ser256 and Ser319 by PKB (Rena, Guo et al. 1999). Phosphorylation of Foxo1 results in nuclear exclusion and prevents its negative regulation of transcriptional activity. In β cells Foxo1 has been shown to block the positive transcriptional regulator Foxa2 from increasing expression of Pdx1, which is a key transcription factor for β cell differentiation and insulin gene expression (Kitamura, Nakae et al. 2002). In addition to its

role in the regulation of cell differentiation, Foxo1 target genes also inhibit cell survival. These include TNF-related apoptosis-inducing ligand (TRAIL) (Modur, Nagarajan et al. 2002), FasL (Brunet, Sweeney et al. 2004), Bim (Gilley, Coffer et al. 2003) and $BclX_L$ (Tang, Dowbenko et al. 2002).

5.2.3 Specific role of PKB in pancreatic β cell homeostasis

A large number of studies have been performed investigating the specific role of PKB in both survival and function of β cells.

Aikin et al demonstrated PKB phosphorylation in isolated human islets following overnight culture, which appears to be due to autocrine activation by insulin. This PKB phosphorylation was suppressed by treatment with anti-insulin antibody and insulin receptor inhibitors, resulting in increased islet death (Aikin, Hanley et al. 2006). Activation of PKB in human islets by simvastatin has also been shown to increase islet viability via decreases in caspase-9 activity and Bad phosphorylation (Contreras, Smyth et al. 2002). In addition, glucose-mediated activation of PKB has been observed in both MIN6 cells and cultured mouse islets, with culture in high glucose concentrations resulting in a threefold decrease in apoptosis in both cell types. This glucose mediated protection was lost in insulinoma cells expressing a kinase-dead PKB, suggesting this is the pathway regulating the effect (Srinivasan, Bernal-Mizrachi et al. 2002). Further work has shown, surprisingly, that infection of human islets with adenoviral vectors expressing luciferase or green fluorescent protein results in PKB activation and subsequently in increased islet cell proliferation (Icyuz, Bryant et al. 2009). The explanation of why infection with adenoviruses encoding non physiological proteins should result in PKB activation is not clear.

Activation of PKB by IGF-1 via PI3-K has also been shown to protect canine islets against cytokine mediated cell death (Aikin, Maysinger et al. 2004) and to reduce serum deprivation

induced cell death in both MIN6 and INS-1 insulinoma cell lines (Liu, Chin-Chance et al. 2002). However, of note IGF-1 activates a number of other signalling pathways including PKA, MAPK, ERK and CREB. Although these alternative pathways appear not to contribute significantly to IGF-1 mediated cell survival (Liu, Chin-Chance et al. 2002) it has not been conclusively shown that this effect is mediated solely via PKB activation.

Further evidence for the key role of PKB in β cell homeostasis has been obtained from studies using constitutively active mutants of PKB. Transgenic mice expressing a constitutively active PKB exhibit significant increases in β cell mass resulting from both an increase in β cell number and size (Bernal-Mizrachi, Wen et al. 2001, Tuttle, Gill et al. 2001). This manifested in significantly higher plasma insulin levels, improved glucose tolerance and resistance to streptozotocin induced diabetes. Expression of constitutively active PKB has also been shown to protect against fatty acid (Wrede, Dickson et al. 2002), cytokine (Collier, Fueger et al. 2006)and AMPK (Cai, Wang et al. 2008) mediated cytotoxicity in INS-1 cells, a rat β cell line and primary rat β cells respectively.

Conversely, studies in transgenic mice lacking PKB show significantly higher blood glucose levels, lower insulin levels and impaired glucose tolerance. These metabolic disturbances appear to result from dysregulation of insulin exocytosis rather than abnormalities in glucose signalling or voltage-gated calcium channel function (Bernal-Mizrachi, Fatrai et al. 2004). Further studies in transgenic mice lacking the PKBβ isoform have shown significantly raised blood glucose levels accompanied by markedly raised plasma insulin levels suggesting peripheral insulin resistance (Cho, Mu et al. 2001).

It is clear from the above studies that PKB plays a central role in regulating survival in pancreatic β cells, but also plays a role in insulin secretion. Therefore, the question arises whether the detrimental effects of rapamycin reported in chapter 4 are mediated via PKB. As

discussed, prolonged rapamycin treatment has been observed to inhibit mTORC2 activity and subsequent PKB activity in a number of cell types, but not in pancreatic β cells. This was therefore the focus for these investigations into the mechanisms of rapamycin toxicity in pancreatic β cells.

5.3 Experimental Results

5.3.1 Effects of rapamycin on phosphorylation of rpS6 in MIN6 cells

As expected, treatment of MIN6 cells with 200nM rapamycin resulted in significant reductions in the phosphorylation of rpS6 at Ser240/244 after only 1 hour treatment (figure 5.2), indicating successful inhibition of mTORC1.

5.3.2 Effects of rapamycin on PKB activity in MIN6 cells

Prolonged rapamycin treatment has been shown to inhibit PKB in a number of cell types, but not previously in β cells (Sarbassov, Ali et al. 2006). To investigate this MIN6 cells were incubated in 200nM rapamycin for up to 72 hours and PKB phosphorylation investigated by Western blotting. This has shown that rapamycin treatment for 24 hours and beyond results in significant reductions in the phosphorylation of PKB at Ser473, the residue phosphorylated by mTORC2 (figure 5.3). This is not accompanied by any significant changes in total PKB expression.

In addition, investigation of downstream targets of PKB showed significant reductions in the phosphorylation of GSK-3α/β at Ser21/9, confirming that prolonged rapamycin treatment also inhibits substrates of PKB (figure 5.4). Interestingly, no effect was seen on the phosphorylation of Mdm2, another known downstream target of PKB. The reason for this discrepancy is not clear, but it has been shown that although phosphorylation of PKB at Ser473 and Thr308 is required for high levels of kinase activity, phosphorylation of either

residue alone confers about a five-fold increase in basal kinase activity (Alessi, Andjelkovic et al. 1996). Although Ser473 phosphorylation is lost in the presence of rapamycin, the residual kinase activity from phosphorylation at Thr308 may be sufficient to maintain the phosphorylation of MDM2. On the other hand, there may be alternative rapamycin-insensitive pathways which can also result in MDM2 phosphorylation.

It is also not clear why there is a time lag between the effects of rapamycin on PKB phosphorylation, which is evident after 24 hours, and the effects on phosphorylation of GSK-3α/β, which appear after 72 hours rapamycin treatment. This may be a result of low turnover of GSK-3α/β.

It is therefore clear that prolonged treatment with 200nM rapamycin inhibits PKB in MIN6 cells. However, this is a supra therapeutic dose of rapamycin. I have also shown that significant reductions in Ser473 phosphorylation of PKB can be seen following 24 hours rapamycin treatment with concentrations as low as 10nM (see figure 5.5). This concentration of rapamycin is comparable to those levels measured in the portal vein of islet transplant recipients (Desai, Goss et al. 2003), and as such, the concentration which transplanted β cells are subjected to.

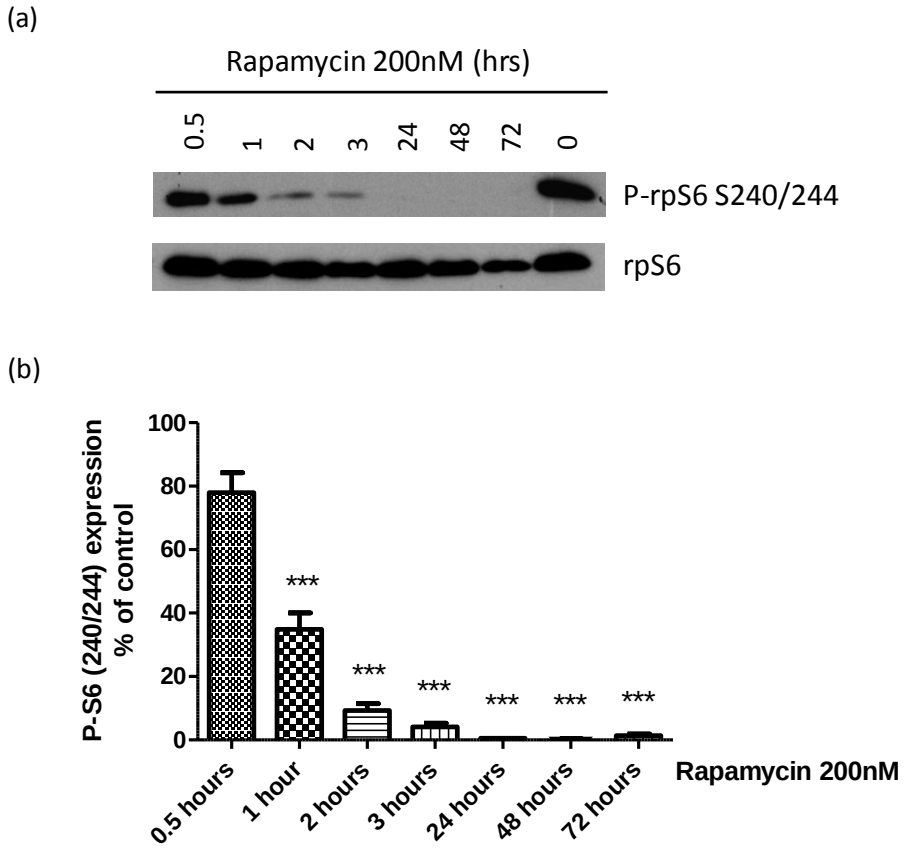


Figure 5.2 Effects of rapamycin on phosphorylation of rpS6 in MIN6 cells. MIN6 cells were treated with 200nM rapamycin for the indicated time periods. (a) Following cell lysis proteins were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P) rpS6 on Ser240/244 (P-rpS6 S240/244), and as a loading control, total rpS6. (b) Quantified data of P-rpS6 S240/244 from 3 independent experiments shown as mean ± SEM. ***P<0.001 vs. 0.5hr timepoint using one-way ANOVA plus Bonferroni's post hoc test.

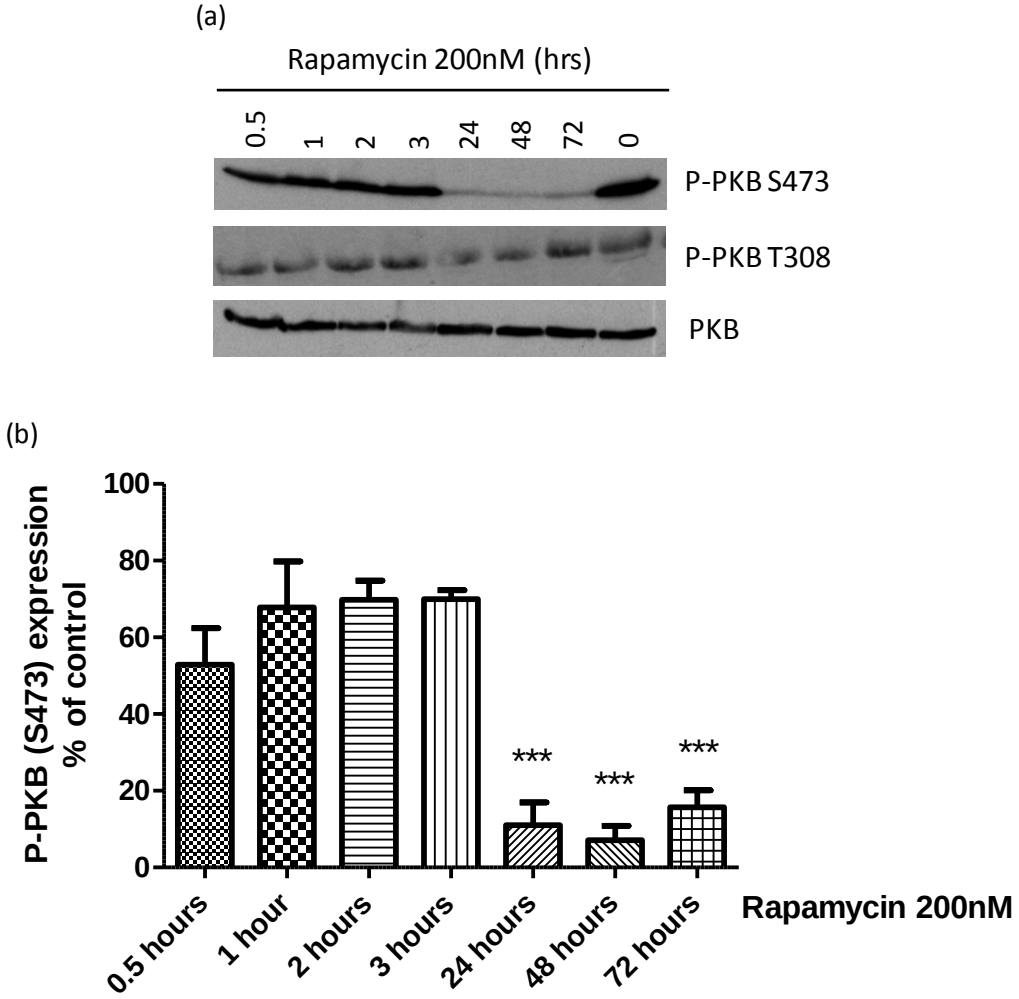


Figure 5.3 Effects of rapamycin on phosphorylation of PKB in MIN6 cells. MIN6 cells were treated with 200nM rapamycin for the indicated time periods. (a) Following cell lysis proteins were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P) PKB on Ser473 (P-PKB S473) and Thr308 (P-PKB T308), and as a loading control, total PKB. (b) Quantified data of P-PKB S473 from 3 independent experiments shown as mean ± SEM. ***P<0.001 *versus* 0.5-3hr timepoints using one-way ANOVA and Bonferroni's post hoc test.

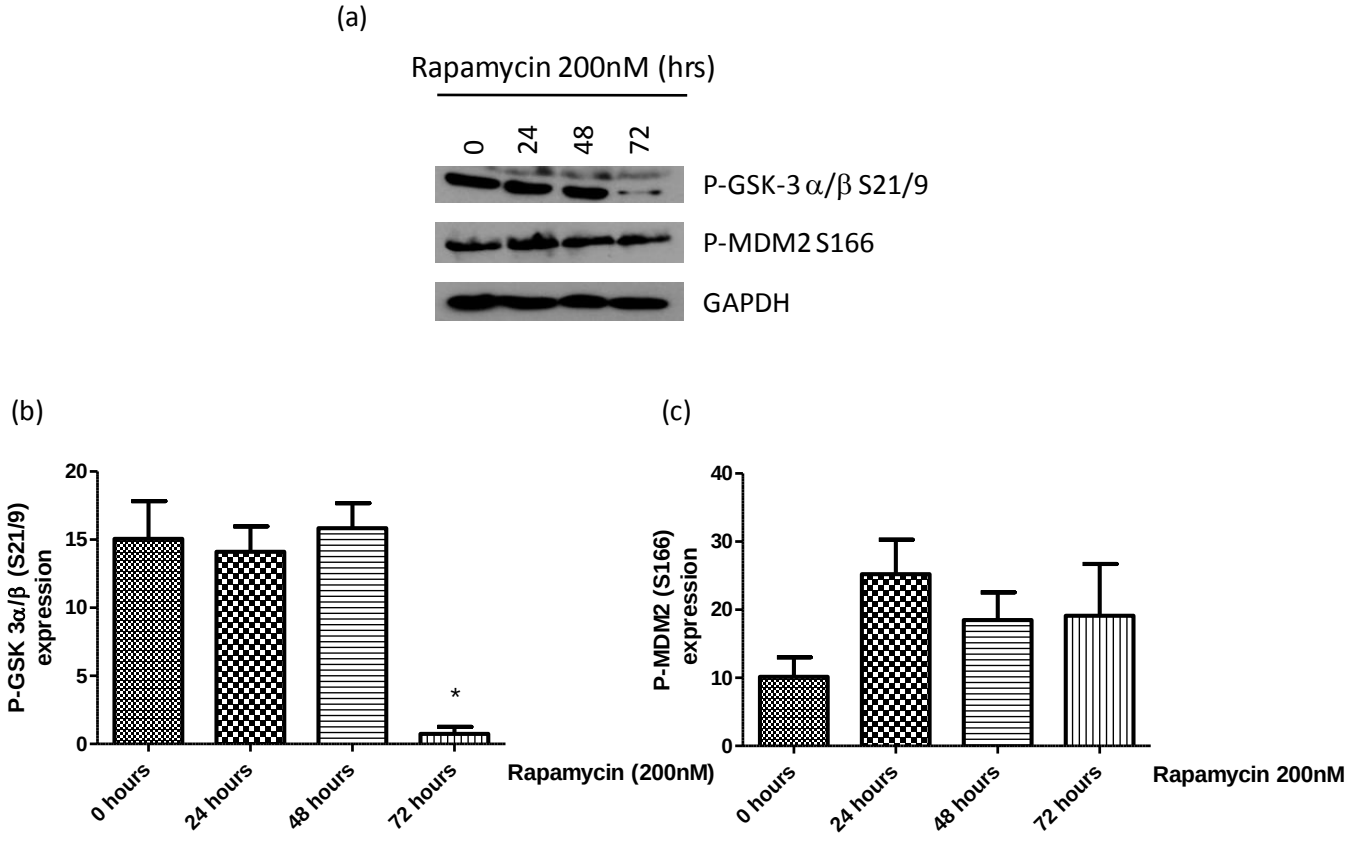


Figure 5.4 Effects of rapamycin on phosphorylation of downstream targets of PKB in MIN6 cells. MIN6 cells were treated with 200nM rapamycin for the indicated time periods. (a) Following cell lysis proteins were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P) GSK-3 α/β on Ser21/9 (P-GSK-3 α/β S21/9), P-MDM2 on Ser166 (P-MDM2 S166) and as a loading control, GAPDH. (b) Quantified data of P-GSK-3 α/β S21/9. *P=0.01-0.05 *versus* other timepoints using one-way ANOVA plus Bonferroni's post hoc test. (c) Quantified data of P-MDM2 (S166). No significant differences between groups using one-way ANOVA. All data are shown as mean ± SEM and are representative of 3 independent experiments.

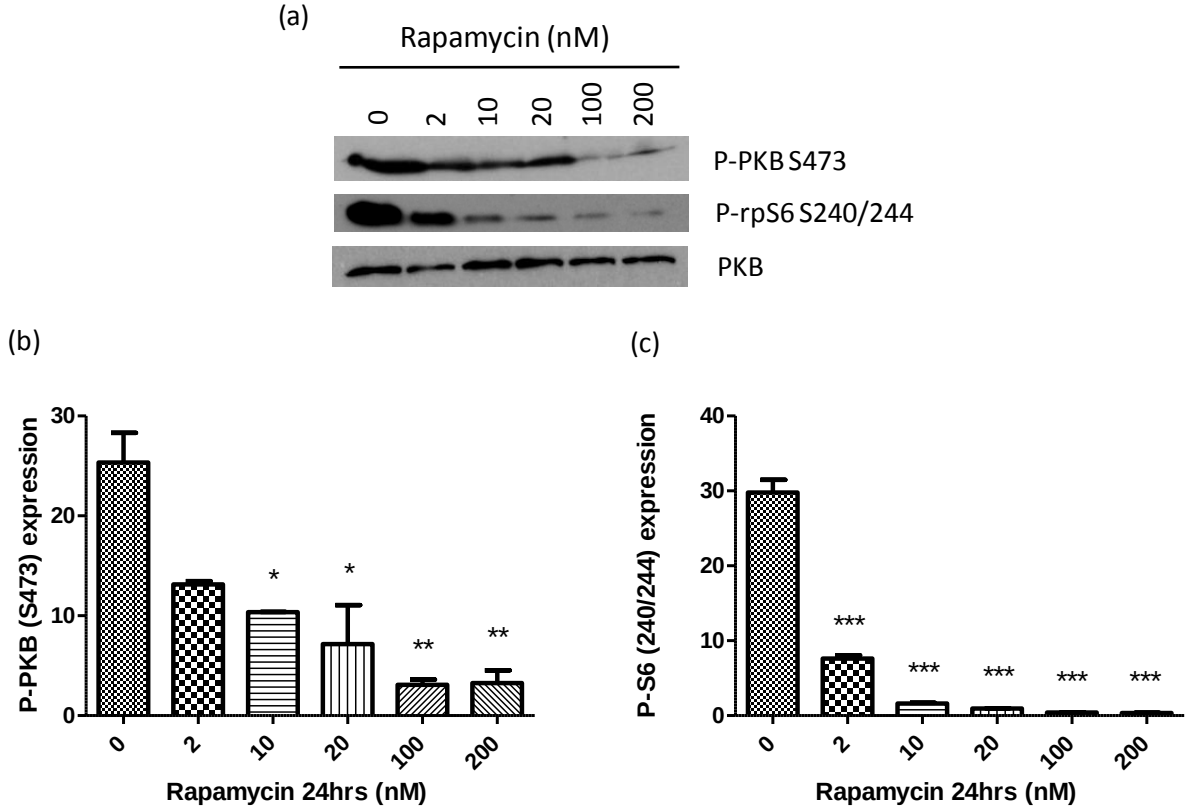


Figure 5.5 Dose-response effects of rapamycin on phosphorylation of PKB and rpS6 in MIN6 cells. MIN6 cells were treated for 24hrs with rapamycin at the indicated concentrations. (a) Following cell lysis proteins were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P) phosphorylated PKB on Ser473 (P-PKB S473) and P-rpS6 on Ser240/244 (P-rpS6 S240/244), and as a loading control, total PKB. (b) Quantified data of P-PKB S473 shown as mean ± SEM. *P=0.01-0.05, **P=0.01-0.001 *versus* control using one-way ANOVA with Bonferroni's post hoc test. (c) Quantified data of P-rpS6 S240/244 shown as mean ± SEM. ***P<0.001 *versus* control using one-way ANOVA with Bonferroni's post hoc test. All data shown are from 3 independent experiments.

5.3.3 Effects of rapamycin on PKC phosphorylation in MIN6 cells

Prolonged treatment with 200nM rapamycin for 72 hours also resulted in reduced phosphorylation of PKCα/βII at Thr638/641 (see figure 5.6). Phosphorylation of this residue has been shown to be dependent on mTORC2 (see section 2.3.3) and these findings, along with reduced PKB phosphorylation at Ser473, strongly suggest that prolonged rapamycin treatment is causing mTORC2 inhibition in MIN6 cells.

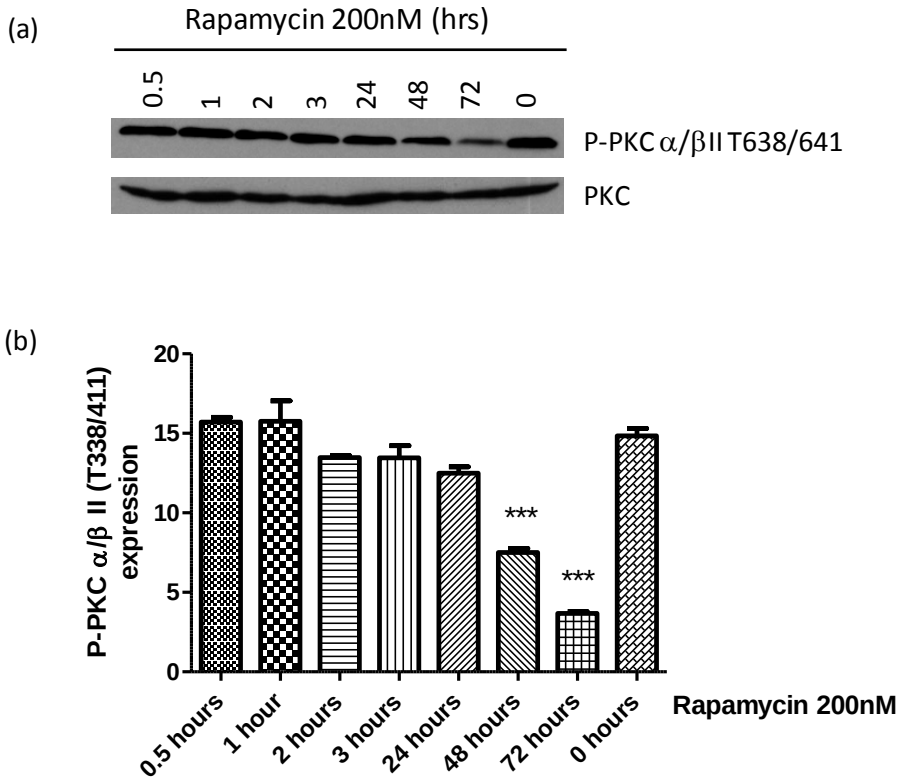


Figure 5.6 Effects of rapamycin on phosphorylation of PKCα/β in MIN6 cells. MIN6 cells were treated with 200nM rapamycin for the indicated time periods. (a) Following cell lysis proteins were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P) PKC α/βII on Thr638/641 (P-PKC α/βII T638/641) and as a loading control, total PKC. (b) Quantified data of P-PKC α/βII T638/641. ***P<0.001 *versus* other control using one-way ANOVA plus Bonferroni's post hoc test. All data are shown as mean ± SEM and are representative of 3 independent experiments.

5.3.4 Effects of rapamycin on mTORC2

Both PKB phosphorylation at Ser473 and PKCα/βII phosphorylation at Thr638/641 result from the kinase activity of mTORC2. The effects of rapamycin on PKB and PKC phosphorylation seen above could be a result of either reduced expression of mTORC2 components or of disturbances in the assembly of mTORC2. This work has shown that prolonged treatment with rapamycin 200nM has no effects on the expression of the main components of either mTORC1 (mTOR and Raptor) or mTORC2 (mTOR and Rictor) (figure 5.7).

Previous work in other cell types has shown that prolonged rapamycin treatment inhibits mTORC2 assembly. This work has shown that prolonged treatment with 200nM rapamycin also inhibits mTORC2 assembly in MIN6 cells (figure 5.8). Immunoprecipitation of mTOR in the absence of rapamycin results in the co-precipitation of Rictor and vice versa. However, following 72 hours rapamycin treatment immunoprecipitation of mTOR does not co-precipitate rictor. Therefore, rapamycin is either causing dissociation of mTOR from Rictor, or inhibiting their association. Previous work in HeLa and HEK293 cells has shown that the FKBP12/rapamycin complex is unable to bind to preformed mTORC2 (Sarbassov, Ali et al. 2004, Jacinto, Loewith et al. 2004), but can bind to free mTOR (Sabatini, Erdjument-Bromage et al. 1994). This has not been demonstrated in β cells, but it remains most likely that rapamycin binding to free mTOR prevents the formation of new mTORC2. However, this exact mechanism has not been confirmed by this study.

This therefore demonstrates that prolonged rapamycin treatment inhibits mTORC2 and subsequent PKB activity in MIN6 cells. However, it does not necessarily follow that the detrimental effects of rapamycin are due to this PKB inhibition.

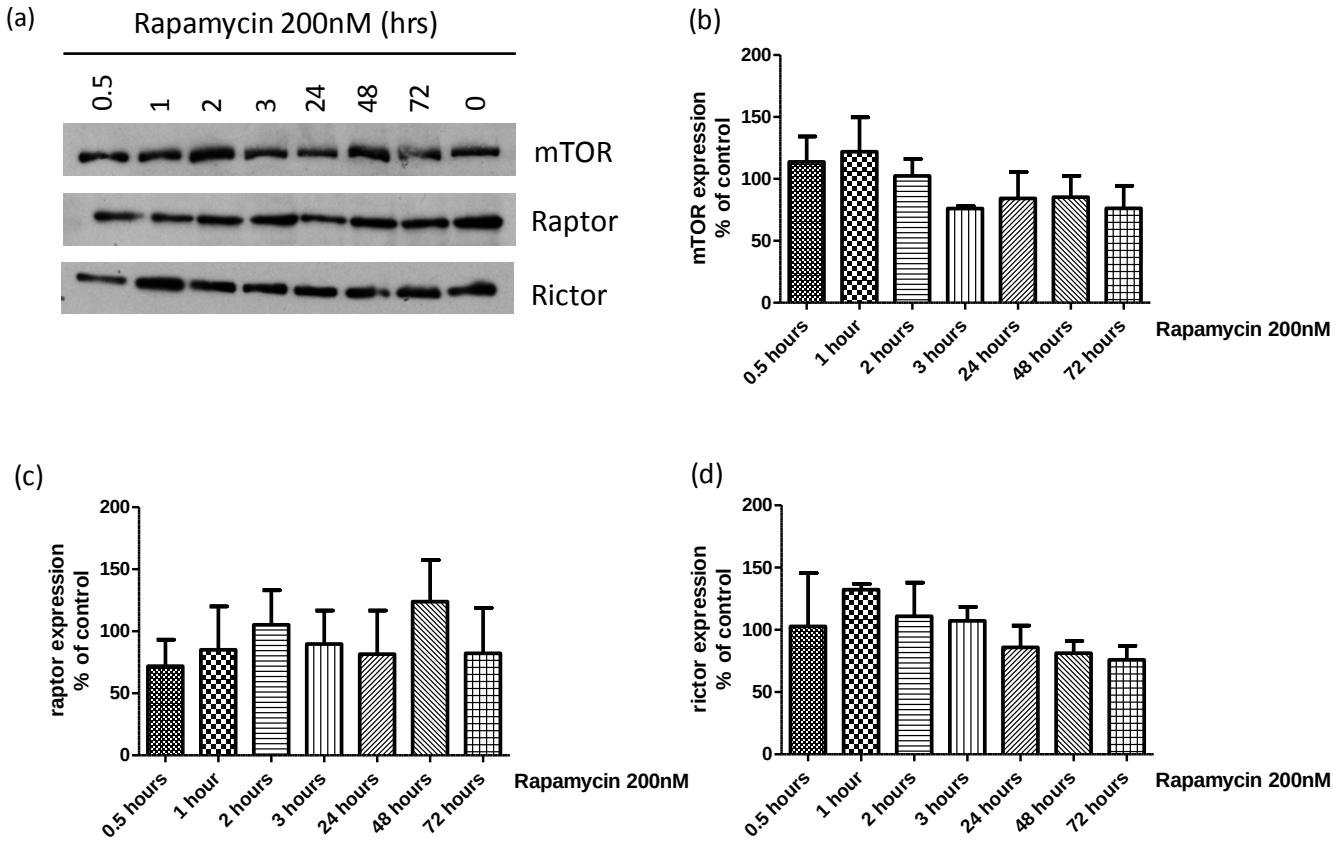


Figure 5.7 Effects of rapamycin on mTOR, Rictor and Raptor expression. MIN6 cells were treated with 200nM rapamycin for the indicated time periods. (a) Following cell lysis proteins were resolved on SDS-PAGE and immunoblotted using antisera against mTOR, Rictor and Raptor. (b) Quantitative data of mTOR expression from 3 independent experiments shown as mean ± SEM, no significant differences seen. (c) Quantitative data of Raptor expression from 3 independent experiments shown as mean ± SEM, no significant differences seen using one-way ANOVA. (d) Quantitative data of Rictor expression from 3 independent experiments shown as mean ± SEM, no significant differences seen using one-way ANOVA.

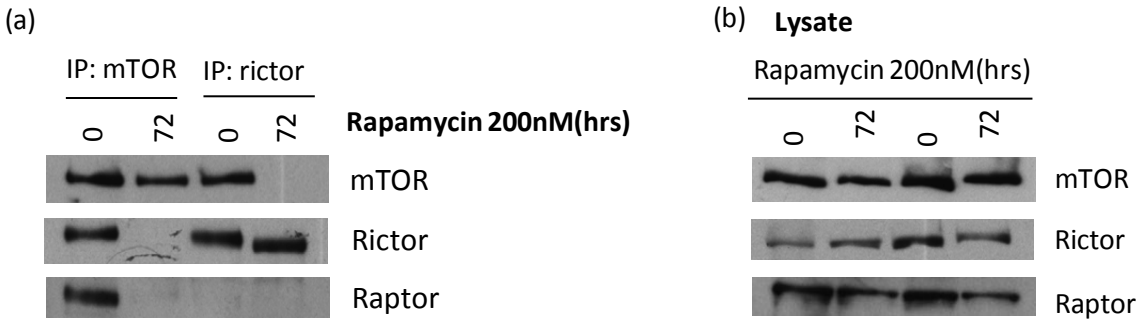


Figure 5.8 Effects of prolonged rapamycin treatment on assembly of mTOR complex 2. Following treatment with rapamycin as indicated, cells were lysed in CHAPS-containing lysis buffer. Immunoprecipitation was performed using anti-mTOR and anti-Rictor antibodies prebound to protein G sepharose beads. Immunoprecipitates (a) and lysates (b) were resolved on SDS-PAGE and immunoblotted using antisera against mTOR, Rictor and Raptor. Results are representative of 3 independent experiments.

5.3.5 Infection of MIN6 cells with constitutively active PKB

In order to investigate further the role of PKB in rapamycin toxicity in β cells, I utilised a recombinant human adenovirus type 5 encoding a constitutively active form of PKB (AdCaPKB). The PKB mutant is rendered constitutively active by the addition of a c-Src myristoylation sequence to the N terminus of PKBα. This myristoylation sequence forces PKB translocation to the cell membrane where it is constitutively activated (Bellacosa, Chan et al. 1998).

Expression of constitutively active PKB (CaPKB) in β cells allows elucidation of the differential roles of mTORC1 and mTORC2 inhibition in rapamycin-mediated toxicity. As PKB activity will remain high despite mTORC2 inhibition by rapamycin, any effects on viability or function can be presumed to be mediated via inhibition of mTORC1. This makes the assumption that CaPKB remains constitutively active in the presence of rapamycin. Nevertheless, figure 5.9b demonstrates that PKB phosphorylation at Ser473 is maintained in MIN6 cells infected with AdCaPKB at a multiplicity of infection (MOI) of 500, despite treatment for 72 hours with 200nM rapamycin. In addition, phosphorylation of GSKα/β at Ser21/9 downstream of PKB is also maintained in AdCaPKB MIN6 cells subjected to prolonged rapamycin treatment. An MOI of at least 500 is required, as below this rapamycin treatment results in loss of PKB phosphorylation despite constitutive activation (figure 5.9a).

It is also of importance that rapamycin mediated inhibition of mTORC1 is sustained in MIN6 cells infected with AdCaPKB. PKB lies upstream of mTORC1 and activation of PKB results in activation of mTORC1 via TSC1/2 and Rheb. Conceivably, a constitutively active PKB mutant could result in sufficient stimulation of mTORC1 to overcome the inhibitory effects

of rapamycin. However, I have shown that rapamycin induced dephosphorylation of rpS6 at Ser240/244 is maintained in MIN6 cells infected with AdCaPKB (figure 5.9b).

In addition, using a radioactive PKB kinase assay demonstrates that treatment of MIN6 cells with 200nM rapamycin for 24 hours results in significant reductions in kinase activity ($P=0.0491$) (see figure 5.9). Infection with AdCaPKB results in kinase activity comparable to control levels despite treatment with rapamycin.

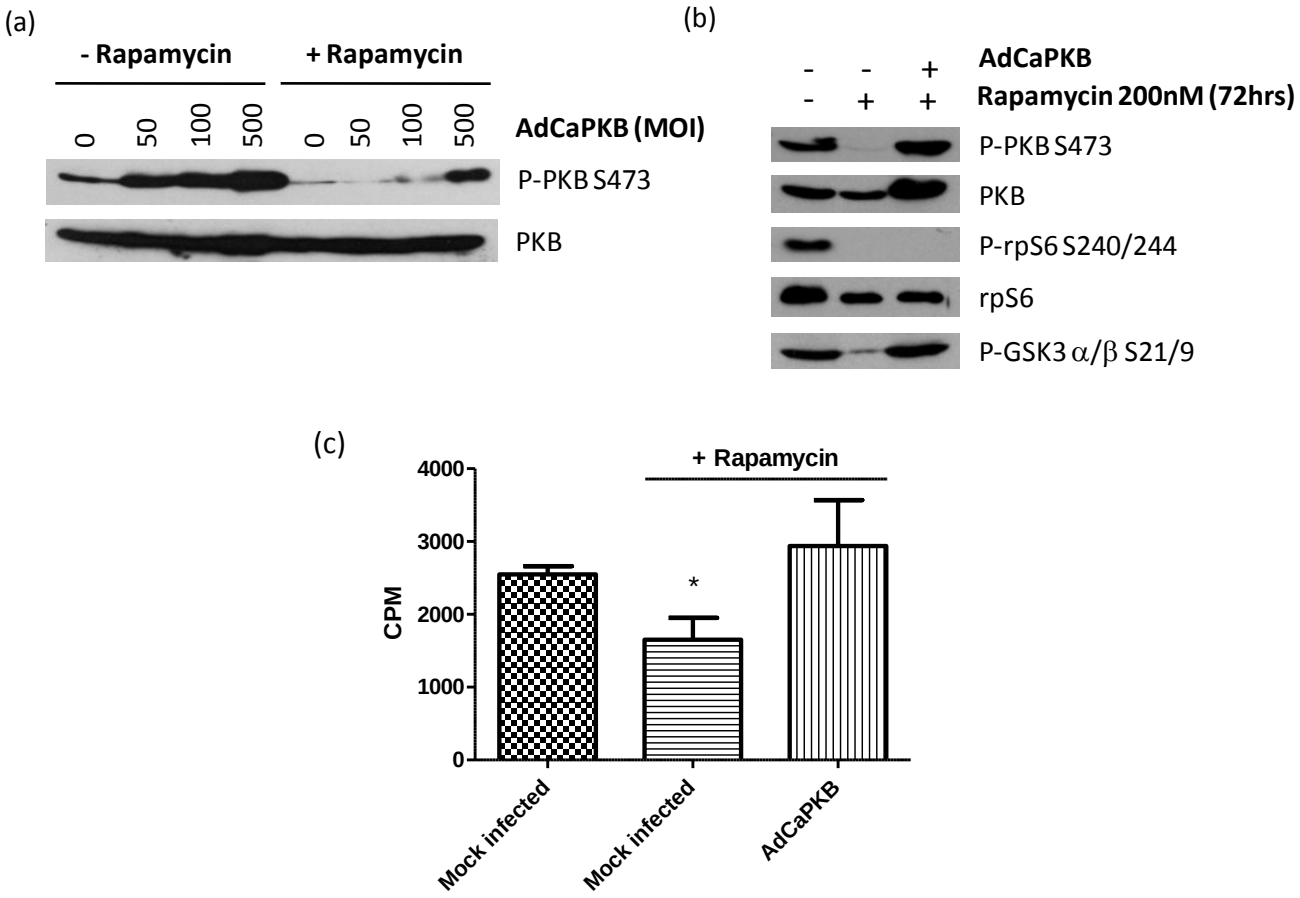


Figure 5.9 Characterisation of AdCaPKB infection in MIN6 cells. (a) MIN6 cells were mock infected or infected with recombinant adenovirus expressing constitutively active PKB (AdCaPKB) at the MOI indicated for 24hrs and incubated for a further 24hrs in the presence or absence of 200nM rapamycin as indicated. Cell lysates were resolved on SDS-PAGE and subject to immunoblotting with antisera against phosphorylated (P) PKB on Ser473 (P-PKB S473) and as a loading control, total PKB. (b) Cells were infected with AdCaPKB at a MOI of 500 for 24hrs, followed by incubation for 72hrs in the presence or absence of rapamycin 200nM. Following cell lysis proteins were resolved on SDS-PAGE and immunoblotted using antisera against P-PKB S473, P-rpS6 on Ser240/244 (P-rpS6 S240/244), P-GSK3 α/β on Ser21/9 (P-GSK3 α/β S21/9) and as loading controls, total PKB and total rpS6. (c) Following cell lysis, PKB was immunoprecipitated using immobilized antibody and kinase acivity determined using γP32-ATP and Crosstide as the substrate. Data are shown as mean ± SEM. *P=0.01-0.05 *versus* control using Student's t-test. All results are representative of 3 independent experiments.

5.3.6 Effect of AdCaPKB on rapamycin induced MIN6 cell dysfunction

MIN6 cells infected with AdCaPKB were treated with rapamycin 200nM for 24 hours and glucose stimulated insulin secretion measured. As shown in figure 5.10, there was no difference in the fold change in insulin secretion in AdCaPKB infected cells treated with rapamycin, as compared to that in control cells and untreated AdCaPKB infected cells (mock *versus* AdCaPKB *versus* AdCaPKB+rapamycin = 1.93(±0.06) *versus* 2.05(±0.38) *versus* 2.18(±0.35) respectively, P=0.848).

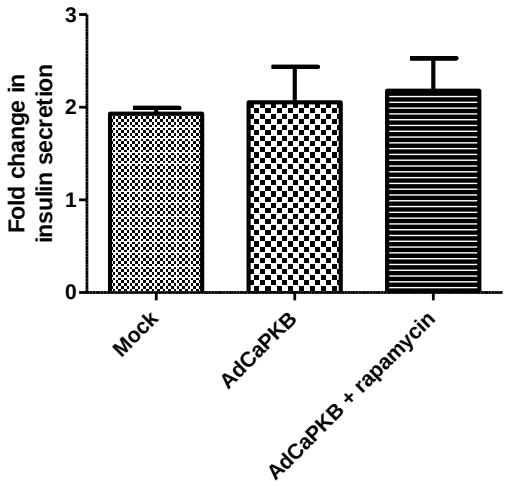


Figure 5.10 Effects of rapamycin on insulin secretion in MIN6 cells infected with AdCaPKB. MIN6 Cells were mock infected or infected with recombinant adenovirus expressing constitutively active PKB (AdCaPKB) at an MOI of 500 for 24hrs and incubated for a further 24hrs in the presence or absence of 200nM rapamycin as indicated. Following rapamycin treatment cells were incubated in KRB containing 1mM glucose for 1hr followed by KRB containing 20 mM glucose for a further 1hr. Supernatants were collected and assayed for insulin concentration using ELISA. Results are expressed as fold change in stimulated insulin secretion over basal insulin secretion (mean ± SEM) and are of 3 independent experiments. No significant differences seen using Student's t-test.

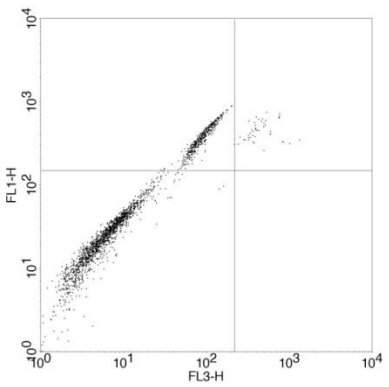
5.3.7 Effect of AdCaPKB on rapamycin induced MIN6 cell death

MIN6 cells infected with AdCaPKB were treated with rapamycin 200nM for 24 hours and apoptosis assessed by propidium iodide/annexin V staining and quantification by flow cytometry. This shows that infection of MIN6 cells with AdCaPKB protects against rapamycin induced apoptosis (% of cells undergoing apoptosis: mock *versus* AdCaPKB *versus* mock+rapamycin *versus* AdCaPKB+rapamycin = 19.79(±0.74) *versus* 14.64(±1.98) *versus* 55.39(±4.19) *versus* 18.48(±2.25)%) and cell death (% of cells undergoing necrosis: mock *versus* AdCaPKB *versus* mock+rapamycin *versus* AdCaPKB+rapamycin = 2.61(±0.34) *versus* 2.03(±0.34) *versus* 7.93(±1.09) *versus* 2.25(±0.09)%) (see figure 5.11).

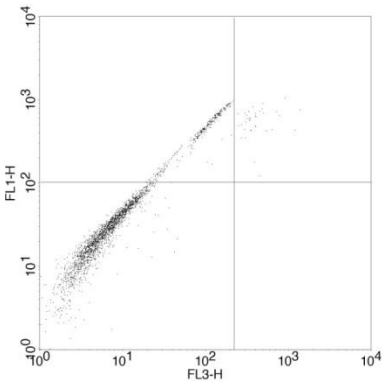
These findings, along with those seen for glucose stimulated insulin secretion suggest that the toxicity of rapamycin in MIN6 cells is predominantly due to mTORC2 inhibition rather than mTORC1 inhibition, as when mTORC2 inhibition is overcome using AdCaPKB no detrimental effects of rapamycin are seen.

MIN6 cells are derived from a tumour cell line, which may potentially have abnormal cell physiology. This is of particular relevance when investigating PKB as disruption of normal PKB signalling, including PTEN mutations and PKB overexpression, occurs in many human cancers including, amongst others, ovarian (Saito, Okamoto et al. 2000, Sun, Wang et al. 2001), breast (Sun, Wang et al. 2001, Sun, Paciga et al. 2001), colonic (Chang, Chen et al. 1999)and lung (Forgacs, Biesterveld et al. 1998). Therefore, it is important that the findings in the MIN6 cell line are confirmed in primary cells.

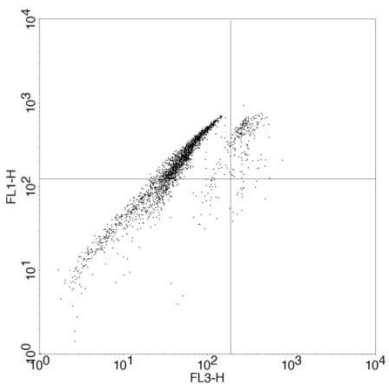
(ai) Mock infected



(aii) AdCaPKB



(aiii) Mock infected + rapamycin



(aiv) AdCaPKB + rapamycin

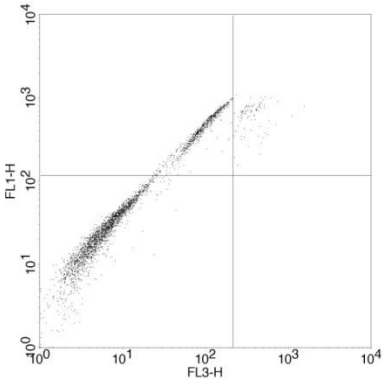


Figure 5.11a Effects of AdCaPKB on rapamycin induced apoptosis and cell death in MIN6 cells. MIN6 cells were mock infected or infected with recombinant adenovirus expressing constitutively active PKB (AdCaPKB) at an MOI of 500 for 24hrs and incubated for a further 24hrs in the presence or absence of 200nM rapamycin as indicated. Following treatment cells were dispersed, stained with annexin V and propidium iodide and analysed by flow cytometry. FL1-H = propidium iodide, FL3-H = annexin V. Results are representative of 3 independent experiments.

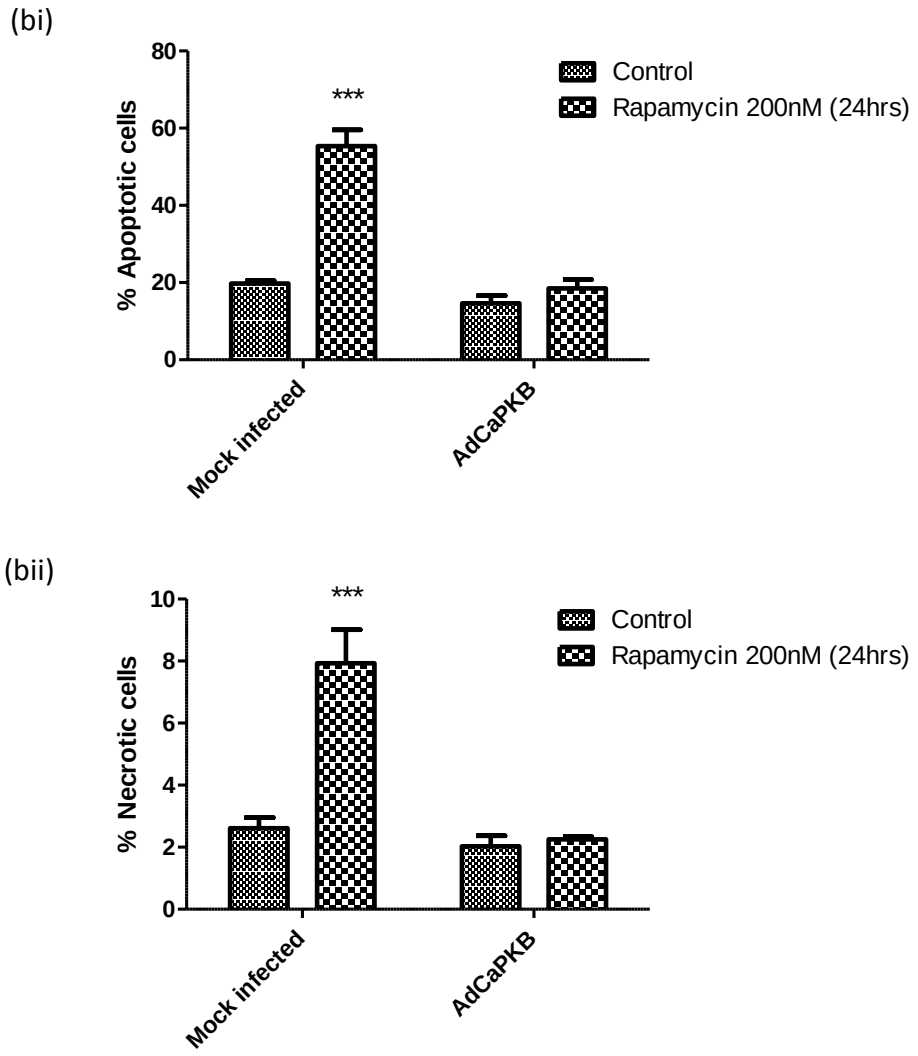


Figure 5.11b Effects of AdCaPKB on rapamycin induced apoptosis and cell death in MIN6 cells. MIN6 cells were mock infected or infected with recombinant adenovirus expressing constitutively active PKB (AdCaPKB) at an MOI of 500 for 24hrs and incubated for a further 24hrs in the presence or absence of 200nM rapamycin as indicated. Following treatment cells were dispersed, stained with annexin V and propidium iodide and analysed by flow cytometry. (i) % of apoptotic cells as quantified by flow cytometry. (ii) % of necrotic cells as quantified by flow cytometry. All results are expressed as mean ± SEM and are of three independent experiments. ***P<0.001 *versus* other groups using one-way ANOVA with Bonferroni's post hoc test.

5.3.8 Effects of rapamycin on PKB phosphorylation in rat islets

Isolated rat islets were treated with 200nM rapamycin for up to 48 hours and phosphorylation of PKB at Ser473 investigated. Corroborating the findings in MIN6 cells, PKB phosphorylation at Ser473 was completely abolished following 48 hours rapamycin treatment (figure 5.12). As expected, phosphorylation of rpS6 at Ser240/244 was also abolished following rapamycin treatment. Rapamycin therefore appears to inhibit both mTORC1 and mTORC2 in rat islets. The assumption is that the mechanism of mTORC2 inhibition in rat islets is the same as in MIN6 cells, namely inhibition of complex assembly, but this would need to be confirmed by further study.

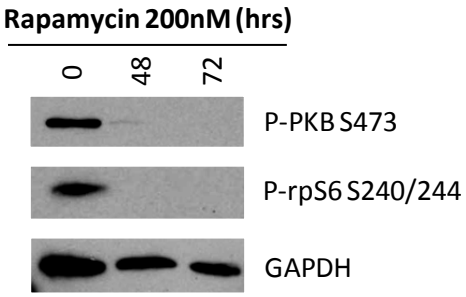


Figure 5.12 Effects of rapamycin on PKB phosphorylation in isolated rat islets. Following isolation, rat islets were treated with 200nM rapamycin as indicated. Following cell lysis proteins were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P) PKB on Ser473 (P-PKB S473), P-rpS6 on Ser240/244 (P-rpS6 S240/244), and, as a loading control, GADPH.

5.3.9 Effect of AdCaPKB on rapamycin induced rat islet dysfunction

Isolated rat islets were infected with AdCaPKB and treated with rapamycin 200nM for up to 72 hours. Glucose stimulated insulin secretion was measured by ELISA. This has shown that AdCaPKB abrogated the reductions in both basal insulin secretion (uninfected 0hrs *versus* infected 0hrs *versus* uninfected 48hrs *versus* infected 48hrs *versus* uninfected 72hrs *versus* infected 72hrs = 301.7(±12.1) *versus* 319.3(±22.8) *versus* 160.9(±15.6) *versus* 199.9(±40.8) *versus* 156.2(±21.1) *versus* 223.8(±39.1) pmol/mg) and stimulated insulin secretion (uninfected 0hrs *versus* infected 0hrs *versus* uninfected 48hrs *versus* infected 48hrs *versus* uninfected 72hrs *versus* infected 72hrs = 543.3(±62.3) *versus* 501.3(±62.4) *versus* 416.1(±1.5) *versus* 516.3(±47.9) *versus* 361.0(±28.9) *versus* 559.5(±25.9) pmol/mg) seen with rapamycin treatment (figure 5.13). This corroborates the findings in MIN6 cells.

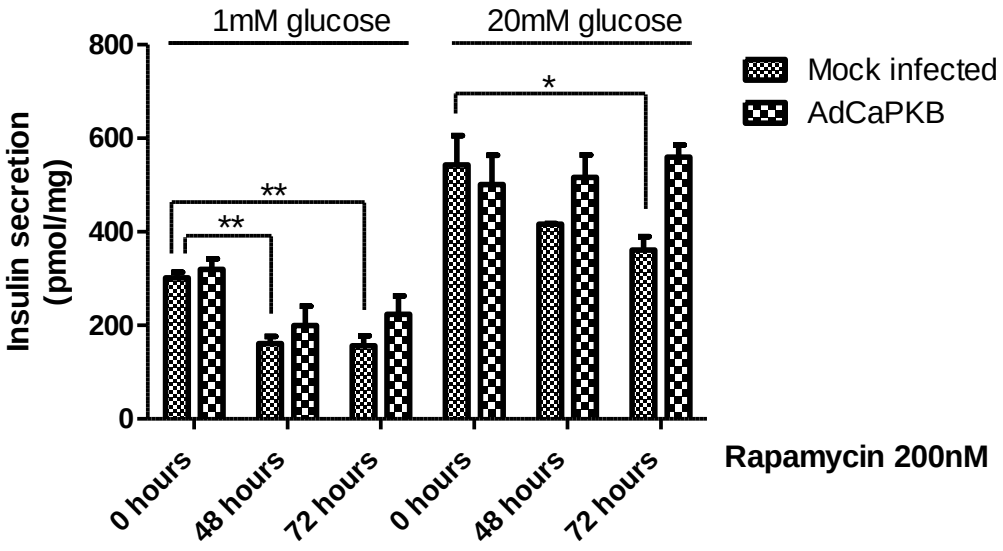


Figure 5.13 Effects of AdCaPKB on function of rapamycin treated isolated rat islets. Following isolation, rat islets were mock infected or infected with recombinant adenovirus expressing constitutively active PKB (AdCaPKB) for 24hrs and incubated in the presence or absence of 200nM rapamycin as indicated. Following rapamycin treatment cells were incubated in KRB containing 1mM glucose for 1hr followed by KRB containing 20 mM glucose for a further 1hr. Supernatants were collected and assayed for insulin concentration using ELISA. All data is displayed as mean ± SEM. *P=0.05-0.01, **=P0.001-0.01 using two-way ANOVA with Bonferroni's post hoc test. Results shown are obtained from 3 independent experiments.

5.3.10 Effect of AdCaPKB on rapamycin induced rat islet death

As dissociation of islets into single cells results in accelerated cell death (Aikin, Hanley et al. 2006), it was not feasible to use flow cytometry to evaluate islet viability. An in-situ cell death detection kit was therefore utilised. This is based on the TUNEL assay (*T*dT-mediated d*U*TP *n*ick *e*nd *l*abelling). DNA fragmentation is one of the hallmarks of apoptosis. As well as producing double stranded, low molecular weight DNA fragments, DNAse activity introduces strand breaks or 'nicks' into high-molecular-weight DNA. These processes can be identified by labelling the free 3'-OH termini with fluorescein dUTP, catalysed by terminal transferase (TdT). The fluorescein dUTP labelling is then quantified using a sandwich ELISA technique.

Isolated rat islets were infected with AdCaPKB and treated with rapamycin 200nM for up to 72 hours. Infection with AdCaPKB resulted in significant (P=0.037) reductions in islet cell death following 48 hours rapamycin treatment, but not following 72 hours treatment (figure 5.14). It is possible that with a higher MOI of AdCaPKB abrogation of rapamycin induced cell death following 72 hours treatment would also be seen, but this higher MOI may itself cause islet loss due to adenoviral toxicity.

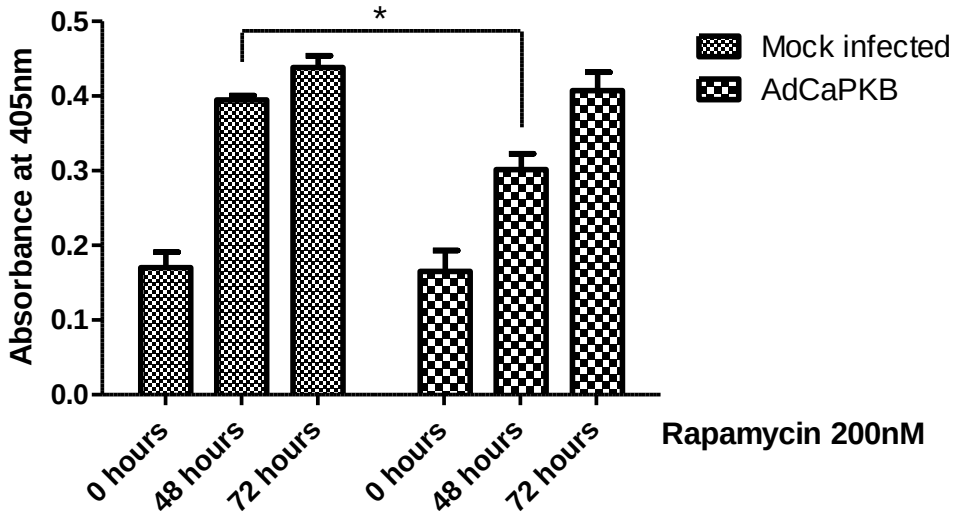


Figure 5.14 Effects of AdCaPKB on viability of rapamycin treated isolated rat islets. Following isolation, rat islets were mock infected or infected with recombinant adenovirus expressing constitutively active PKB (AdCaPKB) for 24hrs and incubated in the presence or absence of 200nM rapamycin as indicated. Following rapamycin treatment islets were lysed and cell viability assessed using an in-situ cell death detection ELISA. All data is displayed as mean ± SEM. *P=0.05-0.01 using two-way ANOVA plus Bonferroni's post hoc test. Results shown are obtained from 3 independent experiments.

5.4 Summary

I have demonstrated that rapamycin inhibits phosphorylation of PKB at Ser473 in both MIN6 cells and rat islets. In MIN6 cells I have shown that this is due to inhibition of assembly of mTORC2. Infection of MIN6 cells and rat islets with AdCaPKB resulted in abrogation of rapamycin induced reductions in insulin secretion and of increases in cell death. As expression of AdCaPKB overcomes rapamycin-mediated mTORC2 inhibition this finding suggests that rapamycin toxicity in pancreatic β cells is predominantly mediated via mTORC2 inhibition rather than mTORC1 inhibition.

CHAPTER 6

Discussion

6.1 Summary of findings

This work is, to my knowledge, the first to investigate the mechanisms of rapamycin toxicity in pancreatic β cells. I have confirmed that rapamycin has deleterious effects on both the survival and function of a mouse insulinoma cell line and isolated rat islets. In addition my novel findings suggest that these detrimental effects are mediated via inhibition of assembly of mTORC2 by rapamycin and subsequent inactivation of PKB. The rapamycin toxicity can be overcome by infecting β cells with an adenovirus encoding constitutively active PKB, providing further evidence that the toxicity is mediated via PKB inhibition.

The observation that rapamycin has detrimental effects on β cells has been reported in a number of other studies using murine cell lines (Fuhrer, Kobayashi et al. 2001, Paty, Harmon et al. 2002) and murine islets (Fabian, Lakey et al. 1993, Bell, Cao et al. 2003, Zhang, Su et al. 2006). Sarbassov et al showed prolonged rapamycin treatment inhibits mTORC2 assembly and PKB in a number of cells including PC3, HeLa, HEK-293 and Jurkat cells (Sarbassov, Ali et al. 2006). However, this is the first study to demonstrate rapamycin inhibits mTORC2 assembly and subsequent phosphorylation of PKB at Ser473 in pancreatic β cells. Of particular note is that this inhibition occurs at rapamycin concentrations seen in the portal circulation of islet transplant recipients.

Other studies have investigated the effects of rapamycin on PKB phosphorylation in β cells and shown no effects. Liu et al investigated the effects of rapamycin on IGF-1 treated INS-1 cells and showed no decrease in PKB phosphorylation following treatment with 10nM rapamycin for 30 minutes (Liu, Chin-Chance et al. 2002). Other work has reported similar findings in human islets, with no change in PKB phosphorylation seen following 30 minutes exposure to 25nM rapamycin (Liu, Remedi et al. 2009). However, it is clear from my findings that inhibition of mTORC2 requires prolonged rapamycin treatment of around 24

hours, and that 30 minutes treatment is not going to result in changes in PKB phosphorylation.

In addition, McDaniel's group investigated the role of GSK-3 in human islets and showed that phosphorylation and subsequent inhibition of GSK-3 via mTOR led to increased DNA synthesis, cell cycle progression and proliferation (Liu, Remedi et al. 2009). This was rapamycin sensitive. They studied the effects of rapamycin treatment on phosphorylation of PKB at Ser473 and saw no decrease in phosphorylation. They therefore conclude that GSK-3 inhibition is not mediated via mTORC2, but via a proposed pathway where overactivation of mTORC1 results in phosphorylation and inhibition of GSK-3. However, whilst the majority of their experiments involved 96 hours rapamycin treatment, the experiment on PKB phosphorylation used 30 minutes rapamycin treatment. Given the clear reduction of PKB phosphorylation with 24 hours rapamycin treatment seen in my work, it is likely they would have seen reduced phosphorylation effects with longer rapamycin treatment. Therefore, it appears their conclusion is misfounded and that the effects of GSK-3 seen could well be mediated via mTORC2 and PKB.

Alongside the findings of the effects of rapamycin on mTORC2 in pancreatic β cells, this work also demonstrates the key role that PKB plays in β cell survival and function. This corroborates with other studies as described in section 5.1.4. Contrary to these findings, Bernal-Mizrachi et al investigated β cell function and survival in transgenic mice expressing kinase-dead PKB (kdPKB), which acts in a dominant negative fashion (Bernal-Mizrachi, Fatrai et al. 2004). Although these mice had impaired glucose tolerance and defective insulin secretion resulting from disruptions in insulin exocytosis, no differences were seen in basal or thapsigargin induced β cell *in vitro* apoptosis as compared to wild type controls. However, an explanation for this disparity is that the kdPKB transgenic mice still retained approximately 20% endogenous PKB activity which may be sufficient to maintain cell

survival. In addition, these mice only lacked one PKB isoform and it is feasible that the different PKB isoforms have differing roles in the regulation of β cell survival and function.

6.2 Strengths and weaknesses

The strengths of my work are that the findings have been replicated in both a cell line and primary cells. In addition the use of AdCaPKB provides further evidence of the mechanism of rapamycin toxicity above and beyond the observation that PKB is inhibited by prolonged rapamycin treatment. Furthermore all the assays used are recognised techniques also reported by other groups conducting pancreatic β cell research.

However, the findings have yet to be confirmed in human islets, which is of obvious importance if they are to have clinical relevance. In addition although the results seen with AdCaPKB support the hypothesis that rapamycin toxicity is mediated via PKB they certainly do not prove it. Despite the complete abrogation of the toxic effects of rapamycin by AdCaPKB it is possible that mTORC1 inhibition does result in some toxicity, but that the over activity of PKB seen with AdCaPKB, and subsequent protective effect, results in amelioration of any detrimental effects of rapamycin mediated via mTORC1 inhibition. This requires further investigation if this is to be disproved.

6.3 Future work

As mentioned above, it is important to confirm the findings of my research in human β cells. This includes investigating the effects of prolonged rapamycin treatment on function and survival of isolated human islets, as well as the effects on PKB phosphorylation.

Further support for my hypothesis would be gained if it were possible to selectively inhibit mTORC1 and mTORC2. It would then be possible to clarify the differential roles of the two complexes in pancreatic β cell function and survival.

Unfortunately, attempts to develop a specific mTORC2 inhibitor, which would be of particular interest as a cancer therapy, have yet to prove fruitful (Sparks, Guertin 2010). However, automated screening has been used to identify potential selective mTORC1 inhibitors. Compounds identified include perhexiline, nicosamide, amiodarone and rottlerin (Balgi, Fonseca et al. 2009). Treatment of MCF-7 cells for 4 hours with these compounds resulting in inhibition of S6K, but not PKB. If further investigation reveals these compounds do indeed selectively inhibit mTORC1 in pancreatic β cells despite prolonged treatment, they could be used to investigate the specific role of mTORC1 in β cell homeostasis.

A different approach is to specifically knockdown mTORC1 or mTORC2 using small interfering RNA (siRNA) directed against Raptor or Rictor respectively and assess the differential effects on pancreatic β cell function and survival. This work is currently being conducted in Dr Herbert's lab and will hopefully provide further confirmation that mTORC2 inhibition is the predominant mechanism of rapamycin mediated β cell toxicity.

6.4 Clinical relevance

The findings described in this thesis have important implications for clinical islet transplantation.

Firstly, it brings into serious question the use of rapamycin as a primary immunosuppressant in islet transplantation. However, there are limited alternatives. Glucocorticoids and tacrolimus have both been shown to have even more significant detrimental effects on β cells. The UK Islet Transplant Consortium (UKITC) has, as part of its immunosuppressive protocol, replaced rapamycin with mycophenolate mofetil (MMF). MMF, also known as Cellcept, is an antiproliferative agent that exerts its immunosuppressive effects via inhibition of inosine monophosphate dehydrogenase, part of the de novo purine synthesis pathway (Ransom 1995). This decision by UKITC does not appear to be evidence based. Indeed, studies in human islets have shown MMF treatment to result in significant reductions in glucose stimulated insulin secretion (Johnson, Ao et al. 2009)and to increase cell death in the RIN-5F rat β cell line (Park, Ahn et al. 2009). However contrary to this, as discussed in section 1.5.3, a protocol utilized by the Minnesota group incorporating induction therapy of ATG and etanercept, maintenance immunosuppression of cyclosporine and everolimus for the first year post transplant, with everolimus then substituted for MMF or MPA has resulted in arguably the most promising long-term survival data reported for islet transplantation to date (Bellin, Kandaswamy et al. 2008).

The key role of PKB in pancreatic β cell function and survival, as shown by this work and that of others, is of importance to those involved in the development of novel immunosuppressive agents for islet transplantation. Ideally, any new agents should not affect PKB activity, given its importance to β cell homeostasis. One potential area for development is mTORC1 specific inhibitors which should retain the immunosuppressive effects of rapamycin, without any mTORC2 mediated toxicity. However, this makes the assumption

that the immunosuppressive effects of rapamycin are indeed mediated solely via mTORC1 rather than mTORC2, which is not currently known.

In addition, the question arises whether *in vivo* activation of PKB might improve the outcome of islet transplantation by improving the function and survival of transplanted β cells. There are a number of potential mechanisms by which PKB can be activated in vivo. These include GLP-1, erythropoietin and statins.

Erythropoietin has been shown to activate PKB in endothelial cells (Akimoto, Kusano et al. 2000, d'Uscio, Katusic 2008), cortical neurons (Sakanaka, Wen et al. 1998, Shang, Wu et al. 2007) and myocardial cells (Mudalagiri, Mocanu et al. 2008, Tramontano, Muniyappa et al. 2003). Although erythropoietin has not been shown to activate PKB in pancreatic β cells, both adenoviral gene transfer of erythropoietin (Fenjves, Ochoa et al. 2004) and direct treatment of cultured human islets with erythropoietin (Fenjves, Ochoa et al. 2003) have been shown to protect against cytokine induced apoptosis. It would be of interest to establish whether this cytoprotection is mediated via activation of PKB. Additionally the availability, low cost and minimal side effects of erythropoietin make it very feasible to conduct a clinical trial of its potential benefits in improving outcomes in human islet transplant recipients.

Both pravastatin and simvastatin have been shown to activate PKB in human islets and increase islet viability (Contreras, Smyth et al. 2002, Favaro, Miceli et al. 2008). Additionally, simvastatin treated human islets showed significantly better function when transplanted in diabetic NOD-SCID mice (Contreras, Smyth et al. 2002) and pravastatin has also been shown to reduce the islet mass required to reverse diabetes in total pancreatectomized dogs undergoing autologous islet transplantation (Arita, Nagai et al. 2002). Despite these compelling findings, a clinical trial of statin treatment in human islet transplant recipients has yet to be conducted.

The incretin human glucagon-like peptide (GLP)-1 is a peptide secreted from the intestinal L cells in response to food ingestion and is known to activate PKB in pancreatic β cells (Buteau, Roduit et al. 1999, Cornu, Yang et al. 2009, Wang, Li et al. 2004). The GLP-1 agonist exenatide has been shown to ameliorate defects in glucose stimulated insulin secretion induced by tacrolimus, rapamycin and MMF in cultured human islets (Johnson, Ao et al. 2009). In addition, transfection of MIN6 cells with the gene expressing GLP-1 improved the function and viability of cells exposed to a cocktail of immunosuppressive drugs comprising rapamycin, MMF and tacrolimus (D'Amico, Hui et al. 2005). Exendin 4, a GLP-1 analogue, has also been shown to protect MIN6 cells and rat islets against rapamycin induced cell death (Kawasaki, Harashima et al. 2010). The authors state that this did not appear to be mediated via PKB, but rather by JNK and p38 signalling. Nevertheless there is cross talk between the PKB and JNK pathways (Aikin, Maysinger et al. 2004) and it is not possible to exclude a role for PKB in the effects of exendin 4. *In vivo* studies in isolated islet and whole pancreas transplant recipients showed that a single infusion of GLP-1 lowered basal glucose via enhanced glucose stimulated insulin secretion and glucagon suppression and increased the glucose infusion rate necessary to maintain hyperglycaemia by augmenting second phase insulin secretion (Rickels, Mueller et al. 2009).

Exenatide has been used as a clinical treatment in islet transplant recipients. The University of Illinois at Chicago (UIC) protocol adds etanercept, an anti-TNFα receptor antibody, and exenatide to the Edmonton protocol. The UIC group have reported a comparison of 6 patients transplanted using their protocol (group 1) with 4 patients transplanted using the Edmonton protocol (group 2) (Gangemi, Salehi et al. 2008). All group 1 patients became insulin independent after their initial islet transplant, whilst all group 2 patients became insulin independent following 2-3 islet infusions. However, at 15 months follow up post transplant whilst all group 2 patients remained insulin independent, only 4 out of the 6 group 1 patients

remained so. Therefore, in this series etanercept and exenatide do seem to lower the number of islets required initially to achieve insulin independence but do not seem to confer long-term protection. It is obviously not possible to elucidate how much of this effect is due to the use of etanercept and how much due to exenatide.

Froud et al from the University of Miami have also reported a non-randomised trial of the use of exenatide in 16 islet transplant recipients with chronic allograft dysfunction requiring exogenous insulin (Froud, Faradji et al. 2008). At six months, the exogenous insulin requirements were significantly reduced and 3 subjects were able to discontinue insulin altogether. However, there is a suggestion that the effects of exenatide may not be durable, with the reduction in insulin requirements decreasing from 40% between 1 and 4 months to 20% between 5 and 6 months of treatment. Others have raised concerns that exenatide increases the incidence of hypoglycaemia, a particular concern given the selection of candidates for islet transplantation is based on the presence of hypoglycaemic unawareness (Rickels, Naji 2009). The effects of GLP-1 also appear to be related to functional β cell mass and it has been proposed that GLP-1 treatment may contribute to exhaustion of an already low engrafted β cell mass (Rickels, Mueller et al. 2009). So the exact role of exenatide in islet transplantation remains to be determined and clearly randomized controlled trials are required to establish any beneficial effects.

There is therefore huge potential for the study of the benefits of *in vivo* PKB activation in improving outcomes of islet transplantation. However, it is also important to bear in mind that PKB was originally identified as a retroviral oncogene product and PKB isoforms have been shown to be overexpressed in ovarian, breast and pancreatic cancers (Cheng, Godwin et al. 1992, Cheng, Ruggeri et al. 1996, Nakatani, Thompson et al. 1999). Long-term overactivation of PKB is therefore not without its potential risks.

6.5 Conclusion

Despite the improvements in short term outcomes of islet transplantation seen since the introduction of the Edmonton protocol, long term outcomes remain disappointingly poor. There is evidence that the graft loss seen is in part attributable to the toxicity of the immunosuppressive agents used. Rapamycin is the primary agent utilised by the Edmonton protocol and there is good *in vitro* evidence of its detrimental effects on both murine and human β cells.

My research has demonstrated clear deleterious effects of rapamycin on both a mouse insulinoma cell line and isolated rat islets. I have shown that prolonged rapamycin treatment results in inhibition of PKB, a key mediator of β cell function and survival. This inhibition is mediated via impaired assembly of mTORC2. In addition, I have demonstrated that these detrimental effects can be abrogated by infection of β cells with an adenovirus encoding constitutively active PKB. This provides further evidence that the toxicity of rapamycin is mediated predominantly via mTORC2 and PKB rather than mTORC1.

These findings not only bring into question the use of rapamycin as an immunosuppressive for islet transplantation but also highlight further the key role of PKB in pancreatic β cell homeostasis. As such, PKB provides a potential therapeutic target for improving outcomes of islet transplantation.

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