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Long-chain fatty acids and endoplasmic reticulum stress in pancreatic beta-cells: the role of Protein Kinase R (PKR)

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**Long-chain fatty acids and endoplasmic
reticulum stress in pancreatic beta-cells: the role
of Protein Kinase R (PKR)**

by

ANGIE COOPER

A thesis submitted to the University of Plymouth in partial fulfilment for
the degree of

DOCTOR OF PHILOSOPHY

School of Biomedical and Biological Sciences

Faculty of Science and Technology

In collaboration with

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Abstract

Angie Cooper

Long-chain fatty acids and endoplasmic reticulum stress in pancreatic beta-cells: the role of Protein Kinase R (PKR)

Type 2 diabetes (T2D) is a growing health-care and economic burden. Obesity is a risk factor for developing T2D, but the underlying molecular mechanisms are not well understood. However, mechanisms such as lipotoxicity, endoplasmic reticulum stress and inflammation are becoming increasingly well-recognised in obesity, and may underlie the development and progression of T2D. A central player in these mechanisms is Protein Kinase R (PKR), proposed to have a role within nutrient- and pathogen-sensing pathways, and is activated by ER stress and lipotoxicity.

A small molecule inhibitor Compound-16, adenoviral vectors and RNAi techniques in BRIN-BD11 rodent pancreatic β -cells, were used to demonstrate that PKR knockdown affords significant protection against palmitate-induced cell death. Furthermore, PKR knockdown potentiates palmitoleate cytoprotection during lipotoxicity, suggesting the cytotoxic and cytoprotective actions of long-chain fatty acid species may function via the PKR signalling pathway.

The use of a novel 1.1B4 human pancreatic β -cell line has shown that important differences exist between human and rodent cell responses to fatty acids *in vitro*. In 1.1B4 cells, long-chain saturated and monounsaturated fatty acids do not provide increasing protection as their chain-length increases, in contrast to rodent cell models. Furthermore, methyl-saturated fatty acid species are well tolerated, and methyl-monounsaturated fatty acids are cytoprotective to 1.1B4 β -cells.

TXNIP overexpression in an INS-TXNIP β -cell model has a proapoptotic role in conditions of glucotoxicity, but not glucolipotoxicity. Furthermore, in this cell model, succinate is cytoprotective against glucotoxicity, but not glucolipotoxicity. By contrast in 1.1B4 β -cells, succinate significantly protects against apoptosis induced by both glucotoxic and glucolipotoxic conditions.

Chronic inflammation has been implicated in the development and progression of T2D. At the centre of this response is the pro-inflammatory cytokine IL-1 β . The cellular origin of IL-1 β is unclear, but IL-1 β secretion has been linked to activation of the NLRP3 inflammasome, recently implicated in pancreatic β -cell death in T2D. Results suggest that IL-1 β is secreted by INS-TXNIP and 1.1B4 pancreatic β -cells under lipotoxic conditions, thus offering a potential role for targeted IL-1 β therapy in T2D.

List of Abbreviations

α -cell	Alpha-cell
δ -cell	Delta-cell
PP-cell	Gamma-cell
ϵ -cell	Epsilon cell
2-AP	2-Aminopurine
Ab	Antibody
ADS	Antibody dilution solution
ADST	Triton-X100 antibody dilution solution
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
ASC	Apoptosis-associated speck-like protein containing a CARD
ASK	Apoptosis signal-regulating kinase
ATF3	Activating transcription factor 3
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
β -cell	Beta-cell
BGL	Blood glucose levels
BMI	Body mass index
BSA	Bovine serum albumin
c-AMP	Cyclic AMP
CARD	Caspase recruitment domain
cDNA	Complementary DNA
CHOP 10	C/EBP (CCAAT/enhancer-binding protein)-homologous protein 10 transcription factor
CLR	C-type lectin receptor
CoA	Co-enzyme A
DAG	Diacylglycerol
DAMP	Danger-associated molecular pattern(s)
DAPI	4',6-diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	Diethylpyrocarbonate
dGTP	2'-Deoxyguanosine 5'-triphosphate
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DOPE	L-dioleoyl phosphatidylethanolamine
DOX	Doxycycline
dsDNA	Double-stranded DNA
dsRBD	Double-stranded RNA binding domain
dsRBM	Double-stranded RNA binding motif
dsRNA	Double-stranded RNA
dTTP	2'-Deoxythymidine 5'-triphosphate
ECL	Enhanced chemiluminescence

EDTA	Ethylenediaminetetraacetic acid
eIF2 α	Eukaryotic initiation factor 2 α
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERS	Endoplasmic reticulum stress response
EV	Empty vector
FA	Fatty acid(s)
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FFA	Free fatty acid(s)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN2	General control non-derepressible-2 kinase (aka eIF2 α kinase 4)
gDNA	Genomic DNA
GDP	Guanosine diphosphate
GRP	Glucose-regulated protein
GSIS	Glucose stimulated insulin secretion
GTP	Guanosine triphosphate
HRI	Haem-regulated inhibitor kinase (aka eIF2 α kinase 1)
HRP	Horseradish peroxidase
IAPP	Islet amyloid polypeptide
IFN- γ	Interferon-gamma
IL-1 β	Interleukin-1 beta
IoL	Islets of Langerhans
IRE	Inositol requiring ER-to-nucleus signal kinase
JNK	c-jun N-terminal kinase
KD	Knockdown
LC	Long-chain
LC-CoA	Long-chain acyl-CoA
LDS	Lithium dodecyl sulphate
LPS	Lipopolysaccharide
LRR	Leucine rich repeat(s)
MAPK	Mitogen activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
miRNA	Micro RNA
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
MUFA	Monounsaturated fatty acid(s)
NACHT	Nucleotide binding and oligomerization domain [NOD] domain
NF- κ B	Nuclear Factor-Kappa B
NLR	Nucleotide-binding domain (NOD)-like leucine-rich repeats
NLRP3	NLR-protein-3
NO	Nitric oxide
NOD	Nucleotide-binding domain
PACT	Protein activator of PKR
PAMP	Pathogen-associated molecular pattern(s)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PERK	Double-stranded ribonucleic acid (dsRNA)-dependent protein kinase R (PKR)-like ER kinase (aka eIF2 α kinase 3)
PI	Propidium iodide

PKR	Protein kinase R (aka eIF2 α kinase 2)
PM	Plasma membrane
PRR	Pattern-recognition receptor(s)
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
PYD	Pyrin domain
RIG	Retinoic-acid-inducible protein
RLR	Retinoic-acid-inducible protein (RIG)-like receptor(s)
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
SCD1	Stearoyl-CoA desaturase 1
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SFA	Saturated fatty acid(s)
shRNA	short hairpin RNA
siRNA	short interfering RNA
SOCS	Suppressor of cytokine signalling
ssDNA	Single-stranded DNA
SV	Scrambled vector
T1D	Type 1 Diabetes mellitus
T2D	Type 2 Diabetes mellitus
TAE	Trizma/Acetic acid/EDTA
TG	Triglyceride
TBE	Trizma/Borate/EDTA
TBS	Trizma buffered saline
TET	Tetracycline
TF	Transcription factor
tGFP	Turbo Green Fluorescent Protein
TIR	Toll-IL1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF- α	Tumour necrosis factor alpha
TRAF	TNF receptor-associated factor
TTBS	Tween Trizma buffered saline
TXNIP	Thioredoxin-interacting protein
UPR	Unfolded protein response
XBP-1	x-box binding protein 1

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Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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Relevant scientific seminars and conferences were regularly attended where work was presented.

Publications:

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Structure of thesis

The objective of this work was to investigate whether lipotoxicity-induced pancreatic β -cell death is mediated by activation of an ER stress response, centred on alterations to PKR activity. The availability of a newly developed human pancreatic β -cell line 1.1B4 extended the main objective, to include investigations which characterised responses to FA species in this cell model. Additional studies considered the role of TXNIP as a proapoptotic factor during lipotoxic conditions in a TET-induced TXNIP overexpression INS-1 cell model. A role for succinate as a cytoprotective agent in glucotoxic and glucolipotoxic conditions was also investigated in this cell model. Mediators of inflammation and inflammasome activation, and the effect of endogenous pro-inflammatory cytokines in pancreatic β -cells were investigated during glucotoxic and glucolipotoxic conditions, in 1.1B4 cells and INS-TXNIP cells.

Chapter 1

This introduction chapter covers an overview of T2D within the scope of this study, including the link between obesity and T2D; mechanisms of lipotoxicity; FA effects on β -cell function; ER stress; and PKR as a mediator of apoptosis. The introduction also considers the role of inflammation in obesity and T2D, specifically inflammasome activation and the involvement of IL-1 β as an underlying apoptotic mediator in T2D. To conclude, this section considers the historical limitations of human pancreatic β -cells for *in vitro* use, and introduces a new human pancreatic β -cell line 1.1B4.

Chapter 2

This chapter details an overview of the general methods adopted throughout the study, the materials used and the protocols followed in each chapter.

Chapter 3

PKR has been identified as a potential mediator of pro-apoptotic responses in pancreatic β -cells exposed to lipotoxicity. A small molecule inhibitor, DN adenoviral vectors and shRNA methods were used in this chapter to target PKR in rodent BRIN-BD11 pancreatic β -cells, to determine how altered kinase activity affected cell viability and markers of ER stress in response to FA species.

Chapter 4

The timely publication reporting a novel human pancreatic β -cell 1.1B4, allowed the opportunity to conduct functional studies to investigate the cytotoxic or cytoprotective effects of various FA species. This chapter focussed on viability studies to investigate 1.1B4 *in vitro* responses to FAs, to consider the suitability of this cell model for the study of β -cell dysfunction during lipotoxicity.

Chapter 5

This chapter considered the role of TXNIP as a proapoptotic factor in conditions of glucotoxicity and glucolipotoxicity, using an INS-1 cell line with a TET-inducible stable-INS-1 TXNIP overexpression vector. We investigated the cytoprotective role of succinate on TXNIP overexpression, and considered mediators of inflammasome activation in the INS-1 TXNIP cell line and human 1.1B4 cells

during lipotoxic and pro-inflammatory conditions. This chapter also considered the effect of endogenous pro-inflammatory cytokines on 1.1B4 β -cell viability.

Chapter 6

This final section provides a brief overview of each chapter, the key findings in terms of novel results, limitations of the studies undertaken and areas for future work.

Chapter 1 Introduction

1.1 Diabetes mellitus

In 2011 the World Health Organisation (WHO) estimated that 346 million people worldwide had diabetes mellitus (DM). Diabetes mellitus is defined as a metabolic disorder characterised by chronic hyperglycaemia. There are now several recognised categories of DM, but the two main forms are known as type 1 diabetes (T1D) and type 2 diabetes (T2D). Both types of diabetes are a major cause of morbidity and mortality with the cost of treating these diseases and their associated complications representing an ever-escalating economic and health-care burden (Ryan, 2009).

Type 1 diabetes accounts for approximately 10% of all diagnosed DM cases, and is caused by an autoimmune-mediated mechanism of unknown pathoetiology. This disease involves a process known as insulinitis (Gepts, 1965): recent work investigating insulinitis has more clearly defined the cellular profile of the inflammatory infiltrate of the islets of Langerhans (IoL) within the pancreas. These cells have been found to be predominantly cytotoxic T-cells and macrophages, although there are also a smaller number of other immune cells present. Insulinitis leads to pancreatic beta-cell (β -cell) destruction and complete loss of insulin secretion (Gepts, 1965; Willcox *et al.*, 2009). Type 1 diabetes typically develops in children and juveniles, and requires daily exogenous insulin therapy (Zhong *et al.*, 2012).

The majority of DM cases globally have T2D, and there has been an alarming rise in the number of diagnosed cases in recent decades. This is suggested to be attributable to the increasing prevalence of obesity due to the widespread

adoption of Westernised diets high in saturated-fat, combined with a more sedentary lifestyle (Hu, 2011). Being chronically overweight or obese can cause adverse metabolic effects such as increased circulating cholesterol and triglyceride (TG) levels. These effects result in elevated plasma lipid concentrations which are suggested to underlie insulin deficiency (decreased insulin secretion by the β -cells), or a reduced physiological response to insulin secretion (insulin resistance), or indeed a combination of both mechanisms. These mechanisms collectively result in diminished utilisation of glucose within peripheral tissues, greatly increasing the risk of the development of DM (Cunha *et al.*, 2008; Cnop *et al.*, 2007; Leibowitz *et al.*, 2011).

Type 2 diabetes has historically been considered a 'late-onset' disease. However, in the past couple of decades, there has been a clear reduction in the mean age of diagnosis (52.0 to 46.0 years, $p < 0.05$), and a tendency towards higher frequency of diagnosed cases among children and younger adults (Peterson *et al.*, 2007; Koopman *et al.*, 2005; Cree-Green *et al.*, 2013). Furthermore, racial or ethnic groups that had a higher risk of developing T2D compared to Caucasian populations, now no longer show an ethnic or race difference in risk (Koopman *et al.*, 2005; Ford *et al.*, 1997).

1.2 The link between obesity and T2D

Obesity occurs when there is a long-term excess of nutrient intake, to the extent that health and well-being are affected. In particular, obesity is associated with increased risk of hypertension, hyperinsulinaemia, glucose intolerance, some common cancers and cardiovascular disease (Vernochet *et al.*, 2009). Importantly, there is a clear link between chronic obesity and an increased risk of

developing T2D (Ford *et al.*, 1997; Gwiazda *et al.*, 2009). Obesity is now considered the predominant metabolic 'disease' globally (Golay and Ybarra, 2005). A WHO consultation on obesity defined pre-obesity (overweight) as a body mass index (BMI) of ≥ 25 , obesity (class 1) as a BMI ≥ 30 , class 2 obesity as a BMI of ≥ 35 and class 3 obesity as a BMI ≥ 40 (Barlow and Dietz, 1998). Studies linking obesity and T2D collectively suggest that between 60 – 90% of patients with T2D have a BMI greater than 23.0 kg/m^2 (Wannamethee and Shaper, 1999; Golay and Ybarra, 2005; Haslam, 2010). It has also been suggested that for every kilogramme of body weight gained, the risk of developing T2D increases between 4.5 - 9% (Ford *et al.*, 1997). This risk is further increased when there is an established family history of DM, gestational diabetes or weight gain particularly in childhood (Wannamethee and Shaper, 1999; Haslam, 2010). Recent change in lifestyle habit, particularly less exercise and increased intake of energy-dense foods, as seen more commonly in Westernised or rapidly-developing third world countries, leads directly to increased adipose tissue deposition, which if persistent, may eventually cause metabolic dysfunction (Vernochet *et al.*, 2009).

A primary purpose of adipose tissue is to store energy in the form of TG within lipid droplets, which can be easily utilised by activation of lipolysis depending on the energy requirements of other tissues and organs. Adipose tissue can secrete proteins, such as leptin and adiponectin that impact on energy homeostasis (Vernochet *et al.*, 2009; Ravussin and Smith, 2002). Dysregulation of these processes is suggested to trigger reduced insulin sensitivity (Despres and Lemieux, 2006). Furthermore, large adipocytes within subcutaneous deposits are less insulin responsive than smaller adipocytes (Lafontan, 2012). In addition,

it has been suggested that adipocytes present in larger adipose mass are subject to elevated stress, possibly as a result of mechanisms such as hypoxia, lipotoxicity and low-grade chronic inflammation (Gregor and Hotamisligil, 2007), therefore suggesting a mechanism between obesity and the progression of T2D.

1.3 Lipotoxicity

Obesity has been shown to create an altered plasma lipid profile, specifically elevated concentrations of plasma free fatty acids (FFAs). Free fatty acids, such as palmitate or palmitoleate, are derived from TG and circulate in the plasma bound to albumin. They are recognised to be the vehicle by which TG is transported from storage within adipose tissue, to target sites for utilisation (Karpe *et al.*, 2011). If an elevated FFA profile persists in a chronic state, it is thought to underlie the progression of insulin resistance and subsequent insulin deficiency, resulting in reduced glucose utilisation within peripheral tissues (Eizirik *et al.*, 2008; Cnop *et al.*, 2005; Maedler, 2008). Chronically elevated plasma FFAs concentrations are proposed to exert a deleterious effect on both β -cell function and survival via a mechanism termed 'lipotoxicity' (Newsholme *et al.*, 2007; Wei *et al.*, 2006). Lipotoxicity as observed *in vivo*, is defined as an excess of fatty acids (FAs) in non-adipose tissue (such as the pancreas), over and above their metabolic requirement. This has a detrimental effect on the cells resulting in metabolic dysregulation, rather than specific cellular insult such as radiation or infection (Schaffer, 2003; Bergman and Ader, 2000; Eitel *et al.*, 2003; El-Assaad *et al.*, 2003; Moffitt *et al.*, 2005). It is this process of metabolic dysregulation within the pancreas that is suggested to underlie the long-term reduction in β -cell mass seen in T2D (Butler *et al.*, 2003). Interestingly, several groups propose that lipotoxicity is unlikely to adversely affect β -cell viability unless chronic

hyperglycaemia is already established. This suggests that β -cell loss may be induced by a combination of both chronic hyperglycaemia and lipotoxicity, via a mechanism perhaps more aptly termed 'glucolipotoxicity' (Fig. 1.1) (El-Assaad *et al.*, 2003; Poitout and Robertson, 2008).

1.3.1 Mechanisms of lipotoxicity

Many mechanisms have been suggested to explain the underlying molecular pathway that drives lipotoxicity, however, to date this process is still to be fully elucidated. What is clear is that there are differences between *in vivo* and *in vitro* responses to FA: for example, the acute reduction of FFAs *in vivo* leads to a drop in insulin response to glucose (Boden *et al.*, 1998). Conversely, *in vitro* studies have shown that the acute reduction of FFAs may result in exceptionally high levels of GSIS (Straub and Sharp, 2004). Furthermore, the loss of β -cell viability occurs much more quickly in *in vitro* models than when compared to *in vivo* models (Diakogiannaki *et al.*, 2007; Dhayal *et al.*, 2008). Nonetheless, the varied responses observed in β -cells during chronic exposure to saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), suggest that different FA species may exert their effects via different mechanisms (Welters *et al.*, 2004; Eitel *et al.*, 2003; Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Morgan *et al.*, 2008). There have been several hypotheses proposed to explain why this may be the case which are outlined below.

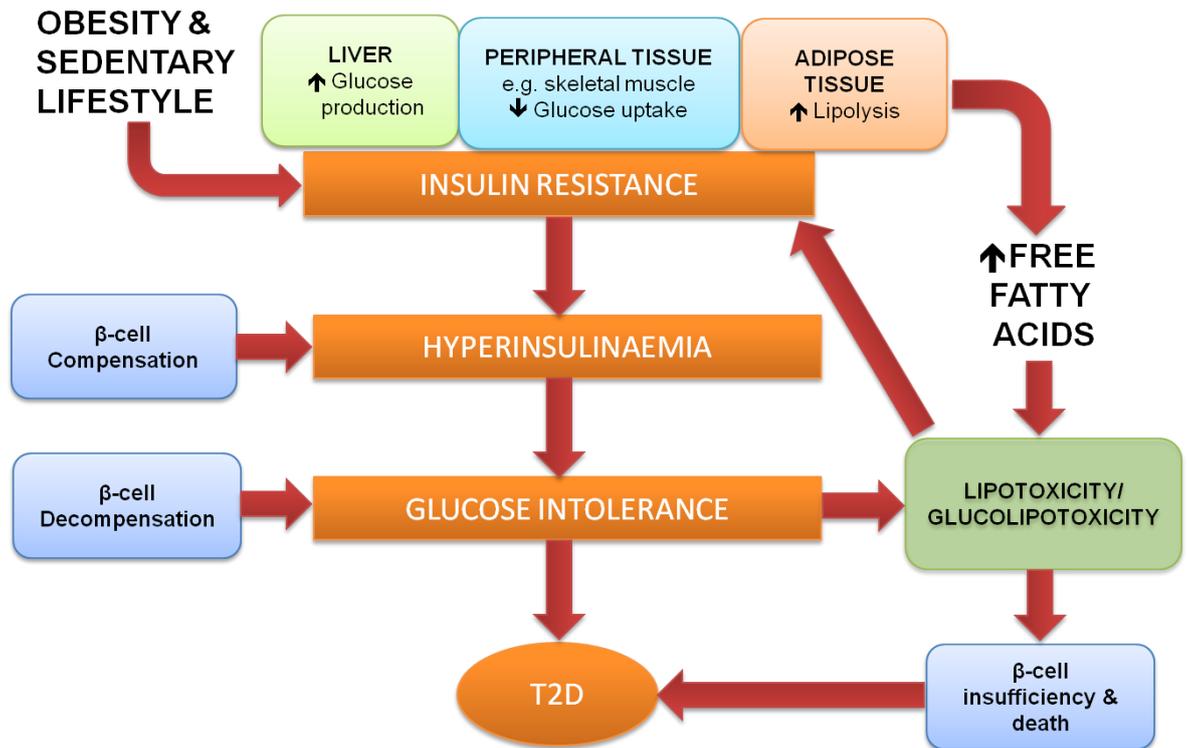


Fig. 1.1 Suggested model for the development of T2D

A sedentary lifestyle and being obese can lead to insulin resistance resulting in the increased production of glucose in the liver, decreased glucose utilisation in peripheral tissues and increased lipolysis and TG deposition in adipose tissue. Pancreatic β -cells initially secrete more insulin to compensate for insulin resistance and maintain normoglycaemia, however, eventually β -cells will be unable to continue with this compensatory mechanism and glucose intolerance occurs. Hyperglycaemia and increased lipolysis drive lipotoxicity and glucolipotoxicity in β -cells, causing decreased β -cell mass and eventual progression to overt T2D (schematic adapted from Yang and Trevillyan, 2008).

1.3.2 Mechanisms of lipotoxicity: ceramide formation

Palmitate, as one of the predominant FFA species present in human serum, is readily accessible to pancreatic β -cells *in vivo*. Furthermore, palmitate has been identified as one of the main precursors required for *de novo* ceramide synthesis (Orrenius, 2004). It has been suggested that the abundant availability of palmitate as a preferentially-metabolised SFA species may drive increased ceramide production (Wei *et al.*, 2006; Kelpke *et al.*, 2003; Maedler *et al.*, 2001). Ceramide has an important role as a second signalling molecule, but is also known to be a potent apoptotic mediator in response to diverse stress stimuli, such as nutrient overload or withdrawal (Ruvolo *et al.*, 2001). Therefore, increased ceramide formation is suggested to initiate apoptosis in pancreatic β -cells subjected to nutrient overload as seen in obesity. Shimabukuro and colleagues (1998) proposed that in part, an increased rate of *de novo* ceramide synthesis was responsible for SFA-induced pancreatic β -cell death seen in *fa/fa* Zucker Diabetic fatty (ZDF) rats.

However, other groups have countered this hypothesis, and shown that in a liver cell model, SFAs significantly increased cell death; however, this was observed independently of ceramide production (Wei *et al.*, 2006). Furthermore, Hardy and colleagues (2003), from their studies in a breast cancer cell line, argued that *de novo* ceramide synthesis was not the mechanism by which palmitate induced β -cell apoptosis. Their use of a specific ceramide-synthesis inhibitor, fumonisin B1, prevented palmitate-induced ceramide synthesis in these cells, but did not prevent apoptosis. This inhibitory effect using fumonisin B1 has also been observed in rat and human islets (Maedler *et al.*, 2001; Shimbukuro *et al.*, 1998). In other studies, fumonisin B1 did not prevent palmitate-induced caspase-3

activity or palmitate-induced cell death. This suggests that the formation or increased production of ceramide, cannot be the sole mechanism responsible for cytotoxic effects seen during palmitate exposure (Beeharry *et al.*, 2004; Hardy *et al.*, 2003; Welters *et al.*, 2004). The exact mechanism whereby changes in ceramide levels may trigger cell death are still unclear and require further investigation (Newsholme *et al.*, 2007).

1.3.3 Mechanisms of lipotoxicity: TG formation

Free fatty acids are transported into cells by membrane-associated fatty-acid binding proteins, such as CD36 (Schwenk *et al.*, 2010). Once inside the cytoplasm, FFAs are activated by acyl-CoA synthetase to form acyl-CoA; this can then be transported into the mitochondria or peroxisomes by carnitine acyl transferase for β -oxidation to release ATP. Alternatively, it may be stored as TG in lipid droplets. Importantly, the metabolism of long-chain acyl-CoA (LC-CoA) from SFA is less efficient than from MUFA, suggested to lead to an accumulation of ceramide, and diacylglycerols (DAGs) (another second messenger molecule), (Shimabukuro *et al.*, 1998; Nolan and Larter, 2009; Schaffer, 2003). Some groups have shown that blocking β -oxidation using the FA oxidative inhibitor etomoxir, (which specifically blocks carnitine palmitoyltransferase-1, a mitochondrial enzyme that mediates the transport of LC-FAs across the membrane), markedly enhanced palmitate-induced cytotoxicity in rodent β -cells and human islets (El-Assaad *et al.*, 2003; Hardy *et al.*, 2003; Cnop *et al.*, 2001). However, results using this inhibitor to block β -oxidation are conflicting as heightened palmitate-induced cytotoxicity is not universally observed (Busch *et al.*, 2005; Welters *et al.*, 2004).

One hypothesis is that the process of TG accumulation may function as a defence mechanism and might therefore be beneficial to β -cell survival, by sequestration of excess FFAs that would otherwise have toxic effects (Nolan and Larter, 2009; Ricchi *et al.*, 2009; Cnop *et al.*, 2001; Cnop *et al.*, 2002). Conversely, others suggest that TG formation due to elevated levels of FFAs is detrimental to β -cells through physiochemical disruption of cells as observed *in vitro* using INS-1 cells (Moffitt *et al.*, 2005). Elevated SFA incorporation within the cell is suggested to impact on cell membrane rigidity and fluidity due to altered lipid deposition. It is this physical action which may ultimately have a detrimental effect on mechanisms such as cell signalling, the secretion and trafficking of insulin granules, protein processing within the ER and disrupted secretory granule fusion during exocytosis.

Diakogiannaki and colleagues (2007) showed that remodelling and increased TG formation occurs in β -cells incubated with SFAs. However, the use of methyl-palmitoleate in the presence or absence of palmitate, failed to increase TG or to modify the amount of TG formation seen in the β -cells treated with palmitate, suggesting TG formation is unlikely to be the cause of cytotoxicity. The addition of a methyl group on the functional FA carboxyl group blocks the initial esterification step in the β -oxidation pathway, and as such, FA methyl-esters prevent FA oxidation. Methyl-palmitoleate did however, display cytoprotective effects, suggesting that cytoprotection is not via altered TG formation or composition.

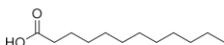
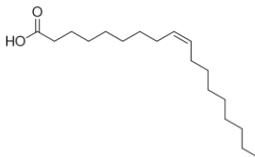
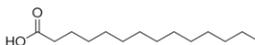
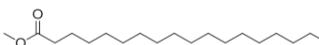
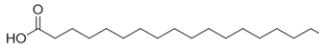
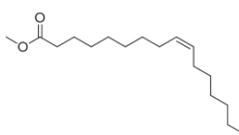
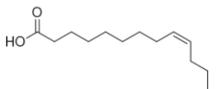
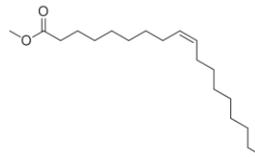
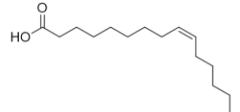
Moffitt and colleagues (2005) stated that using a single SFA species within an *in vitro* system affects TG storage resulting in disruption of normal cell morphology,

in a manner that may not typically occur *in vivo*, where equal concentrations of unsaturated FAs would also be present. On this basis, they argue that lipotoxicity is an *in vitro* artefact observed when culturing β -cells with exogenous FAs, and suggested that this presents limitations when using cell models to investigate the effects of SFAs.

While it is probably accepted that *in vitro* models may not be completely representative of *in vivo* conditions, it is also acknowledged that *in vitro* findings are likely to be reflective of *in vivo* pancreatic β -cell responses to circulating FFAs. This suggests that the prevailing ratio of plasma FFA species within the pancreatic β -cell environment at any time is a relevant and important factor with regard to long-term β -cell survival. Consequently, *in vitro* models may still offer critical information regarding the pathological mechanisms underlying lipotoxicity (Newsholme *et al.*, 2007).

1.4 Fatty acid effects on β -cell function

Key observations regarding the molecular mechanisms underlying the effects of FA have been gleaned using *in vitro* culture of pancreatic β -cell lines (Newsholme *et al.*, 2007; Welters *et al.*, 2004; Eitel *et al.*, 2003; Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Morgan *et al.*, 2008). What is clear from these studies is that not all FA species exert similar effects on β -cells during chronic exposure. The degree of toxicity observed in BRIN-BD11 and INS-1 β -cells varies markedly according to the carbon chain length, degree of saturation and configuration of FA species (Table 1.1).

Table 1.1 Fatty acid nomenclature			
Fatty acid	Structure	Fatty acid	Structure
Lauric acid (laurate) C12:0 (med-chain) • Saturated • No double bond		Oleic acid (oleate) C18:1 (long-chain) • Unsaturated • One double bond • <i>Cis</i> configuration	
Myristic acid (myristate) C14:0 (long-chain) • Saturated • No double bond		Methyl-palmitate C16:0 (long-chain) • Saturated • No double bond	
Palmitic acid (palmitate) C16:0 (long-chain) • Saturated • No double bond		Methyl-stearate C18:0 (long-chain) • Saturated • No double bond	
Stearic acid (stearate) C18:0 (long-chain) • Saturated • No double bond		Methyl-palmitoleate C16:1 (long-chain) • Unsaturated • One double bond • <i>Cis</i> configuration	
Myristoleic acid (myristoleate) C14:1 (long-chain) • Unsaturated • One double bond • <i>Cis</i> configuration		Methyl-oleate C18:1 (long-chain) • Unsaturated • One double bond • <i>Cis</i> configuration	
Palmitoleic acid (palmitoleate) C16:1 (long-chain) Unsaturated • One double bond • <i>Cis</i> configuration			

Long-chain SFA (LC-SFAs) such as palmitate (C16:0) or stearate (C18:0) exert a detrimental effect on cell viability, whereas medium-chain and/or shorter-chain SFAs (C14:0 or less) are well-tolerated and do not affect cell viability (Welters *et al.*, 2004; Eitel *et al.*, 2003; Dhayal *et al.*, 2008). Long-chain monounsaturated fatty acids (LC-MUFAs) such as palmitoleate (C16:1) or oleate (C18:1) are also well-tolerated by rodent β -cells. However, most importantly, the *in vitro* co-incubation of LC-MUFAs with LC-SFAs effectively attenuates the cytotoxic effects seen when β -cells are incubated with LC-SFAs alone (Karaskov *et al.*, 2006; Welters *et al.*, 2004). It has been demonstrated that this protective effect of LC-MUFAs is also observed when rodent β -cells are exposed to pro-inflammatory cytokines, or when the cells are subjected to serum withdrawal (Welters *et al.*, 2004). Palmitoleate was able to ameliorate cytotoxic effects in BRIN-BD11 cells when it was added up to 10 h after cells were initially incubated with the SFA palmitate. This suggests that the protective actions of LC-MUFAs are exerted rapidly, as cytotoxicity due to palmitate-induced toxicity would already have been initiated.

The configuration of the FA species also impacts on the cytoprotective action of LC-MUFAs. Again using BRIN-BD11 and INS-1 β -cells, it has been demonstrated that LC-MUFAs in the *cis* configuration effectively protect cells against the cytotoxic effects of LC-SFAs. However, substituting a *cis* double bond with a *trans* double bond results in significantly reduced cytoprotective potency (Dhayal *et al.*, 2008).

It has been suggested that the SFA palmitate must be metabolised to LC-CoA to exert a cytotoxic effect, as studies using a non-metabolisable palmitate analog,

2-bromopalmitate, did not promote cell death, nor evoked such a cytotoxic response as palmitate (Diakogiannaki *et al.*, 2007; Hardy *et al.*, 2003; El-Assaad *et al.*, 2003; Gwiazda *et al.*, 2009). However, it has also been proposed that altered lipid-partitioning (when lipid is stored in areas other than sub-cutaneous tissue) (Weiss, 2007), not β -oxidation, enhances the proapoptotic effects of palmitate. Poorly-metabolised methyl-FA derivatives such as 2-bromopalmitate, have also been used to demonstrate that the cytoprotection afforded by the addition of LC-MUFA species is *not* via their metabolism, prompting suggestions that the cytoprotective action may be mediated by a FA receptor (Diakogiannaki *et al.*, 2007). However, while there has been great interest in the relatively newly discovered class of lipid-responsive G-protein-coupled receptors with regard to mechanisms of FA action, there is no clear consensus about a single receptor that may satisfactorily explain the cytotoxic or cytoprotective effects of individual FFAs (Morgan and Dhayal, 2009; Morgan, 2009).

1.5 The ER: function and implications of FFA-mediated stress

Whilst the exact molecular mechanisms that may mediate the proapoptotic or protective effects of FFAs in β -cells are still unclear, the last few years has seen increased interest in the possibility that the decline in β -cell numbers observed during the progression of T2D may be attributable to ER stress (Qu *et al.*, 2009; Breckenridge *et al.*, 2003; Laybutt *et al.*, 2007). The ER is a highly dynamic organelle that plays a key role in the routing of secretory proteins through eukaryotic cells (Schröder, 2008). It is a primary site for synthesis, folding and post-translational modification of secretory and membrane proteins and is also the location where disulphide bonds are formed. In addition, the ER has a central role in lipid, sterol and protein synthesis and is involved in the storage and

regulation of intracellular calcium (Ca^{2+}) (Qu *et al.*, 2009; Eizirik *et al.*, 2008; Scheuner and Kaufman, 2008; Schröder, 2008). Another important ER function is its strict quality control role, whereby slowly-folding or folding-incompetent proteins are retained and subsequently targeted for proteolytic degradation via the ER-associated protein degradation (ERAD) pathway (Schröder, 2008). Appropriate Ca^{2+} concentrations ($[\text{Ca}^{2+}]$) and a suitable redox state within the ER lumen are essential factors for competent protein folding and chaperone function. Imbalances can lead to an accumulation of misfolded proteins, triggering an ER stress response (ERS), which in turn triggers an unfolded protein response (UPR) to re-establish homeostasis (refer to Section 1.8 for more detail). It has been proposed that some metabolic disease states can impair ER equilibrium, initiating ER stress, a subsequent UPR and eventual apoptosis (Xu *et al.*, 2005; Lindholm *et al.*, 2006; Kin *et al.*, 2008). One mechanism suggested to mediate this process is a depletion of ER Ca^{2+} concentration (rather than increased $[\text{Ca}^{2+}]_{\text{cyt}}$) due to activation of the UPR, leading to dysfunctional processing of luminal proteins and apoptosis (Eizirik *et al.*, 2008; Gwiazda *et al.*, 2009; Michalak *et al.*, 2002; Nakano *et al.*, 2006).

Some groups have shown that palmitate causes a marked increase in cell death, due to upregulation of certain ERS proteins such as p-eIF2 α , ATF4 and CHOP10 (known as the p-eIF2 α /ATF4/CHOP10 apoptotic pathway) (Eizirik *et al.*, 2008; Özcan *et al.*, 2004). This establishes a link between underlying pathophysiological perturbations observed in obesity such as increased plasma FFA, and the progression of T2D via β -cell ER stress-mediated apoptosis (Laybutt *et al.*, 2007; Karaskov *et al.*, 2006; Breckenridge *et al.*, 2003; Wei *et al.*, 2009). However, it is interesting that Karaskov and colleagues (2006) found no

change in the level of the ER chaperone protein GRP78/BiP expressed in INS-1 cells that had been treated with palmitate, which would be reasonably expected in response to an ERS.

1.6 The pancreas: cells and function

The pancreas functions as both an endocrine and exocrine organ. The majority of the pancreas consists of acinar cells: these are found clustered around small ducts that ultimately coalesce to form the single large pancreatic duct. The acinar cells have an important role in digestion and metabolism as the enzymes secreted from these cells, such as carbohydrases, lipases and proteases within pancreatic juice, are responsible for the digestion of all the major food groups. The endocrine cells of the pancreas are spread around the entire organ, in small clusters known as I α L. The islets consist of distinct endocrine cell types that secrete different hormones with various functions. The β -cells secrete insulin, while the alpha-cells (α -cells) secrete glucagon. These two cell types are predominantly responsible for glucose homeostasis: in simple terms, insulin decreases blood glucose levels (BGL) whereas glucagon increases BGL. The delta-cells (δ -cells) secrete somatostatin which inhibits the secretion of both insulin and glucagon; the gamma cells (also known as PP-cells) secrete pancreatic polypeptide which has an appetite-suppressing role. Finally, the epsilon cells (ϵ -cells) secrete ghrelin, which is implicated in increasing appetite (Fig. 1.2) (Hohmeier and Newgard, 2004).

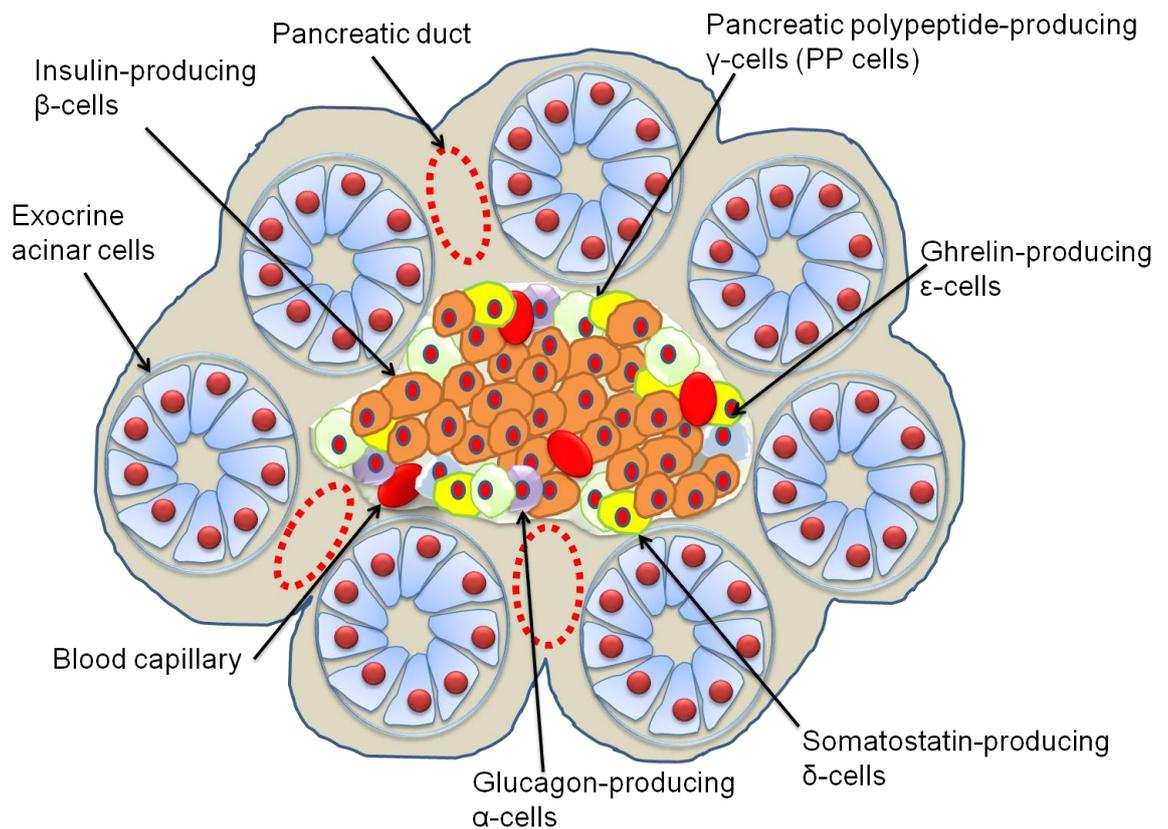


Fig. 1.2 Cell anatomy and function of the pancreas

The pancreas consists predominantly of acinar cells which secrete digestive enzymes. The IOL are interspersed throughout the pancreas, and consist of insulin secreting β -cells (~70%), glucagon secreting α -cells (~20%), somatostatin secreting δ -cells (~5%) and smaller numbers of pancreatic polypeptide secreting PP-cells and ghrelin secreting ϵ -cells (schematic adapted from Efrat and Russ, 2012).

1.6.1 β -cells and susceptibility to ER stress

One of the primary functions of the pancreatic β -cell is the synthesis and secretion of insulin in response to glucose, thereby maintaining normoglycaemia. Beta-cells have a highly developed ER with high secretory capacity necessary to handle insulin secretion load. This is estimated to take up approximately 70% of all protein synthesis within the cell. It is this high secretory load which is believed to be responsible for the particular vulnerability to homeostatic change (Cnop *et al.*, 2007; Cardozo *et al.*, 2005). The ER stress response (ERS) is suggested to have evolved to allow temporal and physiological variations in ER load to be more effectively managed (Butler *et al.*, 2003), making the ERS a critical adaptive signalling response necessary for optimal β -cell function (Cnop *et al.*, 2008; Laybutt *et al.*, 2007; Araki *et al.*, 2003). The growth of a glucose-responsive β -cell phenotype within the pancreas during foetal development is typically subject to strict regulation, as the risk of uncontrolled insulin secretion could lead to severe or persistent hypoglycaemia, for example as seen in persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) (Aynsley-Green, 1981). In addition to tight regulation of function and mass, it is suggested the β -cell phenotype has developed especially sensitive apoptotic susceptibility during developmental differentiation (Mandrup-Poulsen, 2003; Laybutt *et al.*, 2007). It is known that ER stress-susceptibility varies according to cell type, so it is relevant given the aforementioned points, that β -cells are highly susceptible to ER stress which may be regarded as an adaptive 'failsafe' mechanism to eliminate compromised cells (Araki *et al.*, 2003).

1.7 The role of ERS in obesity and T2D

Evidence points strongly towards a role for the ER in cell survival and apoptotic responses, particularly when associated with lipotoxic conditions in individuals with T2D (Mathias *et al.*, 1998; Cnop *et al.*, 2001). Beta-cell ERS occurs as a result of perturbations in normal ER balance due to pathological or physiological insults such as infection, nutrient deprivation or nutrient overload, ER luminal Ca²⁺ depletion, accumulation of misfolded proteins, impaired protein trafficking or inflammatory cytokines. Any of these insults may lead to altered ER functionality resulting in the accumulation of unfolded or misfolded proteins within the ER lumen (Ito *et al.*, 1999; Özcan *et al.*, 2004; Qu *et al.*, 2009; Schröder and Kaufman, 2006; Araki *et al.*, 2003; Lee *et al.*, 2007; Hotamisligil, 2005).

It has already been mentioned that the hyperlipidaemic state accompanying obesity results in an altered circulating lipid profile, which has been shown to be cytotoxic to β -cells under both normal physiological conditions and in obesity-induced diabetes models (Herbert, 2007; Lai *et al.*, 2008; Araki *et al.*, 2003; Cnop *et al.*, 2007; Shimbukuro *et al.*, 1998). This is supported by studies that show heightened expression of certain ER stress marker proteins such as activating transcription factor 4 (ATF-4) and the proapoptotic transcription factor C/EPB [CCAAT/enhancer-binding protein]-homologous protein 10 (CHOP10) (Laybutt *et al.*, 2007). *In vitro* rodent cell and mouse model studies have also shown that elevated plasma FFA concentrations contribute to insulin resistance and impaired insulin secretion, a prerequisite for the development of T2D (Diagkogiannaki and Morgan, 2008; Cunha *et al.*, 2008). Furthermore, the initiation of the β -cell ERS by exposure to LC-SFAs is reported to be necessary for β -cell lipoapoptosis (Araki *et al.*, 2003; Eitel *et al.*, 2003). Taken together, these data suggest that

the persistently high plasma concentrations of FFAs commonly seen in obese individuals may drive a prolonged decline in β -cell mass and the gradual progression to T2D, consequently implicating the ERS in this mechanism. This further implies that, for the long-term survival of β -cells in a lipotoxic environment, the mammalian ERS is critical to alleviate cytotoxic effects by inhibiting global protein synthesis, thus allowing the opportunity for recovery from ER stress and restoration to normal ER homeostasis (Diakogiannaki *et al.*, 2008).

1.8 The ERS pathway

The ERS triggers the initiation of a group of signal transduction pathways collectively called the UPR, which attempt to reduce ER burden and restore homeostasis. These collective stress responses are initiated and mediated by three specific transducer proteins, whose role are to collectively transmit stress signals from the ER to the nucleus, under the activated UPR pathways that mediate adaptive cell survival responses (Qu *et al.*, 2009; Fonseca *et al.*, 2009; Cnop *et al.*, 2008; Araki *et al.*, 2003; Herbert, 2007). The three ER-membrane localised components are the α -subunit of inositol requiring ER-to-nucleus signal kinase (IRE-1), activating transcription factor 6 (ATF-6) and the double-stranded ribonucleic acid (dsRNA)-dependent protein kinase R (PKR)-like ER kinase (PERK).

1.8.1 The UPR

To date, there are four known functionally co-ordinated and distinct transcriptional and translational responses associated with the ERS (Fig. 1.3). The first response to disrupted ER function is the initiation of a molecular chaperone system, through upregulation of ER chaperone genes encoding GRP78 (also

known as the immunoglobulin heavy-chain binding protein, BiP) and GRP94, thereby preventing protein agglutination and also enhancing protein folding capacity within the ER (Araki *et al.*, 2003). Secondly, attenuation of protein translation is mediated by the phosphorylation of a key serine residue (Ser51) on the α -subunit of eukaryotic initiation factor 2 (eIF2). This prevents the exchange of eIF2-GDP for eIF2-GTP required to bind and deliver the initiator met-tRNA to the translation machinery. This reduces synthesis of new proteins and prevents additional protein overload in the already compromised ER (Raven and Koromilas, 2008). Thirdly, increased proteasomal degradation via the ERAD pathway facilitates the removal of folding-incompetent or slowly-folding proteins, also helping to relieve protein overload in the ER (Eizirik *et al.*, 2008; Breckenridge *et al.*, 2003; Laybutt *et al.*, 2007; Araki *et al.*, 2003). Finally, a paradox of the UPR is that if prolonged or persistent ER stress continues unabated in cells and cannot be relieved by other compensatory UPR mechanisms, the initiation of apoptosis occurs (Qu *et al.*, 2009; Fonseca *et al.*, 2009; Scheuner and Kaufman, 2008). Apoptotic cell death is induced via transcription factors (TFs) that include activating transcription factor-6 (ATF-6), ATF-4, ATF-3 and their downstream target CHOP10 (explained more fully in Section 1.9). These TFs elude the effects of translation inhibition imposed by eIF2 α phosphorylation as they are further downstream in the signalling pathway (Scheuner and Kaufman, 2008; Okada *et al.*, 2002). Despite evidence clearly implicating a role for ER stress within β -cells in the pathogenesis of T2D, it is still unclear which of the activated ERS pathways are most critical in β -cell death, specifically with regard to β -cell exposure to LC-SFAs such as palmitate.

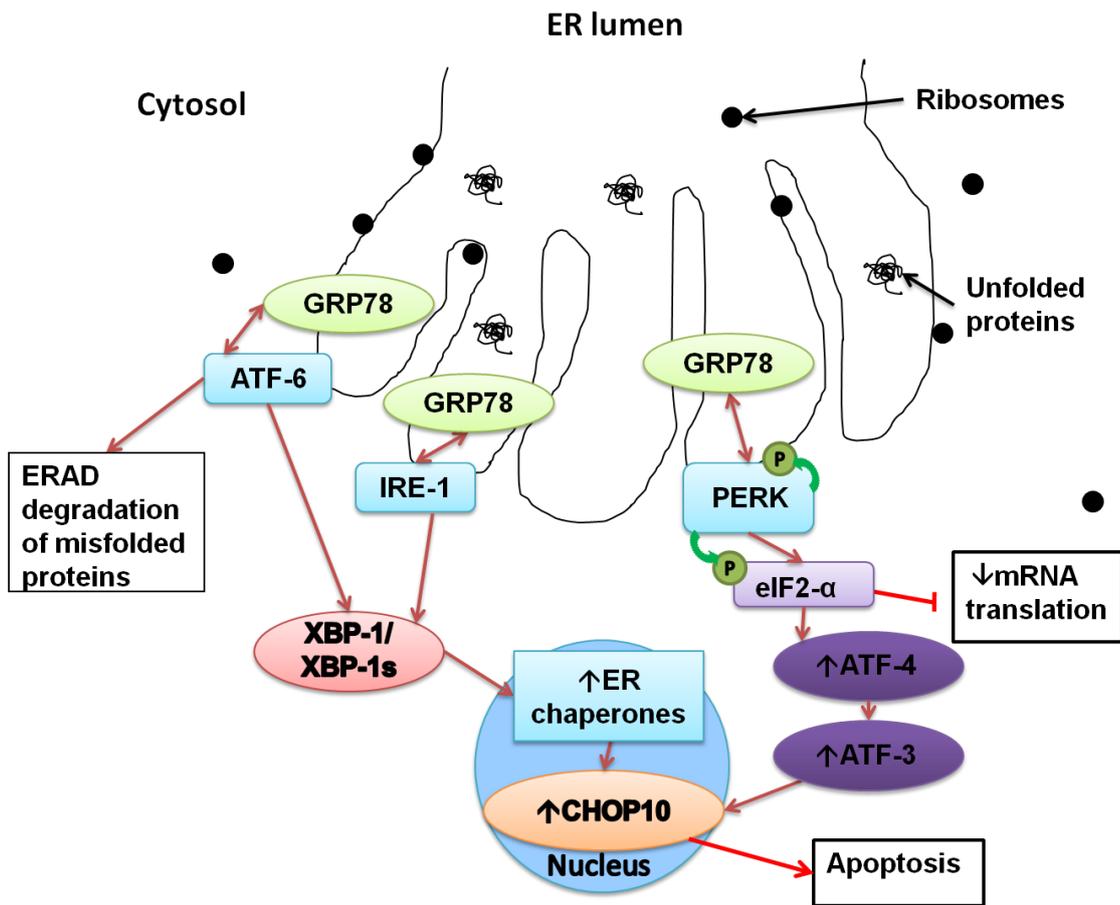


Fig. 1.3 The ERS pathway

The ERS consists of three ER-localised proteins that mediate stress responses: IRE-1, ATF-6 and PERK. All three proteins typically associate with the chaperone GRP78 under non-stress conditions, and are located within the ER lumen. However, during cell stress GRP78 dissociates from the ER lumen and interacts with intraluminal unfolded proteins, initiating downstream signalling cascades. ATF-6 facilitates upregulation of the GRP78/GRP94 chaperone system, which prevents a build-up of misfolded proteins and enhances folding capacity. ATF-6 also enhances the TF XBP-1 mRNA expression and directs degradation of misfolded proteins via the ERAD. IRE-1 principally cleaves XBP-1 which directs gene expression involved in protein folding, helping to reduce ER protein load. PERK auto-phosphorylation in turn phosphorylates eIF2 α , inhibiting mRNA and thereby protein translation. PERK also regulates the TFs ATF-4 and ATF-3 that can eventually trigger apoptosis during persistent stress conditions (schematic adapted from Breckenridge *et al.*, 2003).

1.8.2 Key players of the UPR

The IRE-1 α protein is expressed constitutively in most cells, with particularly high levels of expression in pancreatic tissue. Once activated, IRE-1 α acts as an endoribonuclease, cleaving the mRNA encoding X-box binding protein 1 (XBP-1), generating a spliced XBP-1 variant termed XBP-1s. XBP-1s is a potent TF for genes that are involved in ER protein maturation, folding and export, and targeted mRNA degradation, all of which contribute to reduced protein load in the ER (Breckenridge *et al.*, 2003; Cnop *et al.*, 2008). ATF-6 is an ER-bound, constitutively expressed basic leucine zipper (bZIP) type II transmembrane protein, involved in the upregulation of the GRP78/GRP94 chaperone system and also acts to augment XBP-1 mRNA expression (Eizirik *et al.*, 2008; Wek *et al.*, 2006). PERK is an ER-resident type I transmembrane kinase protein (Butler *et al.*, 2003) that recognises misfolded proteins in the ER, triggering its own activation via *trans*-autophosphorylation under stress conditions, and is proposed to upregulate in sequence: ATF-4, ATF-3 and CHOP10 (Lee *et al.*, 2007; Boyce and Yuan, 2006). Under non-stress conditions, each ER stress protein remains in an inactive state, bound and associated to GRP78 located within the ER lumen. However, GRP78 dissociates from the ER membrane to bind unfolded intraluminal proteins during cellular stress, thereby releasing the transducer proteins and consequently activating downstream ER signalling cascades (Cnop *et al.*, 2008; Qu *et al.*, 2009).

1.9 ERS apoptotic pathways

The exact mechanism whereby persistent ER stress eventually triggers β -cell death apoptosis is unclear, although so far there have been several individual apoptotic signalling pathways identified during ER stress conditions (Lee *et al.*,

2007). These involve the PERK/eIF2 α -dependent induction of CHOP10, Ca²⁺ release from the ER, IRE1 α -mediated activation of the cJUN NH₂-terminal kinase (JNK) pathway and cleavage and activation of caspase 12. Typically, CHOP10 is barely detectable under normal physiological conditions. It is strongly induced under prolonged stress conditions however, where translocation to the nucleus initiates proapoptotic factors that ultimately lead to the death of the cell (Scheuner and Kaufman, 2008). Targeted disruption or knockout of the CHOP10 gene can protect against ER stress conditions in both mice and β -cell models, by improving β -cell function and promoting cell survival (Song *et al.*, 2008; Zinszner *et al.*, 1998; Jiang and Wek, 2005; Marciniak *et al.*, 2004; Oyadomari and Mori, 2003).

Transcriptional activation of CHOP10 is mediated by the three upstream ERS proteins: IRE1 α , PERK and ATF6, although at present there is little known regarding potential downstream targets of CHOP10 in apoptosis. The JNK family is comprised of signal transduction proteins that regulate not only gene expression, but also survival or apoptotic responses in cells. The activated IRE1 α subunit recruits both tumour necrosis factor (TNF) receptor-associated factor (TRAF) 2 and apoptosis signal-regulating kinase (ASK) 1 proteins, forming an IRE1 α /TRAF2/ASK1 complex that triggers JNK activation. Caspase 12 activation has been demonstrated during ER stress in mice, and although it is also expressed in humans, its exact role is not yet clear. This third apoptotic pathway needs further research to clarify the associated molecular mechanisms, although it does not appear to involve either death receptor-mediated or mitochondria-targeted apoptotic signals (Araki *et al.*, 2003). Additionally, research shows that FFA induce activation of both JNK and Caspase 12 (Cunha *et al.*, 2008), however,

it is not yet clear which of these pathways or molecular mechanisms is the most important response under lipotoxic conditions (Lee *et al.*, 2007).

1.10 PERK: function and role in ER stress

The PERK protein has been extensively studied and is known to play a critical role in the proliferation and differentiation of pancreatic β -cells, as evidenced by PERK knockout mice (Herbert, 2007). PERK's role links ER stress to arrest of ER translation. It is a serine/threonine kinase that has long been proposed to directly phosphorylate eIF2 α under ER stress conditions (Kapoor and Sanyal, 2009), thus inhibiting global protein synthesis via regulation of selected mRNAs. Somewhat paradoxically, certain mRNAs such as ATF-4 (whose downstream targets include ATF-3 and CHOP10) continue to be translated when eIF2 α is phosphorylated (Kapoor and Sanyal, 2009; Wek *et al.*, 2006). PERK is typically held docked on the ER membrane via interaction with GRP78. It recognises misfolded proteins within the ER and inhibits protein translocation into the ER lumen, collectively reducing ER protein load during stress conditions (Butler *et al.*, 2003; Scheuner and Kaufman, 2008). ER stress stimuli trigger the release of PERK from its interaction with GRP78, allowing activation of the kinase via autophosphorylation (Lee *et al.*, 2007; Harding *et al.*, 2000; Ron, 2002).

A critical early step and one of the arms of the ERS, is the arrest of bulk protein synthesis, mediated by the phosphorylation of eIF2 α , thereby preventing additional protein overload in the already compromised ER. Exposure of SFA to pancreatic β -cell lines has been shown to result in augmentation of eIF2 α phosphorylation, followed by a marked increase in ATF-4 and subsequently CHOP10, both consistent with activation of this arm of the ER stress signalling

cascade (Diakogiannaki and Morgan, 2008; Diakogiannaki *et al.*, 2008; Eizirik *et al.*, 2008; Ito *et al.*, 1999; Qu *et al.*, 2009; Harding *et al.*, 2000; Herbert, 2007). Induced CHOP10 expression in certain pancreatic β -cell lines during palmitate exposure is also associated with apoptotic progression under the PERK-dependent arm of ER stress, as cited by many groups (Diakogiannaki and Morgan, 2008; Diakogiannaki *et al.*, 2008; Cnop *et al.*, 2008; Morgan *et al.*, 2008; Scheuner and Kaufman, 2008; Laybutt *et al.*, 2007; Song *et al.*, 2008). As such, activation of the PERK signalling cascade has been well established under ER stress conditions and to date, it has been generally accepted that the PERK protein is responsible for this eIF2 α phosphorylation step (Cunha *et al.*, 2008; Eizirik *et al.*, 2008; Qu *et al.*, 2009; Harding *et al.*, 2000; Herbert, 2007). However, new research now questions whether PERK is the sole factor underlying the progression to T2D as studies investigating PERK's involvement have produced somewhat conflicting results (Morgan, 2009; Lee *et al.*, 2007).

Diakogiannaki and colleagues (2008) was unable to observe consistent or full activation of PERK in β -cells exposed to palmitate, and although others have cited evidence of PERK activation in response to SFA, the extent of this activation is rather moderate (Laybutt *et al.*, 2007). Furthermore, others have noted that GRP78 levels remain unaltered during palmitate exposure: an unusual result given that upregulation of GRP78 transcription normally occurs as part of the collective ERS (Diakogiannaki *et al.*, 2008; Karaskov *et al.*, 2006; Diakogiannaki and Morgan, 2008). Studies have also suggested that GRP78 over-expression, which in theory should attenuate palmitate-induced ER stress, has a limited effect at best, or in some cases, has proven to be completely ineffective (Laybutt *et al.*, 2007; Morgan, 2009; Lai *et al.*, 2008). All considered, these results suggest that

PERK may have a less significant role in ER stress-mediated apoptosis than was previously thought.

1.11 PKR: structure and function

Protein kinase R (PKR) is a soluble serine/threonine kinase that is ubiquitously expressed and constitutively active at low levels in most cell types. Around 80% of PKR is distributed within the cytoplasm and mostly associated with ribosomes (Clemens, 1997). PKR has been implicated in many cellular processes such as signal transduction, apoptosis, tumour suppression and regulation of growth and differentiation (Patel and Sen, 1998), as well as ascribed functions in certain disease processes (Cole, 2007; Clemens, 1997; Lee *et al.*, 2007; Lemaire *et al.*, 2005; Sadler and Williams, 2007).

Both PKR and PERK belong to a small family of protein kinases, which respond to a variety of environmental stressors via phosphorylation of eIF2 α , and it has been noted that there is a degree of redundancy in their responses (Sadler and Williams, 2007). In addition to PKR (eIF2 α kinase 2) and PERK (eIF2 α kinase 3), there is also an eIF2 α kinase 4 (also known as the general control non-derepressible-2 kinase [GCN2]) which is induced under amino acid deprivation or UV irradiation conditions and eIF2 α kinase 1 (or haem-regulated inhibitor [HRI] kinase) which is activated by haem deprivation, oxidative stress or heat stress in erythroid tissue (Wek *et al.*, 2006). All of these kinases are able to phosphorylate eIF2 α , thus potentially acting to regulate the distal signalling cascade of the ERS. Wek and colleagues (2006) considered GCN2 an unlikely candidate kinase because although it does phosphorylate eIF2 α in response to nutrient deprivation or UV irradiation, it does not lead to CHOP10 upregulation, a mechanism which

has been closely linked with β -cell apoptosis during exposure to SFA (Morgan, 2009). It is with interest then, that recent work by Lee and colleagues (2007) highlighted the potential of PKR as an alternative candidate for eIF2 α phosphorylation and a driver of apoptosis under the ERS.

The human PKR gene encodes a 551 amino acid (513 in rat, 515 in mouse) protein which consists of two functionally distinct domains: an N-terminal regulatory dsRNA-binding domain (dsRBD) and a C-terminal catalytic kinase domain. The N-terminal dsRBD contains two dsRNA-binding motifs (dsRBM) of around 65 amino acid residues that adopt similar $\alpha\beta\beta\alpha$ folds. These dsRBMs are separated by a 20 amino acid linker, which is made up of a random coil conformation, suggested to offer plasticity so the RBD can optimally bind around a dsRNA helix (Feng *et al.*, 1992; Nanduri *et al.*, 2000) (Fig. 1.4).

PKR was initially discovered as an interferon-induced mediator of cellular antiviral responses (Sonenberg, 1990), where it is activated by ds-RNA, highlighting the critical role of PKR in the immune response to viral infection in higher eukaryotes. PKR is induced by type I interferons in a latent state. Upon binding of dsRNA, PKR undergoes autophosphorylation and activation, phosphorylating the α -subunit of eIF2 thereby inhibiting protein synthesis and viral replication in infected cells. PKR is typically activated by dsRNA with sequences of 30 base pairs or greater, however, it is known that shorter dsRNA sequences are also capable of activating PKR, although it is speculated that this may occur by a different mechanism (Cole, 2007; Samuel, 2001).

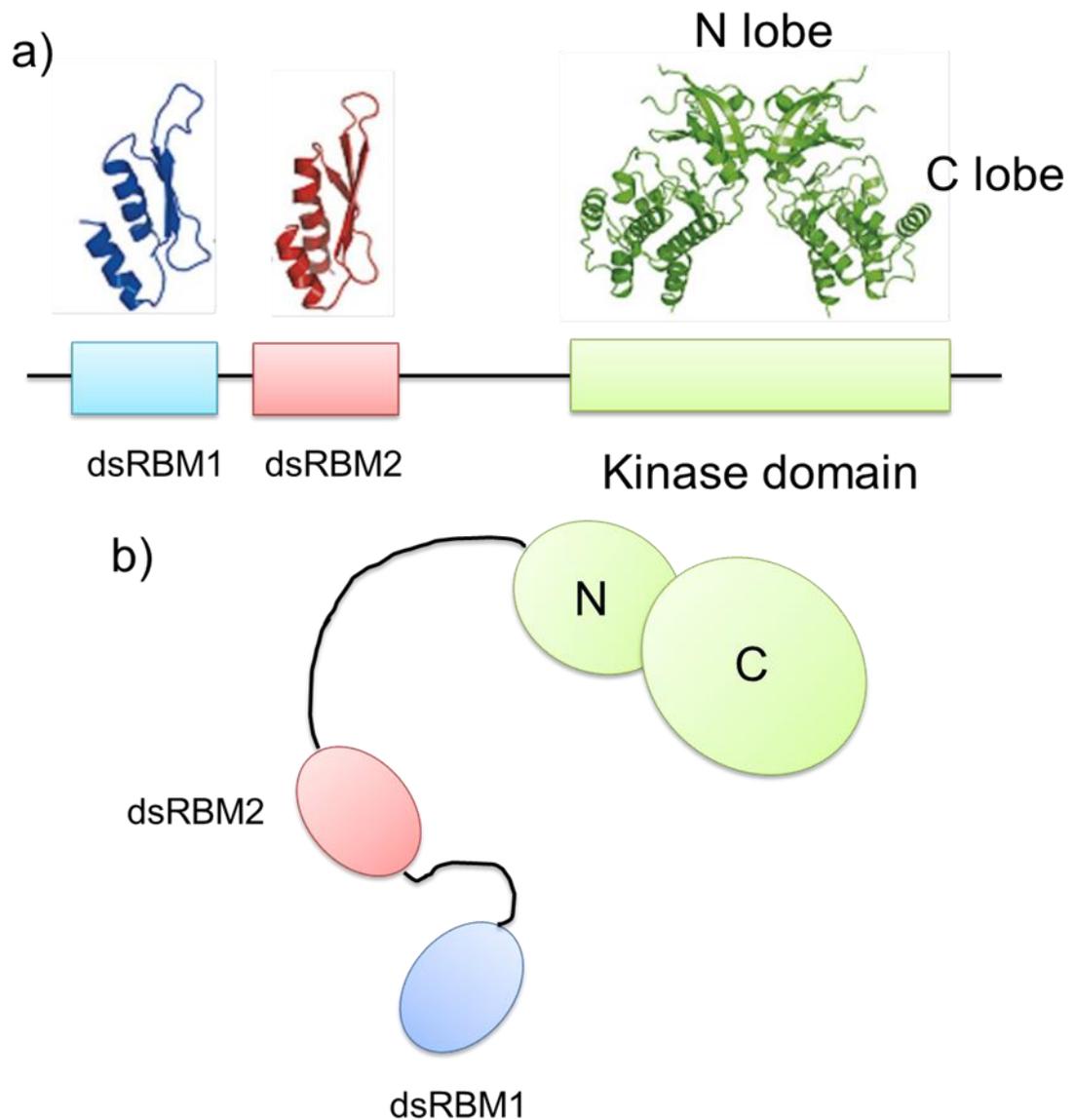


Fig. 1.4 PKR structural domain

a) PKR structural domain consists of an N-terminal regulatory domain comprising two dsRNA-binding motifs: dsRBM1 and dsRBM2, and a C-terminal kinase domain, as represented schematically in b) (schematic adapted from Cole, 2007).

1.11.1 Proposed models of PKR activation

Despite great interest in the PKR protein over the last couple of decades, a detailed molecular understanding of how PKR is activated is lacking. There are currently two suggested models for activation of PKR (Fig. 1.5). The catalytic domains of different protein kinases assume remarkably similar structures in their active state. In contrast, there is marked plasticity in these catalytic domains when the kinases are inactive. Tight regulation of protein kinases is critical, and intramolecular interactions between the catalytic and regulatory domains are often observed as part of this regulatory mechanism. This acts to inhibit kinase activity by allosterically inducing an inactive conformation, or by physically inhibiting substrate binding (Huse and Kuriyan, 2002). In the autoinhibition model (Fig. 1.5a)), evidence suggests that latent PKR exists in a closed formation, where the dsRBD interacts with and blocks the kinase domain, preventing its interaction with its substrate. Binding of dsRNA releases the kinase domain, thereby activating PKR (Nanduri *et al.*, 2000; Wu and Kaufamn, 1997; Bischoff and Samuel, 1985; Spanggord *et al.*, 2002; Dey *et al.*, 2005; Vattem *et al.*, 2001; Wu and Kaufman, 2004; Saelens *et al.*, 2001).

In the second, more favoured dimerisation model (Fig. 5b)), latent PKR is suggested to exist in a monomer-dimer equilibrium, where ATP can bind both enzyme forms. Double-stranded RNA binding induces and enhances PKR dimerisation via the catalytic domain and subsequent autophosphorylation, resulting in PKR activation (Robertson and Mathews, 1996; Lemaire *et al.*, 2005; Gabel *et al.*, 2006; Tian and Mathews, 2001). Biophysical and structural data do not support the physical blockade of the kinase active site by the dsRBD, as it has been demonstrated that the kinase active site is readily accessible in both

latent and activated PKR. Importantly, work has also demonstrated that latent PKR can be activated by dimerisation even in the absence of RNA (Lemaire *et al.*, 2005), contrary to the suggested mechanism of action proposed in the autoinhibition model. Nevertheless, the proposed dimer model for PKR activation is further complicated by the fact that PKR contains multiple phosphorylation sites and by evidence suggesting that PKR undergoes both *cis* and *trans* autophosphorylation when activated by dsRNA (Cole, 2007; Sadler and Williams, 2007; Lemaire *et al.*, 2005; Williams, 1999; Galabru and Hovanessian, 1997; Samuel, 2001).

1.11.2 PKR dsRNA-independent activatory mechanisms

Work has shown that PKR can also be activated by dsRNA-independent mechanisms (Proud, 1995), for example, in direct response to cellular stressors, which include lipid derivatives such as ceramide. This mechanism is mediated by way of a directly upstream regulatory molecule, named protein activator of PKR (PACT) (Patel and Sen, 1998; Ruvolo *et al.*, 2001; Sadler and Williams, 2007). Furthermore, recent work has demonstrated a pivotal role for PKR within nutrient- and pathogen sensing pathways, and as a mediator of inflammatory responses within metabolism (Nakamura *et al.*, 2010). This mechanism has for the first time, linked elevated levels of FFA with activation of PKR and β -cell death in metabolic disease (Shimabukuro *et al.*, 1998; Newsholme *et al.*, 2007; Wei *et al.*, 2006).

1.11.3 PKR as a proapoptotic molecule

To date, the best-characterised role of PKR is the inhibition of translation via eIF2 α phosphorylation. However, work published by Lee and colleagues (2007) established that PKR is simultaneously but independently activated alongside PERK under lipotoxic-mediated ER stress conditions. It was demonstrated that PERK phosphorylated around 50% of eIF2 α ; however, PKR was responsible for as much as 30 – 40% of eIF2 α phosphorylation. This study also suggested that PKR plays the predominant role in β -cell apoptotic mechanisms, by inducing proapoptotic downstream eIF2 α targets such as ATF4, ATF3 and CHOP10 (the p-eIF2 α /ATF4/CHOP10 apoptotic pathway). These findings support conclusions reached by other research groups, in that PERK and PKR appear to play different roles in lipotoxic conditions. PERK's principal role is suggested to influence pro-survival mechanisms by mediating translational arrest, but the role of PKR role

may mediate cell-death related mechanisms through the upregulation of the proapoptotic TF CHOP (Harding *et al.*, 2000; Cullinan and Diehl, 2004; Cullinan *et al.*, 2003; Lee *et al.*, 2007).

There is much additional evidence to support the idea of PKR as a proapoptotic molecule. For example, PKR has been found to become associated with ASK1 (mentioned previously in JNK activation as one of the ERS apoptotic pathways), STAT1 (signal transducer and activator of transcription) necessary for caspase-3 expression and also the tumour suppressor p53 (Sadler and Williams, 2007). More recent work has associated PKR activation with plaque formation in certain central nervous system disorders, such as Huntington's, Parkinson's and Alzheimer's diseases (Peel *et al.*, 2001; Peel and Bredesen, 2003). Notably, similar plaque formation is also known to be a characteristic feature of T2D (in the form of IAPP) (Potter *et al.*, 2009), thereby implying PKR may act as a potential pathogenic factor in T2D progression (Sadler and Williams, 2007). When considering this evidence, it is undoubtedly feasible that PERK may not be the most critical factor in mediating death in β -cells during lipotoxicity. Evidence clearly points to a role for PKR in general ERS and during β -cell exposure to elevated levels of SFAs. This mechanism is known to underlie the progression to T2D, suggesting that PKR may potentially mediate predominantly apoptotic cell signalling outcomes (Fig. 1.6).

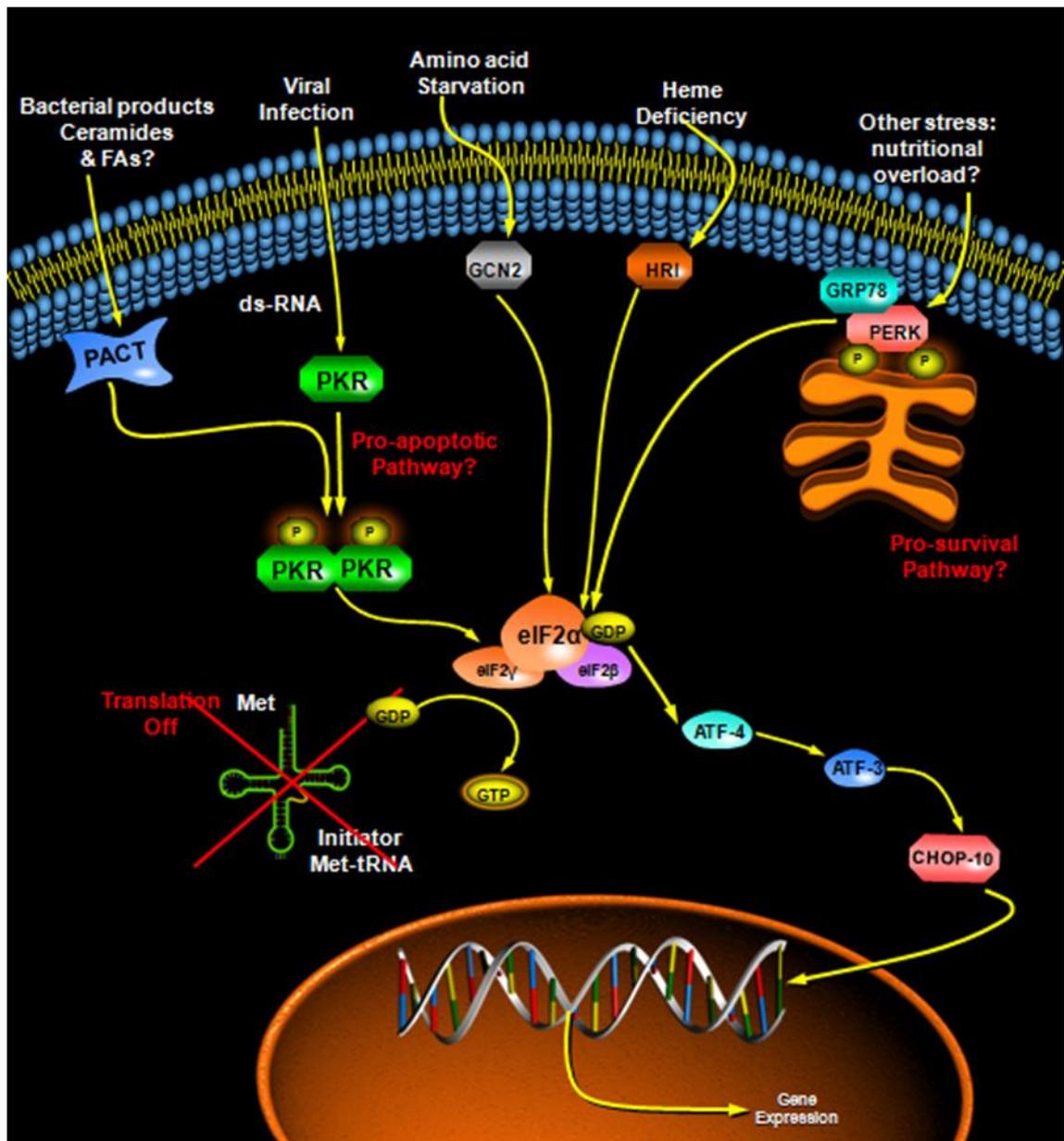


Fig. 1.6 Proposed PKR activity in stress response signalling pathways

PKR is induced by IFN- γ in response to dsRNA, and phosphorylates the α -subunit of eIF2, inhibiting global protein translation and preventing replication of virally infected cells. PKR is also activated by dsRNA-independent mechanisms: ceramide and FAs are suggested to activate PKR via the upstream regulator PACT, via a predominantly apoptotic pathway (schematic adapted from Protein Lounge, 2012).

1.12 Innate immunity and inflammation in obesity and T2D

Activation of the innate immune system was originally believed to occur solely in response to noxious insults, for example, microbial or physical stimuli. As part of this activation response and as a means of protection, inflammation is considered as the first line of defence to counter potential infection or tissue injury. However, it is now clear that a prolonged or chronic inflammatory response can be deleterious and exacerbate tissue damage. Interestingly, chronic inflammation has been recently implicated in the development and progression of certain diseases such as T2D, gout and rheumatoid arthritis (Dinarello, 2010). It is becoming apparent that under certain circumstances, the innate immune system can be activated in the absence of an exogenous trigger. As such, increasing evidence has pointed to a role for innate immunity as a custodian for sensing metabolic stress (Menu and Vince, 2011; Tannahill and O'Neill, 2011; Yang *et al.*, 2012; Ciraci *et al.*, 2012; Hotamisligil and Erbay, 2008; Kaufman *et al.*, 2002).

The innate immune system is thought to play a key pathogenic role in a variety of diseases where underlying chronic low-level inflammation is observed, such as insulin resistance, obesity and T2D, in a mechanism termed 'metainflammation'. Studies have identified a strong association between low-grade inflammatory markers such as circulating pro-inflammatory cytokines, chemokines and acute phase proteins, and metabolic dysfunction (Calle and Fernandez, 2012; Donath, 2011; Lavelle, 2008; Hotamisligil, 2006). Chronic systemic inflammation observed in obesity and T2D is a well-established factor contributing to the upregulation of SOCS (suppressor of cytokine signalling) proteins, which have been recognised to reduce insulin receptor signalling, thereby conceivably driving the progression of insulin resistance within metabolic

dysfunction (Ueki *et al.*, 2004). Chronic nutrient overload is now suggested to result in a sterile inflammatory process, otherwise termed an autoinflammatory response. Furthermore, evidence has shown that metabolic stress can elicit an autoinflammatory reaction specifically in pancreatic islets (Böni-Schnetzler *et al.*, 2009; Dinarello *et al.*, 2010; Donath *et al.*, 2009; Maedler *et al.*, 2002; Masters *et al.*, 2010; Masters *et al.*, 2011). Consequently, there has been great interest in the suggestion that inflammation may well be a common link between metabolic diseases and the driving factors underlying their pathogenesis (Böni-Schnetzler *et al.*, 2009; Donath, 2011; Donath and Shoelson, 2011; LaValle, 2008; Masters *et al.*, 2011; Wen *et al.*, 2012).

1.13 The link between ER stress, inflammation and metabolic disease

With the prevalence of obesity reaching epidemic proportions, it is becoming increasingly important to understand how excess adiposity driven by chronic nutrient excess can affect metabolic homeostasis. It is becoming clear that within this metabolically driven dysfunctional state, ER stress and activation of an ERS are critical players (Hummasti and Hotamisligil, 2012). Sequestration of excess FFAs as TG is a principal metabolic event in response to nutrient surplus: TG is typically deposited in adipocytes, but chronic calorific overload can drive TG deposition in other tissues, which can result in lipotoxicity. Beyond a critical threshold, cells begin to exhibit signs of stress, such as disrupted mitochondrial function, altered lipid, cytokine and adipokine signalling, hypoxia, production of reactive oxygen species (ROS), inflammation and initiation of ER stress resulting in apoptosis (Gregor and Hotamisligil, 2007; Hotamisligil, 2010; Cnop *et al.*, 2011).

It is becoming apparent that many of these mechanisms are interconnected and can influence the function of each other (Hotamisligil, 2010; Cao *et al.*, 2008). Metabolism, the immune system, inflammation and ER stress are tightly regulated adaptive responses that are absolutely critical to survival, and they are dependent on the availability of adequate energy to function properly. It is therefore logical that there is extensive integration and communication between these energy-requiring mechanisms. Equally this also links dysfunctional immune responses such as autoinflammation and ER signalling with metabolic disease (Hummasti and Hotamisligil, 2012; Wellen and Hotamisligil, 2005; Kaufman *et al.*, 2002; Hotamisligil and Erbay, 2008).

1.14 Inflammation and the role of IL-1 β

At the centre of the autoinflammatory response driven by metabolic dysfunction, activation of the IL-1 β pathway is critical (Böni-Schnetzler *et al.*, 2009; Dinarello *et al.*, 2010; Donath *et al.*, 2009; Maedler *et al.*, 2002; Masters *et al.*, 2010; Masters *et al.*, 2011). It has been established that the production and release of IL-1 β is not only triggered by exogenous infectious danger signals, such as bacterial lipopolysaccharide (LPS) or viral ds-RNA, but also by endogenous danger signals such as ATP or DNA released from metabolically stressed or dying cells (Goldbach-Mansky, 2012). IL-1 β is known as the archetypal 'endogenous pyrogen' (Ehnes *et al.*, 2009; Larsen *et al.*, 2007; Sauter *et al.*, 2008) and as such, it is highly regulated at all levels of production and secretion. Pro-inflammatory cytokine secretion results in the activation of innate immune system Toll-like receptors (TLRs) or nucleotide-binding domain (NOD)-like leucine-rich repeats (NLRs). These receptors in turn induce activation of NF- κ B and subsequent upregulation of pro-IL-1 β (Ozkurede and Franchi, 2012).

A role for IL-1 β in diabetes was first identified when it was established that this cytokine was toxic to pancreatic β -cells (Bendtzen *et al.*, 1986). Although pancreatic β -cell loss occurs at a much later stage in T2D than when compared with β -cell loss in T1D, there are arguments that link IL-1 β involvement with both diseases (Dinarello *et al.*, 2010). Studies have demonstrated that pancreatic islet exposure to IL-1 β either alone, or in combination with other pro-inflammatory cytokines such as IFN- γ or TNF- α , can trigger impaired β -cell function that is progressive and may eventually lead to β -cell apoptosis (Eizirik and Mandrup-Poulsen, 2001). IL-1 β has been shown to activate the c-jun N-terminal kinase (JNK) pathway, which constitutes a stress-activated member of the mitogen activated protein kinase (MAPK) family of threonine/serine kinases. These participate in stress and apoptotic signalling in many cells, and as such play a critical role in cytokine-mediated apoptosis in pancreatic β -cells, as suggested by the prevention of apoptosis by inhibition of the JNK pathway (Ammendrup *et al.*, 2000). Within the scope of this signalling pathway, both animal and clinical studies have demonstrated that inhibition of IL-1 β secretion can prevent islet inflammation, and in doing so can improve insulin release and glycaemic control. This further argues for inflammation as a critical etiological factor in both obesity and T2D (Ehnes *et al.*, 2009; Larsen *et al.*, 2007; Sauter *et al.*, 2008).

Studies have shown that IL-1 β has a closely linked role with glucose metabolism, where IL-1 β elicits potent effects on decreasing glucose uptake, potentially contributing to insulin resistance (del Ray and Besedovsky, 1987; Ling *et al.*, 1994). Since these studies, further work has demonstrated that the presence of hyperglycaemia, elevated FFAs and human IAPP can induce IL-1 β secretion in β -cells, consequently launching a proinflammatory cytokine response. This may

initiate metabolic dysregulation which may then impair insulin secretion (Wen *et al.*, 2012; Vandanmagsar *et al.*, 2011; Wen *et al.*, 2011; Masters *et al.*, 2012; Zhou *et al.*, 2010; Westermark *et al.*, 2011).

1.15 IL-1 β and the inflammasome

The processing of pro-IL-1 β to its mature active form of IL-1 β is now known to be mediated by inflammasome complexes (Fig. 1.7). Studies have recently provided evidence that in addition to inducing IL-1 β secretion, factors such as hyperglycaemia and IAPP can activate inflammasomes in the pancreatic β -cell (Böni-Schnetzler *et al.*, 2009; Dinarello *et al.*, 2009; Donath *et al.*, 2009). Equally, FFAs and ceramides have been demonstrated to activate inflammasomes in adipose tissue, further integrating mechanisms that underlie metabolic disease with low-grade inflammation (Böni-Schnetzler *et al.*, 2009; Dinarello, 2010; Donath *et al.*, 2009; Donath *et al.*, 2010; Maedler *et al.*, 2002; Masters *et al.*, 2010; Masters *et al.*, 2011). There are three distinct steps required for the secretion of the mature form of IL-1 β . Firstly, cell activation by TLRs in response to exogenous or endogenous danger signals as described previously, leads to the transcription of pro-IL-1 β . This is followed by cleavage of pro-IL-1 β into the biologically active IL-1 β form by the caspase-1 complex, a critical step involving the inflammasome. Finally, mature IL-1 β is secreted, and the inflammasome is thought to also be instrumental in initiating this secretory step (Mankan *et al.*, 2012).

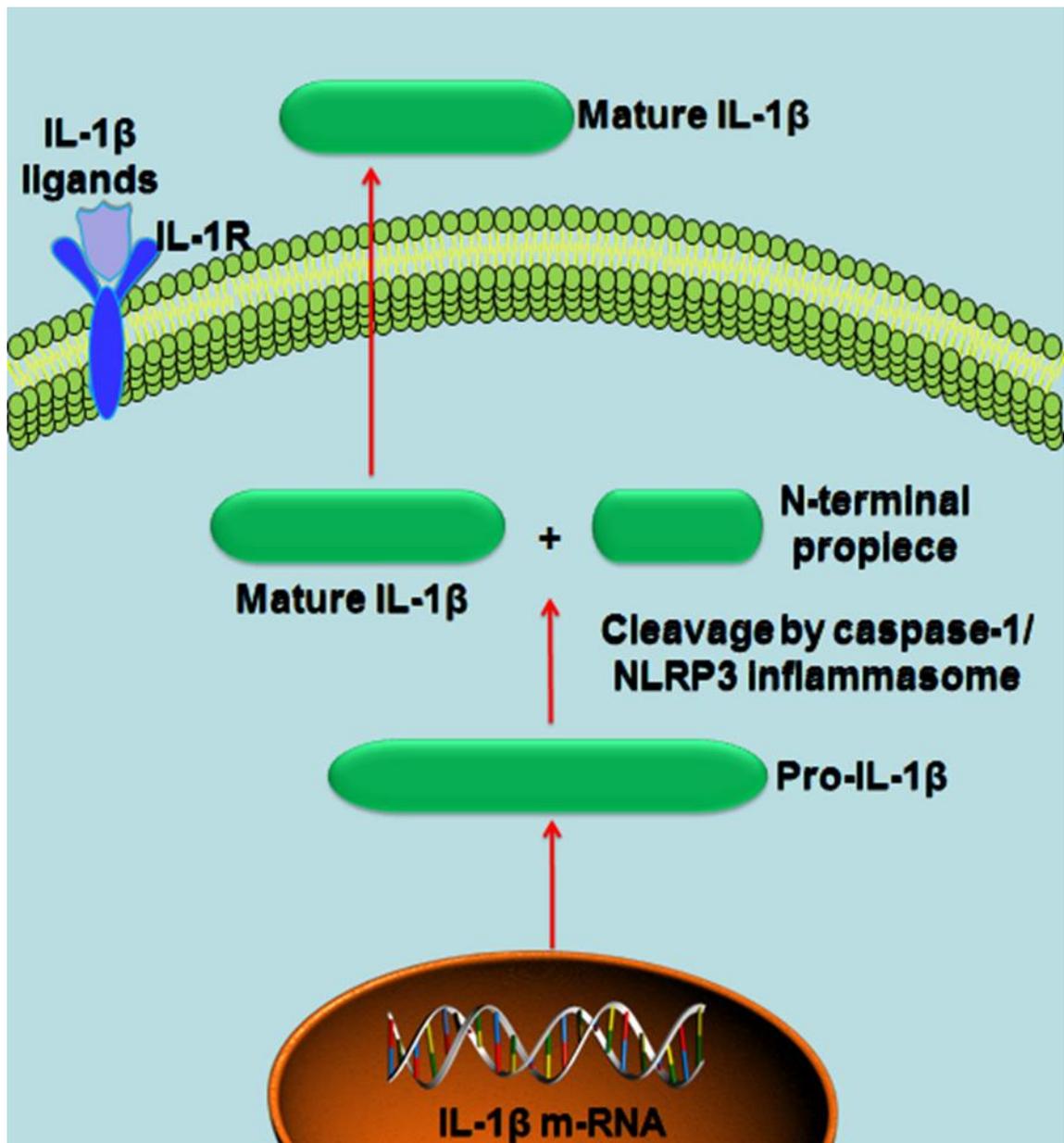


Fig. 1.7 Processing of pro-IL-1 β to mature IL-1 β

Secretion of IL-1 β is triggered by TLR via activation by exogenous or endogenous signals. Transcription of pro-IL-1 β is followed by a cleavage step involving caspase-1 and other components of the inflammasome complex, leading to the biologically active IL-1 β form which is then secreted (schematic adapted from Protein Lounge, 2012 and Gabay & Palmer, 2010).

1.16 The role of adipose tissue in inflammation

Adipose tissue is known to be a major source of proinflammatory cytokines such as IL-1 β , IFN- γ and TNF- α under conditions of stress. Studies have demonstrated that obese individuals have higher numbers of infiltrating macrophages within their adipose tissue compared to non-obese controls, which can trigger inflammatory signalling pathways (Masters *et al.*, 2011; Stienstra *et al.*, 2010; Vandanmagsar *et al.*, 2011; Weisberg *et al.*, 2003; Wen *et al.*, 2011). Infiltrating macrophages are the primary source of TNF- α in adipose tissue, and research has established that this cytokine is secreted at a higher level in obese patients than in their lean counterparts (Kern *et al.*, 1995). TNF- α can promote inflammation via an acute local inflammatory response, with the primary aim of curtailing infection, which in this capacity is beneficial. However, if chronic TNF- α activation occurs as seen in obesity, a detrimental effect on glucose metabolism is observed. The mechanisms by which TNF- α alters glucose metabolism is by altering insulin sensitivity through the attenuation of insulin receptor signalling, by decreasing the glucose transporter GLUT-4 in adipocytes and by the suppression of adiponectin, an important modulator of glucose regulation and fatty acid catabolism (Stephens *et al.*, 1997; Wang and Trayhurn, 2006).

1.17 Inflammasome activation by the innate immune system

In part, activation of the innate immune system is achieved by the recognition of highly conserved pathogen-associated molecular patterns (PAMPs), by means of a small number of receptors known as pattern-recognition receptors (PRRs) (Tannahill and O'Neill, 2011). There are four major PRR families that can independently or co-operatively detect danger signals. These can be broadly characterised into membrane-associated PRRs, namely the TLRs and C-type

lectin receptors (CLRs), and cytosolic PRRs comprised of the NLRs and the retinoic-acid-inducible protein (RIG)-like receptors (RLRs) (Mankan *et al.*, 2012).

Both TLRs and NLRs have been implicated in initiating downstream signalling pathways that can trigger pro-inflammatory responses observed in metabolic dysfunction (Tannahill and O'Neill, 2011). Studies have shown that SFAs can induce a TLR4-induced inflammatory response and obesity-associated insulin resistance (Shi *et al.*, 2006), and a loss of TLR4 function can partially protect against this (Saber *et al.*, 2009).

Toll-like receptors recognise particular molecular components of viruses, bacteria, fungi and parasitic protozoa. Of the 12 TLR members identified in mammals to date, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the surface of various immune cells, whereas TLR3, TLR7, TLR8 and TLR9 are expressed intracellularly, on compartments such as the ER or endosomes. Toll-like receptors are all type 1 transmembrane receptors, characterised by an extracellular leucine rich repeat (LRR) domain, and an intracellular Toll-IL1 receptor-Resistance (TIR) domain. The TIR domain is essential for adaptor molecule recruitment necessary to activate downstream signalling pathways, ultimately leading to the transcription of pro-inflammatory cytokines such as IL-1 β and IL-18 (Tannahill and O'Neill, 2011). The NLRs are activated by a plethora of microbial stimuli, such as bacterial RNA, the double-stranded analog poly I:C, LPS and microbial peptide. However, NLRs are also associated with the recognition of endogenous microbial danger signals, produced during cellular stress or damage, known as danger-associated molecular patterns (DAMPs). These include cholesterol, amyloid deposits and FAs (Franchi *et al.*, 2012;

Mankan *et al.*, 2012; Tannahill and O'Neill, 2011). Activation of NLR subsets drives the assembly of multimolecular inflammasome complexes, which mobilise into a platform allowing activation of proinflammatory caspase-1. It is noteworthy that active caspase 1 has also been shown to be necessary for the production of the mature form of IL-1 β , further pulling together underlying links between inflammation, obesity and T2D. Six inflammasomes have been identified thus far to date: NLRP1, NLRP3, NLRP6, NLRC4, Pyrin and AIM2. However, of particular interest to obesity and T2D is the NLRP3 inflammasome (Tannahill and O'Neill, 2011; Mankan *et al.*, 2012).

1.18 Nod-like receptors: inflammasome-forming complexes

The NLRs are the largest group of inflammasome-forming complexes. In humans, these proteins are characterised by three structural domains (Fig. 1.8). The first domain consists of an N-terminal effector domain, which may be a pyrin domain (PYD) (belonging to the NLRP family), a caspase recruitment domain (CARD) (belonging to the NLRC domain) or a baculovirus inhibitor of apoptosis protein repeat (BIR) domain (belonging to the NLRB family) (Ting *et al.*, 2008). Next follows the intermediate NACHT (nucleotide binding and oligomerization domain [NOD]) domain, a component critical for NLR activation by oligomerization and thereby, formation of the core inflammasome structure. The final component is the C-terminal leucine-rich repeat (LRR) domain, suggested to confer specificity and adopt a regulatory role in inflammasome activation. It is not clear if direct ligand interaction occurs, or if intermediary ligands are necessary (Mankan *et al.*, 2012).

NLRP3 Inflammasome complex

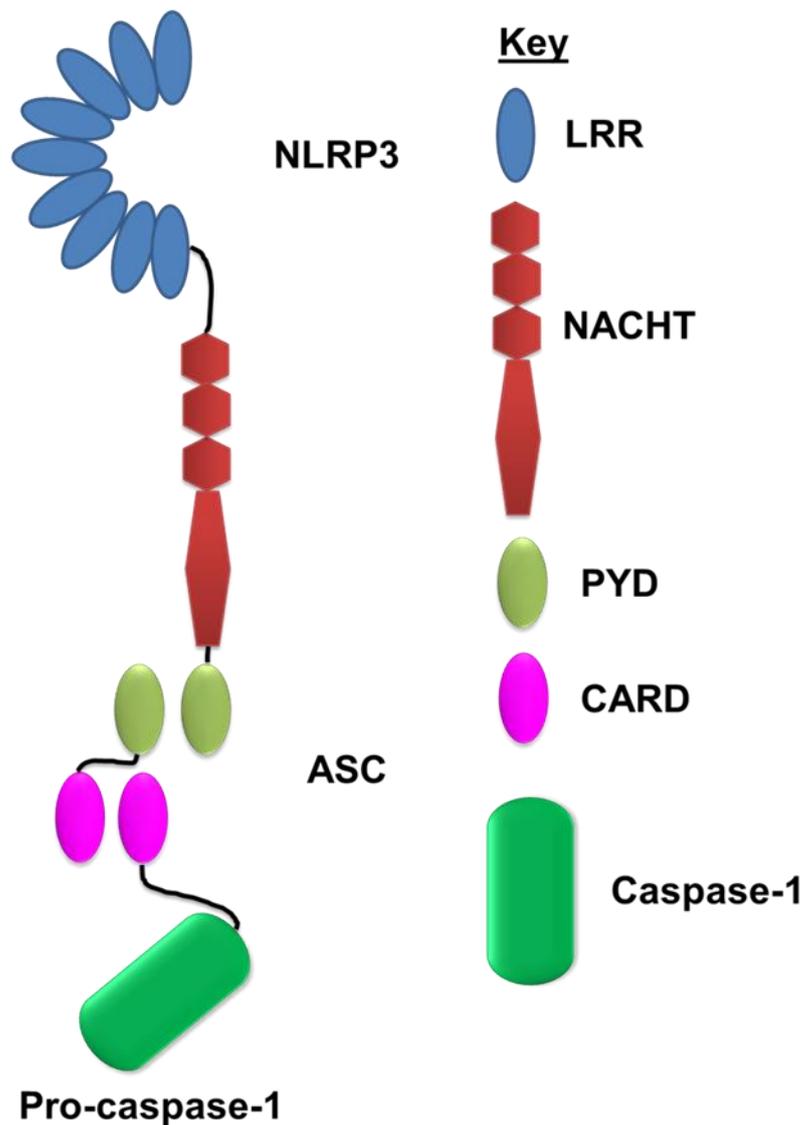


Fig. 1.8 Structural components of the NLRP3 inflammasome complex

The NLRP3 inflammasome is composed of three distinct structural domains: an adaptor protein ASC domain composed of a C-terminal caspase recruitment domain (CARD) and an N-terminal pyrin (PYD) domain belonging to the NLRP family and involved in procaspase-1/caspase-1 interactions; an intermediate nucleotide binding and oligomerization domain (NOD) domain (NACHT), critical for the formation of the core inflammasome structure, and a final C-terminal leucine rich repeat (LRR) domain involved in specificity and regulatory processes (schematic adapted from Cole, 2007).

1.19 Procaspase-1 in inflammasome activation

Procaspase-1 activation is a key downstream consequence of inflammasome activation. In the case of the NLRP3 inflammasome, upon activation the multimolecular structure is believed to directly recruit pro-caspase 1 via a homotypic CARD interaction, or by indirectly recruiting pro-caspase-1 via the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD). ASC has an unusual domain architecture with two death domain superfamily domains: an N-terminal pyrin domain (PYD or DAPIN [domain in apoptosis and interferon response]) and a C-terminal CARD domain. Both domains are involved in protein-protein complex formation via homotypic PYD-PYD or CARD-CARD domain interactions (Richards *et al.*, 2001). The ASC CARD domain interacts with procaspase-1, leading to its activation and processing, which thereby induces subsequent pro-IL-1 β maturation (Srinivasula *et al.*, 2002; Wang *et al.*, 2002; Mankan *et al.*, 2012).

1.20 The NLRP3 inflammasome

NLRP3 has been the most extensively studied inflammasome, and it is unique in the fact that it is the only known inflammasome that can be activated by non-microbial stimuli (Mankan *et al.*, 2012; Franchi *et al.*, 2012). In contrast to other inflammasomes, NLRP3 activation is very tightly regulated to avoid an injurious inflammatory response involving an additional regulatory stage at the transcriptional level (Bauernfeind *et al.*, 2011; Bauernfeind *et al.*, 2009). Also contrary to the other inflammasomes, NLRP3 is endogenously expressed at low levels under resting conditions. A proinflammatory signal is required to induce NLRP3 expression to a high enough level whereby activation is possible. This means that NLRP3 activation requires two distinct steps: firstly, a commonly

called 'priming signal', where the level of NLRP3 expression is amplified, followed by a second 'activation signal' which promotes construction of the multiprotein inflammasome complex (Mankan *et al.*, 2012). Endogenous cytokines such as IL-1 β are sufficient to provide 'signal 1' activation of the NLRP3 inflammasome, whereas 'signal 2' can be in the form of both microbial or non-microbial stimuli (Fig. 1.9) (Harder *et al.*, 2009; Muñoz-Planillo *et al.*, 2009). To date, a vast array of NLRP3 'signal 2' activators have been identified, and it is suggested that this high promiscuity is likely because NLRP3 activation represents a universally broad response mechanism to disturbances in cell integrity. Despite extensive studies, no common upstream molecules have yet been identified which could shed light on this extremely adaptable sensing by the NLRP3 inflammasome (Mankan *et al.*, 2012).

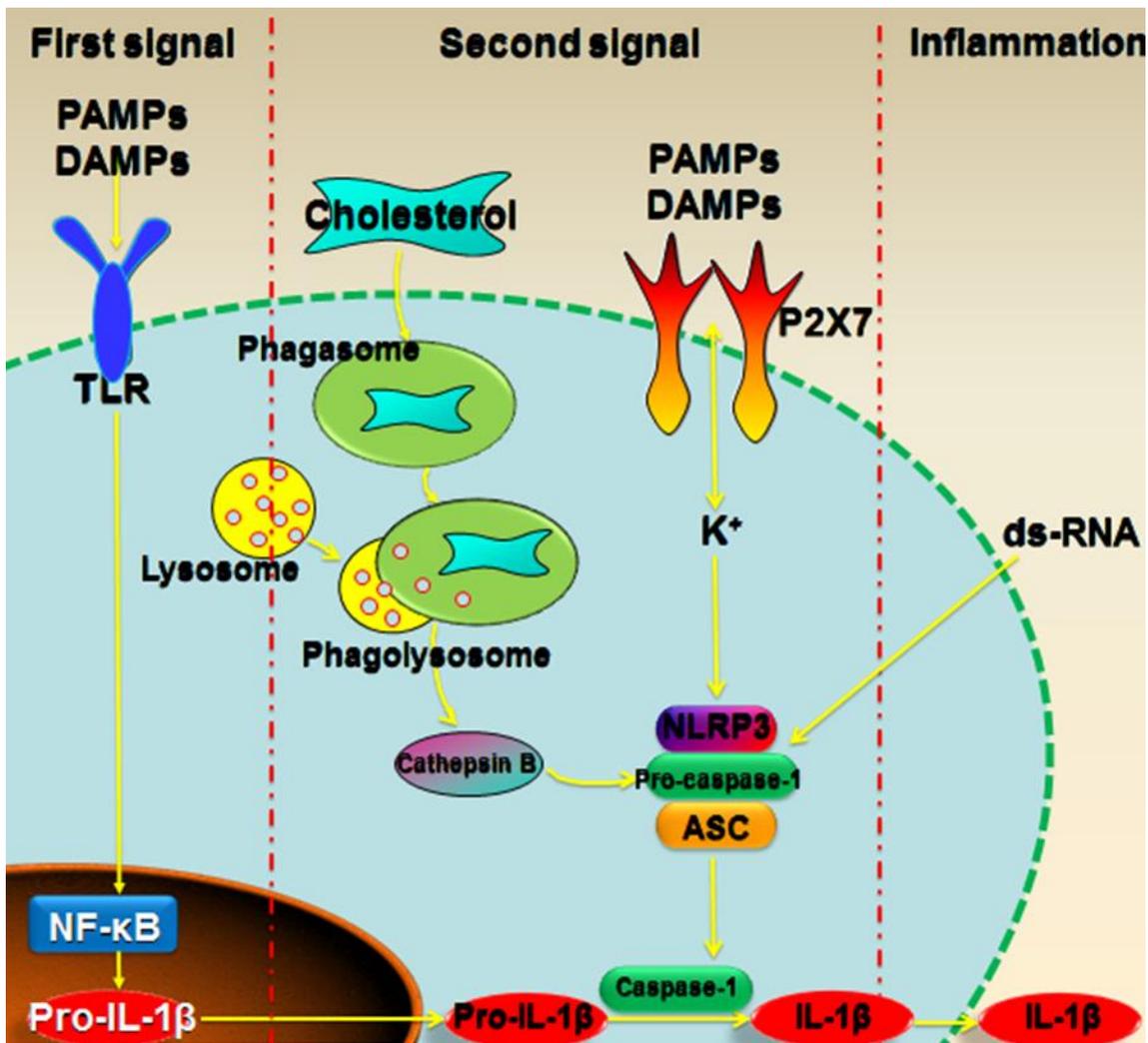


Fig. 1.9 Proposed model of NLRP3 inflammasome involvement in IL-1 β activation.

There are three steps required for the secretion of mature IL-1 β . A two-pronged initiation step by exogenous or endogenous pathogen-associated or danger-associated signals, is suggested to activate the innate immune system via NOD-like or Toll-like receptors. This triggers the initiation of pro-inflammatory TF such as NF- κ B which drives transcription of pro-IL-1 β . Cleavage of pro-IL-1 β into mature IL-1 β is mediated via caspase-1, which is cleaved into its mature form by the NLRP3 inflammasome (schematic adapted from Cole, 2007).

1.21 Proposed mechanisms of NLRP3 activation

Currently, there are three possible mechanisms to explain NLRP3 inflammasome activation: lysosomal breakdown and release of phagocytosed matter; increased ROS production in mitochondrial membranes, and potassium flux by membrane channels. It is possible that these processes act in concert, or they may be sequentially involved in activatory mechanisms (Mankan *et al.*, 2012).

1.21.1 Lysosomal breakdown and phagocytosis

During macrophage phagocytosis, initiation of numerous enzymatic cascades takes place to allow the safe degradation of exogenous material within the lysosomal compartments. However, failure of the proper phagocytic mechanism can lead to lysosomal membrane breakdown. This can result in the leakage of lysosomal contents into the cytoplasm, triggering activation of the NLRP3 inflammasome. It has been shown that inhibition of lysosomal enzymes, in particular cathepsin B, prevented lysosomal rupture and NLRP3 inflammasome activation (Hornung *et al.*, 2008; Halle *et al.*, 2008). However, the addition of a chemical compound that can disrupt lysosomes and thereby the process of phagocytosis did not impede inflammasome activation. This suggests that phagocytosis *per se* is not required to initiate the inflammasome. Additionally, known activators of the NLRP3 inflammasome were not blocked by cathepsin B inhibitors or other lysosomal inhibitors (for example, Bafilomycin A), suggesting this upstream pathway is independent from NLRP3 activation. Furthermore, cathepsin B deficiency appeared to only partially affect NLRP3 activation in response to uptake of phagocytic material. This suggests that there may be a level of redundancy in upstream mechanisms of NLRP3 activation (Banerjee and Saxena, 2012; Halle *et al.*, 2008).

1.21.2 ROS and TXNIP

A link between ROS production and procaspase-1 activation via NLRP3-linked activation has been suggested. Macrophages treated with ATP display elevated ROS production and this initiated phosphatidylinositol 3-kinase (PI3K) activation, in turn triggering procaspase-1 activation and IL-1 β production (Cruz *et al.*, 2007). More recently, TXNIP (thioredoxin (TRX)-interacting protein), a protein involved in the anti-oxidant function of thioredoxin, has been shown to interact directly with NLRP3 in a ROS-sensitive manner (Zhou *et al.*, 2010). TXNIP is the single most highly upregulated transcript under hyperglycaemic conditions in human pancreatic islets (Shalev *et al.*, 2002). The potential for TXNIP being a direct ligand for the NLRP3 inflammasome suggests a mechanism whereby hyperglycaemia may activate the inflammasome, leading to increased IL-1 β levels as observed in T2D. Elevated ROS levels cause a conformational alteration in TXNIP, thereby triggering its dissociation from thioredoxin and subsequent association with NLRP3.

The ROS model has been lately refined however, after studies demonstrated that mitochondria, not phagosomes, were the primary source of ROS production during inflammasome activation. This corresponds with studies that have observed mitochondrial dysfunction in β -cells during T2D (Lowell and Shulman, 2005; Miani *et al.*, 2012; Popov, 2012). ROS inhibitors potently block proinflammatory gene expression, including the NLRP3 inflammasome (Martin *et al.*, 2012). Although NLRP3 is a unique requirement for inflammasome priming (Bauernfiend *et al.*, 2011), the specific blocking activity of ROS inhibitors does not automatically exclude a role for ROS in NLRP3 activation. It may suggest

that ROS mechanisms act upstream of NLRP3 activation, rather than ROS themselves causing direct activation (Yang *et al.*, 2012; Petrilli *et al.*, 2007).

1.21.3 Potassium flux

Extracellular concentrations of ATP may be considered as both endogenous and exogenous danger signals, and may occur due to cellular damage (Paustian *et al.*, 2013). High extracellular ATP concentrations are a potent trigger for IL-1 β processing and release. ATP-triggered activation involves cytosolic potassium efflux, and the purinergic receptors P2X7 are critical for this activation step (Wahli and Michalik, 2012). P2X7 receptor activation causes recruitment of the hemichannel protein Pannexin-1, and this has been suggested to cause the formation of large, non-selective pores which allow the passage of signalling molecules into the cytosol (Miani *et al.*, 2012; Saberi *et al.*, 2009; Shi *et al.*, 2006). However, recent studies suggest that the main role for Pannexin-1 is to release ATP from dying cells. Inhibiting NLRP3 activation can typically be achieved by increasing the extracellular potassium concentration which effectively blocks K⁺ efflux, however, other inflammasomes can also be affected by this mechanism, raising the question of specificity (Arlehamn *et al.*, 2012). Furthermore, these experiments did not address whether potassium efflux was sufficient, or required amongst additional events for NLRP3 inflammasome activation.

1.22 Pancreatic β -cell models used in Diabetes Mellitus research

There may well be a place for animal models but the 3 R's of animal testing: reduction, refinement and replacement, essentially mean that the use of *in vitro* cell models are a preferable replacement to animal models in diabetes research where-ever possible. Cell models are more readily available than animal models with far less legal regulation. As such, they represent a convenient option for studying physiological and pathophysiological processes, and are useful in the development of novel pharmacological targets. Cell lines are cheaper and easier to work with, and they provide the opportunity to acquire faster results that can often be more consistent and reproducible than working with animals due to their natural variability (Skelin *et al.*, 2010).

Over the last few decades, a variety of pancreatic β -cell lines have been established as possible models in which to investigate pancreatic β -cell function. Many have been rodent cell lines, which have provided a valuable insight into functional and molecular studies of rodent islets (Table 1.2) (Asfari *et al.*, 1992; Gazdar *et al.*, 1980; Santerre *et al.*, 1981; McClenaghan *et al.*, 1996; Efrat *et al.*, 1988; Miyazaki *et al.*, 1990).

However, despite the usefulness of rodent cell models, there are of course many differences between rodent and human pancreatic β -cells. Any key findings in rodent cell lines would still need to be confirmed in primary islets and as these have limited availability, much effort has been put into trying to develop human pancreatic β -cell lines from human pancreatic sources (Hohmeier and Newgard, 2004). These sources have included adult islets, foetal pancreases or

insulinomas. Unfortunately, functional problems with these sources have included low insulin secretion, or cells only capable of secreting insulin over a few passages. In the case of adult pancreatic β -cells, supplementation with factors to maintain and promote growth also induces their dedifferentiation (Dufayet de la Tour *et al.*, 2001; Demeterco *et al.*, 2002; Gueli *et al.*, 1987; Levine *et al.*, 1995; Beattie *et al.*, 1999; Russ *et al.*, 2008).

Cell line	Cell origin	Species	Method	Notes	Refs
HIT	Insulinoma	Hamster	SV40 T-antigen transfected	Low insulin content; only HIT-15 responds to glucose.	Santerre et al, 1981
MIN 6	Insulinoma	Mouse	SV40 T-antigen transgenic mouse	Express glucokinase and GLUT-2; nicotinamide makes them glucose responsive.	Ishihara et al, 1993
β TC1	Insulinoma	Mouse	SV40 T-antigen transgenic mouse	Secretes proinsulin and insulin; has hexokinase activity, not responsive to glucose.	Efrat et al, 1988
BRIN-BD11	Insulinoma and normal β -cells	Rat	Electrofusion derived	Expresses glucokinase and GLUT-2; also express glucokinase.	McClenaghan and Flatt, 1999
INS-1	Insulinoma	Rat	Radiation induced	Needs 2-mercaptoethanol during culture.	Asfari et al, 1992
RINm	Insulinoma	Rat	Radiation induced	Insulin content decreases with passage; secretes somatostatin, not responsive to glucose.	Gazder et al, 1980
RINm5F	Insulinoma	Rat	Radiation induced	Inappropriate sensitivity to glucose with abnormal glucose transport.	Gazder et al, 1980
RINr	Insulinoma	Rat	Radiation induced	Inappropriate sensitivity to glucose with abnormal glucose transport.	Gazder et al, 1980
IgSV195	Insulinoma	Mouse	SV40 T-antigen transgenic mouse	Needs supplementation for insulin secretion and not responsive to glucose.	Gilligan et al, 1989
β HC	Hyperplastic islets	Mouse	SV40 T-antigen transgenic mouse	β HC9 express GLUT-2 and glucokinase., and responds to glucose. High hexokinase activity develops.	Radvanyi et al, 1993
NIT-1	Insulinoma	Mouse	NOD/Lt mouse, SV40 T-antigen	High insulin mRNA levels, not responsive to glucose.	Hamaguchi et al, 1991
CRI-G1	Insulinoma	Rat	Radiation induced	Release glucagon in parallel to insulin, not responsive to glucose.	Carrington et al, 1986
In-111	Insulinoma	Rat	BK-virus induced	Not responsive to glucose.	Uchida et al, 1979
CM	Insulinoma	Human	From ascitic fluid of a patient with liver metastasis of malignant insulinoma	Express GLUT-2 and glucokinase, no insulin secretion in response to increasing glucose concentrations.	Baroni et al, 1999
TRM-1	Foetal pancreas	Human	SV40 T-antigen, H-ras ^{val12}	Express GLUT-2, not responsive to glucose.	Wang et al, 1997
Blox5	Foetal pancreas	Human	SV40 T-antigen, H-ras ^{val12} , hTERT oncogene	Exhibits glucose responsive insulin secretion and expresses glucokinase; low insulin content.	Dufayet de la Tour et al, 2001

Limitations of *in vitro* cell lines include their ability to grow indefinitely leading to dedifferentiation over continuous passage. Specifically for pancreatic β -cells, this often results in loss of insulin synthesis and secretory capacity, likely related to their tumoural origin. Cells may have an abnormal chromosome number and/or additional gene mutations that could affect normal protein expression and metabolism (Ulrich *et al.*, 2002). Disruption of cell-cell interactions during treatment and passage may well influence typical cell function, and genetic manipulation of cells likely alters functional responses. These points may explain why many pancreatic β -cells lines have aberrant secretory responses and do not respond to glucose within normal physiological range (Skelin *et al.*, 2010).

An ideal cell model for studying β -cells should have all the characteristics of adult pancreatic β -cells. These features should include high insulin content and the ability to control insulin gene expression, processing and secretion, in response to typical physiological stimuli (Newgard and McGarry *et al.*, 1995). Additionally, the correct expression of the glucose transporter GLUT-1 and the glucose phosphorylating enzyme glucokinase is also essential (Zawalich and Zawalich, 1996). Studies have demonstrated that human pancreatic β -cells predominantly express GLUT-1. However, rodent pancreatic β -cells predominantly express GLUT-2, suggesting that GLUT-2 is unlikely to be the primary glucose transporter in human pancreatic β -cells (De Vos *et al.*, 1995; McCulloch *et al.*, 2011).

In 2011, a paper announced the creation of a new human pancreatic β -cell line, denoted as 1.1B4. The cells were created by electrofusion of human pancreatic epithelial carcinoma cells (PANC-1) with cultured human islets on a 1:1 ratio, resulting in a hybrid that retains characteristics of both cell lines. Colonies were

screened for insulin-positive hybrid cells, and the highest insulin-secreting clones were selected and re-cultured. A final selection of 3 clones with the highest insulin output were selected and the 1.1B4 cells were one of these clones (McCluskey *et al.*, 2011). These human pancreatic β -cells underwent basic studies for characterisation of enzyme production and secretory capacity, and provide a novel resource for additional functional and characterisation studies. These cells have been employed to investigate the *in vitro* responses to FA within the scope of this thesis.

1.23 Summary

The pathogenesis of T2D is complicated. Many risk factors, cellular mechanisms and signalling pathways have been implicated in the progression of the disease that ultimately drives loss of β -cell viability and leads to overt diabetes. Fatty acids have differential effects on β -cells, and these are dependent on the carbon chain length, degree of saturation and configuration of species. The variable responses observed in pancreatic β -cells during chronic exposure to different FA species suggests that they exert their effects by different mechanisms. Chronic β -cell exposure to LC-SFA leads to a loss of viability due to cytotoxic effects, whereas MUFA species are either well-tolerated, can attenuate the cytotoxic effects of LC-SFA during co-incubation, or promote viability in response to other toxic stimuli such as pro-inflammatory cytokines or nutrient deprivation. The mechanisms underlying these toxic or protective mechanisms are not well understood. Recent evidence has linked PKR with nutrient- and pathogen-sensing pathways, suggesting that responses within the innate immune system can trigger metabolic dysfunction such as ER stress and chronic inflammation. These mechanisms are now being regarded as hallmarks of obesity and T2D.

The NLRP3 inflammasome is critical to this chronic inflammatory state, and as such, represents a common pathway linking autoinflammation within the pathogenesis of metabolic dysfunction. Current knowledge regarding direct or indirect ligand activation of NLRP3, modes of activation, or the role of upstream components and modulators are still unclear. Further studies investigating specific activatory and inflammasome assembly mechanisms may allow therapeutic targeting of components with clinical benefit to patients with T2D. A range of pancreatic β -cell lines have been established over the past few decades as models to investigate β -cell function. While providing useful information regarding rodent islet biology, ultimately these cell lines do not compensate for a human pancreatic β -cell line with characteristics and responses of functional adult pancreatic β -cells. Therefore, the availability of any new human pancreatic β -cell provides a novel resource with which to undertake further functional and characterisation studies, to assess the suitability of the cell line for *in vitro* experiments.

1.24 Aims of study

1. To investigate the role of PKR in mediating proapoptotic effects in response to LC-SFAs, by manipulating the activity or expression of PKR in pancreatic β -cells using a putative PKR inhibitor Compound-16, and by generating a stable rodent β -cell line expressing shRNA against PKR.
2. To investigate and characterise the functional responses of a new human β -cell line, to assess their suitability for the *in vitro* study of lipotoxicity.
3. To investigate the role of inflammatory mediators through studying the effects of activatory stimuli, upstream mediators and pro-inflammatory cytokine involvement in rodent β -cell lines, including a β -cell line that conditionally over-expresses TXNIP.

Chapter 2 Materials and Methods

2.1 Chemicals and reagents

All chemicals and reagents were purchased from Sigma (Dorset, UK), unless otherwise specified.

Cell culture materials used such as Roswell Park Memorial Institute (RPMI)-1640 culture medium, Dulbecco's Modified Eagle's Medium (DMEM) culture medium, L-glutamine, penicillin/streptomycin, phosphate buffered saline (PBS) and trypsin-EDTA (ethylenediaminetetraacetic acid) were purchased from Invitrogen (Loughborough, UK). Fetal bovine serum (FBS) and hygromycin were purchased from PAA Laboratories (Yeovil, UK).

All FAs and methyl-FA were purchased from Sigma, except palmitoleate which was purchased from MP Biomedicals (Cambridge, UK) along with FA-free BSA. Table 2.1 shows a complete list of the composition of buffers and solutions used. Table 2.2 provides a complete list of primary and secondary antibodies used, along with dilutions and details of purchase origin.

Pre-cast polyacrylamide Bis-Tris-HCl gels (4-12% depending on protocol), MOPS running buffer and LDS loading buffer used for electrophoresis were purchased from Invitrogen. Polyvinylidene fluoride (PVDF) membrane was purchased from BioRad, while hyperfilm ECL and the developing system for horseradish peroxidase-labelled proteins were obtained from Amersham (via Invitrogen). DNA and protein ladders for electrophoresis, Western blot and PCR were purchased from Fermentas (Thermo Fisher Scientific, Loughborough, UK).

Table 2.1 Composition of buffers/solutions	
Buffer	Composition
Whole cell protein lysis buffer	20 mM Trizma, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, supplemented with 10 µl/ml Sigma protease inhibitor cocktail and 10 µl/ml Sigma phosphatase inhibitor cocktail 1 and 3 just before use to minimise protein breakdown
SDS-PAGE transfer buffer	43.2 g Glycine, 9 g Trizma, 2.4 l dH ₂ O, 0.6 l Methanol (pH 8.3)
5X Trizma buffered saline (TBS)	40 g NaCl, 1 g KCl, 30 g Trizma, 1 l dH ₂ O (pH 7.6)
Tween TBS (TTBS)	TBS & 0.05% Tween 20 (pH 7.6)
Membrane stripping solution	Millipore ReBlot plus Mild Antibody Stripping Solution 10X
TAE buffer	40 mM Trizma, 1 mM EDTA, 100 ml dH ₂ O (pH 8)
TBE buffer	80 mM Trizma, 40 mM Boric acid, 0.2 mM EDTA (pH 8)
PCR Reddy Master mix (Thermo Scientific)	0.625 units/25µl ThermoPrime Taq DNA polymerase, 75 mM Trizma-HCl, 20 mM (NH ₄) ₂ SO ₄ , 1.5 mM MgCl ₂ , 0.01% (v/v) Tween 20. 0.2 mM each of dATP, dCTP, dGTP, dTTP, precipitant and red dye for electrophoresis
BCA protein assay	Reagent A: sodium carbonate, sodium bicarbonate, bicinonic acid and sodium tartrate in 0.1 M sodium hydroxide Reagent B: cupric sulphate
Electrophoresis running buffer	20X NuPAGE MES/MOPS SDS Running buffer diluted 1:20 with dH ₂ O (pH 8.3)
SYBR® Green Supermix	100 mM KCl, 40 mM Trizma-HCl, 0.4 mM each of dATP, dCTP, dGTP, dTTP, 50 units/ml iTaq DNA polymerase, 6 mM MgCl ₂ , SYBR Green I, 20 mM fluorescein and stabilisers
4% Paraformaldehyde (PFA)	PBS, 4% PFA (pH 7.4)
Antibody dilution solution (ADS)	PBS, 0.1 M lysine, 10% donor calf serum, 0.02% sodium azide
ADST	0.2% Triton-X100 in ADS
IL-1β ELISA wash buffer	PBS, 0.05% Tween 20 (pH 7.4)
IL-1β ELISA stop solution	dH ₂ O, 1 M H ₃ PO ₄ (pH 6.7)
Trizma EDTA (TE) buffer	10 mM Trizma, 1 mM EDTA (pH 8.0)

Table 2.2 Dilutions of primary and secondary antibodies			
1° Ab	Dilution of 1° Ab	2° Ab	Dilution of 2° Ab
β-actin (Sigma)	1:10000 (2.5% milk in TTBS)	Goat polyclonal anti-mouse IgG alkaline phosphatase (AP) conjugate (Sigma)	1:20000 (2.5% milk in TTBS)
eIF2α (Cell Signaling/NEB: [Hertfordshire, UK])	1:1000 (TTBS with 1% goat serum)	BioRad Goat Anti-rabbit IgG Horseradish peroxidase conjugate (HRP)	1:20000
p-eIF2α (Cell Signaling/NEB)	1:1000	BioRad Anti-rabbit IgG HRP	1:20000
PKR (Sigma, Abcam [Cambridge, UK], Santa Cruz [Heidelberg, Germany], Pro-Sci [Poway, USA])	1:500 – 2000	BioRad Anti-rabbit IgG HRP	1:2500 – 1:20000
Phospho-PKR (Sigma, Millipore [Watford, UK])	1:500 - 2000	BioRad Goat Anti-rabbit IgG HRP Promega Goat anti-rabbit IgG HRP Sigma Goat anti-rabbit IgG HRP Abcam Sheep anti-rabbit IgG HRP Abcam Donkey anti-rabbit IgG HRP	1:2500 – 1:20000
PERK p-PERK (Cell Signaling/NEB)	1:1000	BioRad Anti-rabbit IgG HRP Goat polyclonal anti-rabbit IgG HRP conjugate (no. 172-1019, Biorad)	1:20000
CHOP10 (Sigma)	1:1000	Promega Goat anti-rabbit IgG AP	1:2500 – 1:20000
IL-1β (Abcam)	1:1000	BioRad Anti-rabbit IgG HRP	1:2500 – 1:20000
NLRP3 (Santa Cruz)	1:1000	BioRad Anti-rabbit IgG HRP	1:500 – 1:20000
TXNIP (Invitrogen)	1:1000	BioRad Anti-rabbit IgG HRP	1:10000
IL-1β for ICC ASC (Alexis, Enzo Life Sciences [Exeter, UK]) for ICC	1:100	Alexa Fluor® 488 goat anti-rat IgG Dapi (1 µg/ml)	1:400 1:1000

2.2 Cell culture conditions

The hybrid rat pancreatic β -cell line BRIN-BD11 was derived by the electrofusion of an immortal rat insulinoma cell line, RINm5F, with a primary culture of New England Deaconess Hospital rat pancreatic islet cells (McClenaghan *et al.*, 1996). The 2-mercaptoethanol-dependent INS-1 rat β -cell line was created by isolation of β -cells from irradiated rat insulinoma (Asfari *et al.*, 1992). The mouse insulinoma β -cell line MIN6 was derived from tumours arising in transgenic mice expressing the SV40 T antigen, under control of the insulin promoter (Miyazaki *et al.*, 1990). The human pancreatic β -cell line 1.1B4 was derived by electrofusion of a primary culture of human pancreatic islet cells with the human pancreatic carcinoma, epithelial-like cell line PANC-1, and hybrid cells were screened for insulin positivity (McCluskey *et al.*, 2011). Conditional over-expression of thioredoxin-interacting protein (TXNIP) was achieved by the stable transfection of parental INS-1 cells with a TXNIP transgene under the control of a tetracycline- (TET) inducible promoter, creating INS-TXNIP cells, along with their control INS-EV (empty vector) cells (Kansikas, 2012). The PKR knock-down clones BRIN-333 and BRIN-335, and their control BRIN-SV (scrambled vector) cells were created by stable transfection of parental rat BRIN-BD11 and INS-1 β -cells expressing a rat Origene shRNA ready-cloned retroviral vector against PKR (as described in detail in Sections 2.6.2 – 2.6.6).

The BRIN-BD11 and 1.1B4 β -cell lines were cultured in RPMI-1640 medium containing 11 mM glucose, and supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. The INS-1 β -cell line was cultured in the same culture medium as BRIN-BD11 cells supplemented with 50 μ M 2-mercaptoethanol. The MIN6 β -cell line was cultured in DMEM medium,

supplemented with 15% FBS, 25 mM glucose, 100 U/ml penicillin, 100 mg/ml streptomycin and 50 μ M 2-mercaptoethanol. The INS-TXNIP/INS-EV cells were cultured in RPMI-1640 medium containing 11 mM glucose, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 μ M 2-mercaptoethanol and 150 μ g/ml hygromycin B. The BRIN-BD11 PKR KD clones BRIN-333, BRIN-335 and BRIN-SV were cultured in RPMI-1640 medium containing 11 mM glucose, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and 1 mg/ml puromycin.

All cells were grown in 75 cm² flasks at 37 °C and with 5% CO₂ at 100% humidity and were used in experiments when approximately 80% confluent; all experiments were performed on cells below passage number 35.

2.2.1 Passaging of cells

All cell lines were sub-cultured when they had reached approximately 80% confluence. Cells were briefly washed in PBS pre-warmed to 37 °C, followed by a 5 min incubation period at 37 °C with 2 ml/75 cm² flask of 0.05% Trypsin-0.53 mM EDTA to assist in the disruption of the adherent cell monolayers from the cell-culture flask. Trypsin-EDTA action was de-activated by the addition of 10 ml of appropriate complete culture medium (containing 10% FBS). Cells were centrifuged at 300 g for 5 min, then approximately one tenth to one twentieth of the original growing population (depending on cell line and growth rate) were re-seeded into fresh 75 cm² cell culture flasks to maintain a stock cell line.

2.3 Treatment of cells with fatty acids

Fifty millimolar stock solutions of laurate (C12:0), myristate (C14:0), palmitate and methyl-palmitate (C16:0), stearate and methyl-stearate (C18:0) were prepared in 50% ethanol by heating to 70 °C. Ninety millimolar stock solutions of myristoleate (C14:1), palmitoleate and methyl-palmitoleate (C16:1), oleate and methyl-oleate (C18:1) were prepared in 90% ethanol at room temperature (RT). These FA preparations were bound to 10% FA-free BSA by incubation for 1 h at 37 °C and then added to RPMI-1640 medium (containing 11 mM glucose) deprived of FBS, making the final concentrations present in the cell environment 1% for BSA and 0.5% for ethanol. All incubations with FA were performed using serum free conditions, as FBS contains FA that could potentially alter final treatment concentrations. Cells were seeded into 6, 12 or 24-well plates at densities of 0.5×10^5 cells/ml and cultured overnight in complete RPMI-1640 medium. The medium was then removed and replaced with relevant FA/BSA complexes in RPMI-1640 medium devoid of FBS for various times. Control groups received BSA and vehicle only.

2.4 Viability assays

2.4.1 Vital dye staining

Trypan blue is a vital dye commonly used to determine the viability of living cells without killing them. Live cells have intact plasma membranes and can selectively extrude certain stains, whereas dead cells retain the dye and are selectively stained when viewed under a light microscope. This dye exclusion test was used to estimate the number of viable cells present in each sample. Following incubation with appropriate treatments, the culture medium containing any potential floating cells was removed and collected, and then attached cells

were harvested after a 5 min incubation with trypsin-EDTA (as previously described in Section 2.2.1). Floating and attached cells were then centrifuged at 300 g for 5 min and re-suspended in 200 µl complete RPMI-1640 and 200 µl Trypan blue (0.4% in PBS) and incubated at room temperature (RT) for 10 min. Both viable and dead cells were counted using a haemocytometer and the proportion of dead cells was expressed as a percentage of the total for each treatment group.

2.4.2 Flow cytometry using propidium iodide staining

Flow cytometry is a fast, accurate and reproducible method that uses the principle of light excitation, emission and scattering of fluorochrome molecules from a focused light source (typically a laser), to measure different cellular properties such as size, granularity and fluorochrome binding from hydro-dynamically focused cells in suspension. Propidium iodide (PI) is a compound that intercalates with the DNA/RNA of cells: PI can only penetrate cells when their membrane integrity has been compromised, such as during apoptosis or necrosis. As such, PI is commonly used to identify non-viable cells within a cell population using flow cytometry. Following incubation with relevant agents, floating and attached cells were trypsinised and collected as described in Section 2.4.1. After the centrifugation step, cells were re-suspended and stained using 200 µl of 1 mg/ml PI and appropriate complete culture medium solution. Cells were incubated for 10 min at 4 °C and then samples were analysed using a BD Biosciences Accuri C6 Flow Cytometer, running the CF-30 CFlow Sample Analysis software.

Gating of cells was initially applied to untreated PI-stained cells (considered to be a negative control treatment group) which encompassed all cell populations, as shown by the red gating shown in the density plot in Figure. 2.1. The forward scatter axis (FSC-A) is able to differentiate cells on the basis of their physical properties such as size, while the side scatter axis (SCC-A) differentiates cells on the basis of their internal granularity (Brown and Wittwer, 2000).

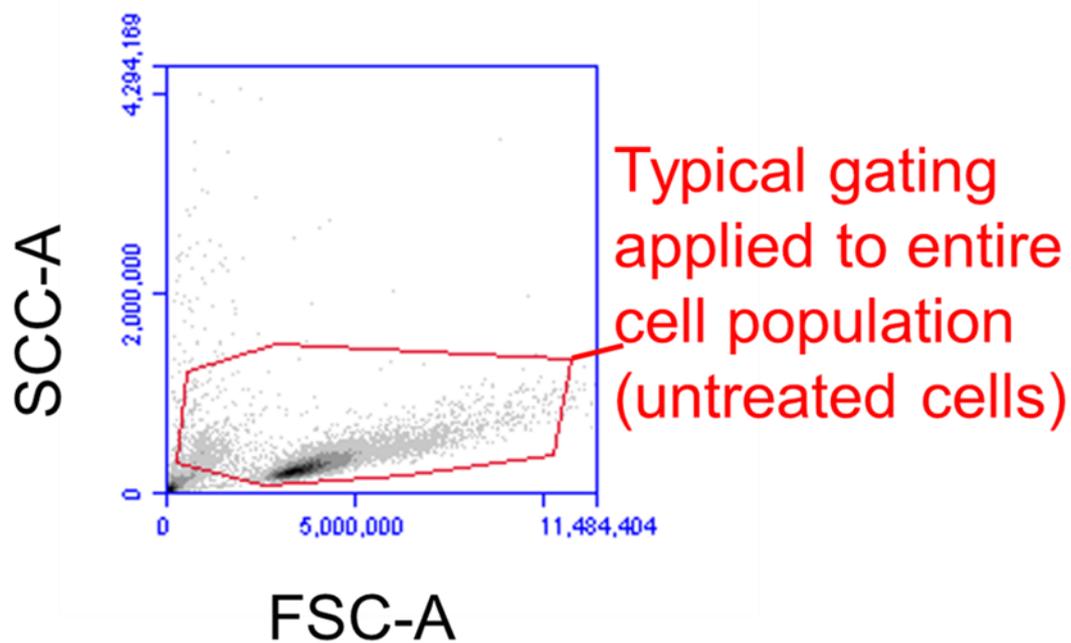


Fig. 2.1 Density plot representing typical gating applied to an untreated cell population

Initial gating of cells was applied to the entire cell population of untreated PI-stained cells (considered to be a negative control treatment group), as depicted by the red gating. The forward scatter axis (FSC-A) differentiates cells on the basis of their size, while the side scatter axis (SCC-A) differentiates cells on the basis of their internal granularity.

Three typical cell populations were identified when using PI staining in the different cell models used. Figure 2.2 represents a typical scatterplot obtained from cells treated with palmitate, showing three distinct cell populations: a healthy cell population, a dead PI positive cell population, and a third population which were reduced size but not PI positive. Preliminary experiments on the Accuri C6 Flow Cytometer have shown that this third cell population were Annexin V positive cells. The Annexin V protein binds to phosphatidylserine (PS) which is typically found predominantly in the inner leaflet of the plasma membrane (PM) when cells are viable. However, once apoptosis is triggered, PS is translocated to the external face of the PM (to allow targeting of cells for phagocytosis), and the PS can be detected using fluorescently labelled Annexin V, marking these cells as early apoptotic (Zhang *et al.*, 1997).

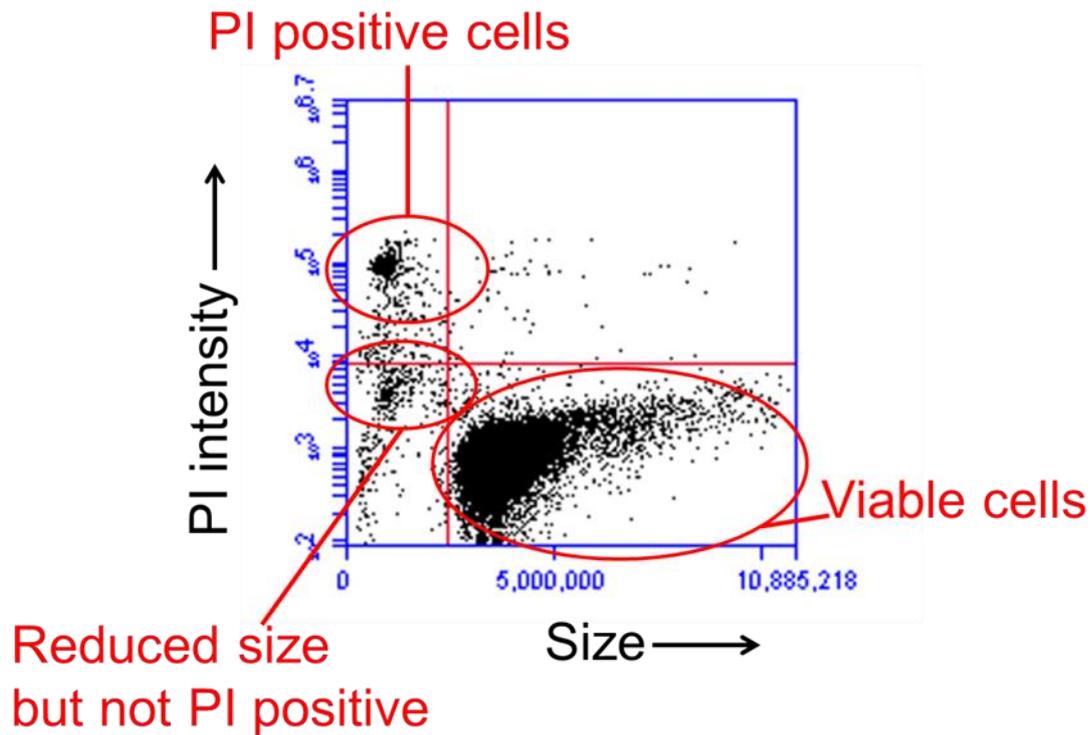


Fig. 2.2 Scatter plot representing cell death quantification using flow cytometry

Cell death was induced by the addition of treatments (e.g. palmitate), and cells were stained with PI to assess viability. The figure represents a typical scatter-plot obtained from treated cells, showing three distinct cell populations: a healthy cell population labelled 'viable cells' (bottom right quadrant), a dead population labelled 'PI positive cells' (upper left quadrant), and a third population labelled 'Reduced size but not PI positive' (bottom left quadrant). This third cell population has been previously analysed in the laboratory and found to be Annexin V positive, which is indicative of early apoptosis. For the purposes of assessing cell viability, only cells within the healthy population were counted; PI-positive and Annexin V positive cells were excluded from the viable cell count.

2.5 Detection of protein expression by Western blotting

2.5.1 Whole cell protein isolation

Cells were seeded at 1×10^5 cell/25 cm² tissue culture flask at 37 °C and with 5% CO₂ and subsequently incubated for varying times depending on the treatment used. Following treatment, cells were washed twice in ice cold PBS, then lysed using 200 µl of lysis buffer (Table 2.1) per 25 cm² flask. Flasks were kept on ice and bathed periodically with lysis buffer for 20 min. The flasks were thoroughly scraped and their contents transferred to microcentrifuge tubes. Each tube was vortexed 6 times for 5 s and incubated on ice between each vortex step, then lysates were clarified by centrifugation at 10000 g for 10 min at 4 °C using a pre-cooled refrigerated centrifuge (ALC, PK121R). The supernatants were retained for Western blotting by transfer to new microcentrifuge tubes and these were stored at -80 °C.

2.5.2 Estimation of protein concentration

Protein within cell lysates were estimated using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Northumberland). The protein of interest reduces Cu²⁺ to Cu⁺ via the interaction of the peptide bonds present in the protein sample, leading to the concentration dependent development of a purple-coloured product with a peak absorbance at 562 nm. The concentration of reduced Cu²⁺ is proportional to the protein concentration present in the sample (Smith *et al.*, 1985). Using a 96 well plate, a range of BSA standards (0-1400 µg/ml) were prepared, in parallel with 10 µl of each sample dilution in duplicate. Reagents A and B (Table 2.1) were mixed in proportions 1:49 and 200 µl of the final BCA solution was added to each standard and each protein sample. These were incubated at RT for 15 min and the absorbance was measured at 562 nm

using a microplate reader (Tecan GENios). The protein concentration of test samples was estimated by comparing their absorbance to that of the known standard concentrations (Fig. 2.3)

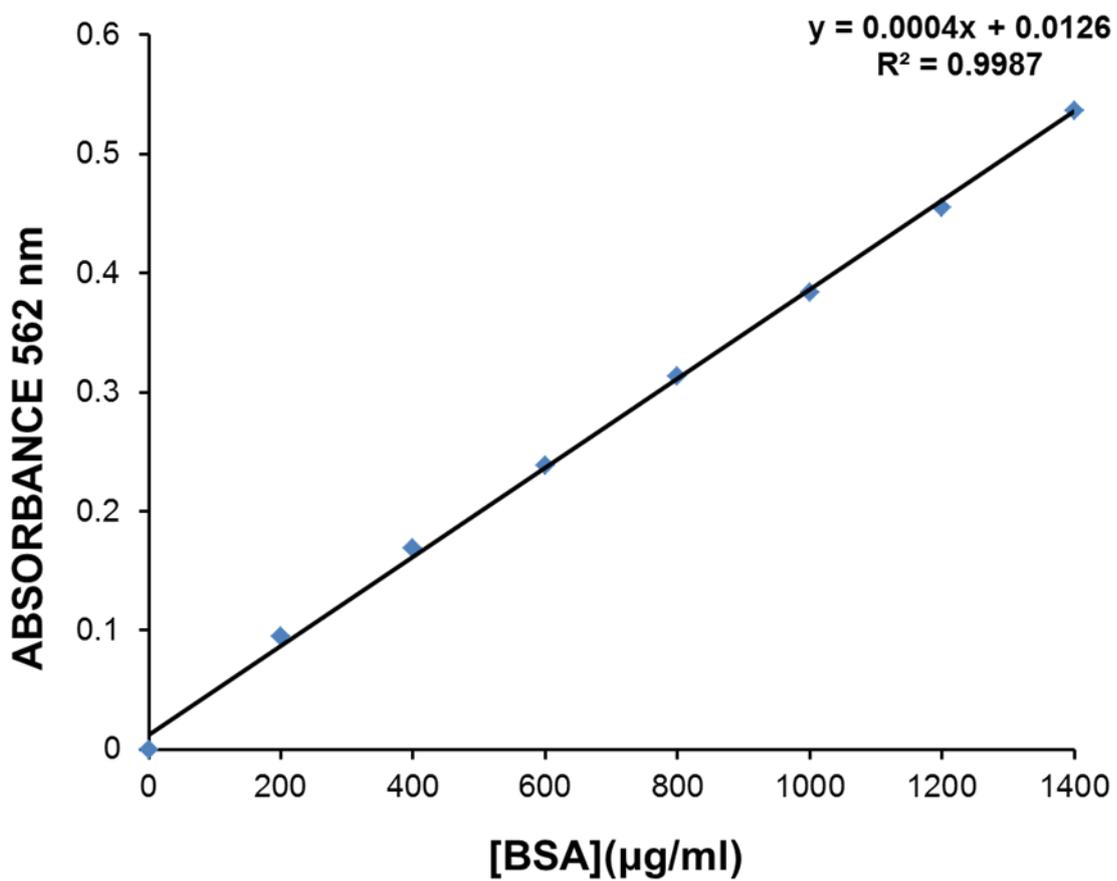


Fig. 2.3 Representative standard curve for calculating protein concentration

2.5.3 Electrophoresis and protein transfer

Gel electrophoresis is a widely used technique that enables the separation of individual proteins within a tissue or cell sample homogenate according to polypeptide length after denaturation. The proteins can then be transferred to a membrane and the addition of specific antibodies (Ab) identify the proteins of interest by Western blotting. This method allows the determination of the molecular weight of a target protein, but can also be used semi-quantitatively to determine amounts of target protein(s) present in different samples. The concentration of protein in each lysate was estimated using the BCA protocol as detailed in Section 2.5.2., and lysates were standardised to a protein concentration of 10-50 µg/19.5 µl loading volume by dilution with lysis buffer, depending on the affinity of each antibody used. Samples were then mixed with 7.5 µl of 4X LDS sample buffer (Invitrogen) and 3 µl of 2-mercaptoethanol (Sigma) and heated to 70 °C for 10 min. Ten microlitres of Spectra multicolour broad range protein molecular weight marker (Fermentas, York) was loaded into the first well of each pre-cast Bis-Tris-HCl gel to monitor protein separation, then 20 µl of each sample was loaded into each subsequent well of the gel. The gel was developed using MOPS SDS running buffer (Invitrogen) at 200 V using a Bio-RAD Power Pac 3000 unit for approximately 1 h, or until the molecular weight protein marker ladder had reached the bottom of the gel.

A piece of pre-cut PVDF membrane was briefly soaked in 100% methanol (Rathburn, Walkerburn), washed three times in dH₂O and soaked in transfer buffer (Table 2.1) until use. Two pieces of blotting paper were soaked in transfer buffer and placed onto the BioRad wet Trans-Blot Cell transfer tank buffer pad, black cassette-side down. A sandwich was made by placing the pre-cast gel on

top of the blotting paper, followed by the PVDF membrane and another two pieces of soaked blotting paper. All air bubbles were removed then the cassette was closed and returned to the transfer tank. The cell was topped up with transfer buffer and connected to a Bio-Rad PowerPac HC Power Supply unit; transfer of proteins from the gel to the PVDF membrane was conducted at 150 mA and 80 V overnight.

2.5.4 Probing with antibodies

Membranes were blocked for 1 h at RT on a roller, in 5% FA-free milk (Sigma) dissolved in TBS (Table 2.1), supplemented with 0.05% Tween® 20 to prevent non-specific binding of Ab to the membrane. Membranes were probed with primary antibodies in 3 ml TTBS (refer to Table 2.2 for individual dilutions and where Abs were purchased from) with 30 µl goat serum and were incubated overnight at 4 °C on a roller. The following morning the membranes were washed three times in TTBS for 30 min. Appropriate secondary antibodies (Table 2.2) were diluted in TTBS containing 2.5% FA-free milk and incubated for 2 h at RT, then washed as previously described. HRP-conjugated proteins were detected using the enhanced chemiluminescence (ECL) Plus (Amersham) substrate system. Briefly, reagent B was mixed with reagent A in a 1: 40 ratio and left to act on the membrane for 5 min at RT. Alkaline phosphatase-conjugated proteins were incubated with 1 ml of CDP-Star chemiluminescent substrate solution for 5 min at RT. Excess liquid was drained, then the membrane was wrapped in Saran Wrap and exposed to X-ray film (Healthcare ECL): exposure time was dependent on the Ab affinity and the protein load. The X-ray film was developed using a Xograph Imaging System Compact X4 Automatic X-ray film Processor (Gloucestershire, UK). Membranes were stripped with stripping buffer (Table 2.1)

for 10 min at RT on a roller, washed 3x for 5 min at RT in TTBS and then re-probed for loading controls using a an anti- β -actin Ab.

2.6 RNAi

RNA interference (RNAi) is a conserved biological process whereby cells control target gene expression at both a transcriptional and translational level. The RNAi pathway is triggered by short non-coding dsRNA molecules known as micro RNA (miRNA), short interfering RNA (siRNA) or short hairpin RNA (shRNA), which initiate the sequence-specific degradation of a target RNA within the cell, leading to inhibition or knock-down of target gene expression. This technique can provide a convenient mechanism to investigate the effects of a specific gene using both *in vitro* and *in vivo* models, and as such can be used to determine key functional pathways in disease states. The use of RNAi technology can lead to transient or stable knock-down of target genes, depending on the vector used (Leung & Whittaker, 2005).

2.6.1 PKR inhibition by adenoviral mutant PKR (Ad- Δ 6PKR)

A dominant negative adenoviral PKR mutant, Ad- Δ 6PKR, was kindly provided by Abujiang Pataer. Ad- Δ 6PKR is a binary adenoviral vector system which contains two adenoviral vectors, often utilised to prevent toxicity that may occur in the packaging cell line (Pataer *et al.*, 2009). Vectors with a dominant negative mutation are typically catalytically inactive, but express a dominant phenotype: this presents a useful method by which to investigate the effects of transient inhibition of PKR expression in selected cell lines. *In vitro* expression of the Δ 6PKR gene product was induced by the co-administration of Ad/GT- Δ 6PKR

(3000 vp/cell) and Ad/GV16 (1500 vp/cell). Ad-luc (3000 vp/cell) was used as a control vector.

BRIN-BD11 cells were seeded into 25 cm² flasks at 1 × 10⁵ cells/flask, in 5 ml of complete culture medium and left to grow for 18 h. Cells were transfected via co-administration of Ad/GT-Δ6PKR (0.5-4 μl/25 cm² flask) and Ad/GV16 (0.25-2 μl/25 cm² flask) or with appropriate Ad-luc control treatments (0.5-4 μl/25 cm² flask) using complete culture medium. Cells were cultured for 16 h, the culture medium was aspirated then cells were treated with FA (as detailed in Section 2.3). Protein was extracted from all treatment groups and used in Western blots.

2.6.2 PERK inhibition by adenoviral mutant PERK (Ad-PerkΔC)

Using the same principles as outlined above in Section 2.6.1, a PERK dominant negative adenoviral mutant (Ad-PERKΔC) kindly provided by Terry Herbert was used to investigate the effects of transient inhibition of PERK expression in the BRIN-BD11 cell line (Gomez *et al.*, 2004). BRIN-BD11 cells were seeded into 25 cm² flasks at 1 × 10⁵ cells/flask, in 5 ml of complete culture medium and left to grow for 18 h. Cells were transfected via co-administration of Ad/GT-Δ6PKR (0.5 μl - 4 μl/25 cm² flask) and Ad/GV16 (0.25 μl-2 μl/25 cm² flask), and/or Ad-PERKΔC (0.5 μl-4 μl/25 cm² flask) or with appropriate control treatments (0.5 μl -4 μl/25 cm² flask) using complete culture medium and cultured for 16 h, before aspirating the culture medium and treating with FA (as detailed in Section 2.3). Protein was extracted from all treatment groups and used in Western blots.

2.6.3 PKR knockdown using shRNA

Using a different strategy, a commercially available rat shRNA ready-cloned retroviral vector (Origene) with four constructs targeted against multiple splice variants of PKR (Table 2.3) was used according to the manufacturer's instruction, to transfect both BRIN-BD11 and INS-1 cells creating stable PKR knock-down cell lines. A scrambled shRNA vector was used as a control. Kanamycin resistance was used as an initial plasmid selection marker, followed by puromycin resistance as a marker of cell transfection.

Table 2.3 PKR shRNA expression vector sequences	
shRNA	Sequence
GI741333 (333)	TAGTCACACGGATGCTTCTGAACAAGGTT
GI741334 (334)	ATCACGCCAACATTGTTCAATACCGAGTT
GI741335 (335)	ACTTTGGCTGAGTGGAAGAACATCTCGGA
GI741336 (336)	TCCACACGACAGAAGGTTTACATTTCAAG

2.6.4 Transformation and plasmid purification using shRNA

For successful transfection of target cell lines, the shRNA plasmids were transformed into competent bacterial cells, which have the ability to take up extracellular DNA. Briefly, this was achieved by mixing 100 ng/ μ l of each plasmid DNA with 65 μ l of competent NEB 5- α *E. coli* (10^9 cfu/ μ g, New England Biolabs, Herts), and incubation on ice for 10 min. The cells were then heat shocked for 30 s at 42 °C and incubated again on ice for 5 min. Super Optimal broth with Catabolite repression (SOC) growth medium (950 μ l) (NEB) was added to each plasmid/bacteria suspension, and these were then incubated on a shaker at 250 RPM for 1 h at 37 °C. From each culture, 25 μ l was streaked over one half of pre-warmed, pre-made kanamycin-(25 μ g/ml) containing liquid broth agar plates (35 g/l). The remaining suspensions were centrifuged for 2 min at 1000 g, then ~ 800 μ l of the supernatant was aspirated and the pellet resuspended in the remaining volume. Twenty-five microlitres of this culture was used to streak the remaining half of each agar plate, and these were incubated overnight at 37 °C. From these initial plates, a single colony was selected for each plasmid using a sterile inoculation loop, and streaked onto a second kanamycin-containing agar plate (as detailed previously), which was incubated overnight at 37 °C. A sterile inoculation loop was used to select a single colony from each plate, and used to inoculate 5 ml LB broth (20 g/l) containing kanamycin (25 μ g/ml). Each culture was incubated in a shaker at 250 RPM and 37 °C for 8 h, and then used to inoculate a further 250 ml LB broth containing kanamycin, and incubated in a shaker at 250 RPM and 37 °C for 18 h. Plasmid extraction was conducted when the *E. coli* were in log-phase growth, using a plasmid maxi prep kit (Qiagen), according to the manufacturer's instructions.

2.6.5 Quantification of plasmid DNA

The DNA from each plasmid was quantified by measuring the absorbance at 260 nm using an Eppendorf Biophotometer spectrophotometer. DNA concentration was calculated by assuming that an absorbance of 1 at 260 nm corresponds to 50 µg of DNA/ml (Barbas *et al.*, 2007).

2.6.6 DNA preparation for transfection

The DNA from each plasmid was precipitated using 0.7 volume of isopropanol, mixed by inversion and immediately centrifuged at 2000 g for 30 min at 4°C. The resulting pellet was washed by centrifugation using room temperature 70% ethanol, at 2000 g for 10 min. The pellet was then air-dried and resuspended in 250 µl TE buffer (Table 2.1).

2.6.7 Generation of stable cell lines expressing shRNA against PKR

BRIN-BD11 and INS-1 cells were seeded into 6 well plates at 3×10^5 cells/well, in 2 ml of complete culture medium and left to grow for 18 h. Cells were transfected using 1 µg of each plasmid DNA, diluted with 3 µl of TransFast™ transfection reagent per well, and mixed with serum free culture medium which was vortexed and incubated at RT for 15 min before use. The transfection mixture was added to the cells at 1 ml/well, then incubated for 1 h at 37 °C. After 1 h, an additional 1 ml of complete culture medium with 20% FBS was added to each well and the cells were cultured for a further 24 h.

TransFast™ transfection reagent is a liposome reagent used to deliver nucleic acids into eukaryotic cells by the process of transfection. TransFast™ is made

from a synthetic cationic lipid (+)-N,N [bis (2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide and the neutral lipid DOPE (L-dioleoyl phosphatidylethanolamine). Incubation of this liposome reagent with DNA causes compaction of the nucleic acid and neutralisation of its negative charge, thereby allowing closer association of the liposome/DNA complex with the negatively charged plasma membrane. The liposome complex enters the cell by endocytosis or by fusion with the plasma membrane.

The transfection mixture was aspirated after 24 h and replaced with complete culture medium for a further 24 h. Following this, puromycin (1 mg/ml) in appropriate complete culture medium was added, and cells were cultured in this medium until small colonies of transfected cells were seen with the naked eye. Only transfected cells should survive, as the puromycin resistance gene was encoded within the plasmids. Individual colonies were picked using cloning cylinders, and passaged into separate wells until growth of cells was such that they could be transferred to 75 cm² flasks. Both RNA and protein was extracted from all constructs (including scrambled vector), and used in Western blots to screen for PKR knockdown.

2.7 Detection of mRNA expression (Reverse Transcriptase-PCR)

2.7.1 DEPC-treated water

The extraction and storage process of RNA required diethylpyrocarbonate (DEPC)-treated water, which inactivates RNase enzyme activity (via the covalent modification of histidine residues) and therefore minimises the risk of RNA degradation. A 0.1% DEPC/H₂O solution was prepared by dissolving the DEPC

in the H₂O: this was then incubated for 12 h at 37 °C, followed by autoclaving for 15 min at 121 °C to remove any remaining DEPC, followed by storage in aliquots at -20 °C until required.

2.7.2 RNA extraction

Cells were seeded at 1×10^5 cell/25 cm² tissue culture flask at 37 °C and with 5% CO₂ and subsequently incubated for various incubation times depending on the treatment used. The culture medium was aspirated from the flasks, then TRIzol® reagent (Invitrogen) was used to lyse and extract cell RNA. TRIzol® reagent maintains the RNA integrity of the cells during the homogenisation process, while simultaneously lysing cells and organelles. Briefly, 1 ml Trizol®/25 cm² tissue culture flask was added and cell lysates were passed through RNase-free sterile filter pipette tips several times to homogenise the sample, before the contents were transferred to sterile microfuge tubes, and allowed to incubate at RT for 5 min. Chloroform (200 µl/1 ml TRIzol® reagent used) was added to the lysate and briefly vortexed, then incubated at RT for 10 min to allow the aqueous and organic phases to separate. Samples were centrifuged at 4 °C, 10000 g for 15 min and the resulting upper colourless phase containing the RNA was carefully aspirated and transferred to a fresh sterile microfuge tube, without disturbing the interface (containing DNA) or the organic lower phase (containing proteins). The RNA was precipitated using 0.5 ml isopropanol/1 ml TRIzol® reagent, briefly vortexed then incubated at -20 °C for 1 h, before being centrifuged again at 4 °C, 10000 g for 20 min. The resulting RNA pellet was washed twice using 75% ethanol and centrifuged at 4 °C, 10000 g for 15 min. Finally, the RNA pellet was air-dried to allow any remaining alcohol to evaporate, and the pellet was resuspended in 25 µl DEPC-treated water and stored at -80 °C until needed.

2.7.3 Quantification of RNA

To determine the RNA yield, the previously frozen RNA/DEPC suspension was quantified using an Eppendorf Biophotometer spectrophotometer. Absorbance readings at 260 nm were taken, using DEPC-treated water as a blank. RNA purity was determined by calculating the absorbance ratio measured at 260/280 nm, where a ratio between 1.8 and 2 signified high purity RNA (Barbas *et al.*, 2007).

2.7.4 cDNA synthesis

One microgramme of extracted RNA/DEPC suspension was mixed to make a total of 10 µl of DEPC-treated water for each reverse transcription (RT) reaction. To this volume, 1 µl of 500 ng/ml random primers (Promega) was added and the mixture heated to 70 °C for 10 min, then incubated on ice. Random primers are used to initiate cDNA synthesis from the total RNA population, by random annealing throughout the RNA. Next, a reaction mix was prepared using 3.5 µl DEPC-treated water, 4 µl 5x RT buffer, 0.5 µl RNasin Plus RNase Inhibitor (40 U/µl), 1 µl dNTP (10mM) mix and 1 µl RT enzyme (all from Promega). Nine microlitres of this mix was added to 11 µl RNA/DEPC/random primer mix. The final mix was incubated at 25 °C for 15 min, at 42 °C for 50 min and finally at 70 °C for 15 min using a BioRad iCycler, to allow synthesis of complementary DNA. A separate mix was prepared using 1 µl nuclease-free dH₂O instead of RT enzyme as a negative control, which when run simultaneously with the RT-positive samples, should not produce any amplified product during the PCR process.

2.7.5 Primer design

Individual primers were designed using the software available online at the Primer3 website (<http://primer3.sourceforge.net/>) (Figure 2.4, 2.5 and 2.6) and were purchased from Invitrogen (Table 2.4).

Rat IL-1 β nucleotide sequence

(<http://www.ncbi.nlm.nih.gov/nuccore/BC091141>)

Accession number NM 031512.21

```
ggctcatctgggatcctctccagtcaggcttcctgtgcaagtgctgaagcagctatggcaactgtccc  
tgaactcaacttgaaatagcagcttcgacagtgaggagaatgacctgttcttgaggctgacagac  
cccaaaagattaaggattgctccaagccctgactgggctgtccagatgagagcatccagctcaa  
atctcacagcagcatctcgacaagagcttcaggaaggcagtgctactcattgtggctgtggagaagc  
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agtttcatattgtgtggatgagagtgttatgaatgaagcacaaagcacatcattttgatgagatgaaata  
aatgtcactaaaacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

IL-1 β FWD PRIMER set 1: **cacctctcaagcagagcacag**

IL-1 β REV PRIMER set 1: gggttccatggtgaagtcaac

Inverse complement of reverse primer: **gttgacttcaccatggaaccc**

Product size: 79 bps

Fig. 2.4 Rat IL-1 β mRNA nucleotide sequence and primers (set 1)

Rat IL-1 β mRNA nucleotide sequence

(<http://www.ncbi.nlm.nih.gov/nuccore/BC091141>)

Accession number NM 031512.21

ggctcatctgggatcctctccagtcaggcttcctgtgcaagtgtctgaagcagctatggcaactgtcc
ctgaactcaactgtgaaatagcagcttctgacagtgaggagaatgacctgttcttgaggctgacag
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ttcattgatctgaaaatgaagagttcatattgtgtggatgagagtgttatgaatgaagcacaagc
acatcattttgatgagtatgaaataaatgtcactaaaacaaaaaaaaaaaaaaaaaaaaaaaaaaaa
aaaa

IL-1 β FWD PRIMER: **caggaaggcagtgctactca**

IL-1 β REV PRIMER: gggatttgcgttgcttgt

Inverse complement of reverse primer: **acaagcaacgacaaaaatccc**

Product size: 339 bps

Fig. 2.5 Rat IL-1 β mRNA nucleotide sequence and primers (set 2)

Human IL-1 β mRNA nucleotide sequence

http://www.ncbi.nlm.nih.gov/nuccore/NM_000576.2

Accession number NM 000576

accaaacctctcgaggcacaaggcacaacaggctgctctgggattctctcagccaatcttcattgctc
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aatgaggatgactgttcttgaagctgatggccctaaacagatgaagtgtcctccaggacctgga
cctctgccctctggatggcggcatccagctacgaatctccgaccaccactacagcaagggctcaggc
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gtgtagatcccaaaaattacccaagaagaagatggaaaagcgatttcttcaacaagatagaaat
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acatgcccgctctcctgggagggaccaaggcggccaggatataactgacttcacctgcaatttgggt
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gtaaagagcctagttttatagctatggaatcaattcaattggactgggtgctctctttaaataagtc
ctttaaataagactgaaaatataagctcagattatttaaataagggaatattataaatgagcaaatatc
actgttcaatgggtctgaaataaacttcaactgaag

IL-1 β FWD PRIMER: agctgatggccctaaacaga

IL-1 β REV PRIMER: tcgttatcccatgtgtcgaa

Inverse complement of reverse primer: ttcgacacatgggataacga

Product size: 260 bps

Fig. 2.6 Human IL-1 β mRNA nucleotide sequence and primers

Table 2.4 PCR Primers		
Gene	Forward Primer	Reverse Primer
Rat IL-1 β set 1	CACCTCTCAAGCAGAGCACAG	GTTGACTTCACCATGGAACCC
Rat IL-1 β set 2	CAGGAAGGCAGTGTCCTCA	GGGATTTTGTCTGTTGCTTGT
Human IL-1 β	TTCGACACATGGGATAACGA	TCTTTCAACACGCAGGACAG

2.7.6 PCR

The reverse transcriptase polymerase chain reaction (RT-PCR) is a universally adopted thermal cycling technique used to amplify a few copies of DNA, by the addition of a heat-stable DNA polymerase (such as Taq polymerase). Such polymerases can construct a new DNA strand by reverse transcribing a single-stranded DNA used as a template, and simultaneously utilising primers to initiate DNA synthesis. An initial high temperature denaturation step separates the two target DNA strands, followed by a lower temperature step which enables the primers to anneal to each separated DNA strand. These individual strands are then used as a template by the DNA polymerase to amplify the target DNA during an extension step. The cycle is then repeated numerous times to amplify the DNA product.

2.7.6.1 RT-PCR reactions

All RT-PCR reactions used 22.5 μ l PCR Master Mix (containing 1.5 mM MgCl₂) (Thermo Scientific) (Table 2.1 for detailed composition) in a BioRad iCycler. One microliter of cDNA and 1 μ l of each forward and reverse custom primer (25 μ M) were added to the Master Mix. The PCR reaction comprised an initial 5 min denaturation step at 95 °C, followed by 35 cycles of amplification consisting of: a 30 s denaturation step at 95 °C, a 30 s annealing step (46-56 °C depending on

primers), and a 30 s extension step at 72 °C. The reaction was completed by a final 5 min extension step at 72 °C. The annealing temperature was determined by the average melting temperature (T_m) – 3 °C of the custom primers used. Detection of the PCR products was carried out by electrophoresis, using 1% agarose (Invitrogen) gel in either Trizma-EDTA-buffer (TAE) or Trizma-BORATE-buffer (TBE) buffer (Table 2.1), containing 3 µl/60 ml agarose Gel Red Nucleic Acid Stain (Biotium). TAE buffer is typically used when the DNA product needs to be isolated for sequencing or for high resolution of larger DNA products, whereas TBE buffer is preferable for the resolution of smaller DNA products and also has better buffering capacity so can be used more than once. Gels using TBE buffer were run at 200 V for 30 min, or 100 V for 1 h using TAE buffer. Gel Red is a fluorescent dye which intercalates with and stains dsDNA, ssDNA or RNA and therefore can be used to visualise the fluorescent DNA product within the gel using an ultraviolet lamp to illuminate the dye (BioDoc-It™ Imaging System). Images were captured using Labworks software. A DNA ladder (Fermentas) was also run simultaneously, to allow determination of the approximate size of visualised bands.

2.7.7 Checking for genomic contamination of cDNA

It is difficult to completely exclude the presence of genomic DNA (gDNA) from RNA samples, so it is important to prepare samples carefully in separate areas and using dedicated equipment and to include proper controls. Typically this would entail using identically-prepared samples that include all the reagents except the reverse transcriptase enzyme, i.e. a negative control, plus a dH₂O control. The presence of an amplified DNA product in the control lanes would be indicative of contaminating gDNA. Depending on the level of contamination, new

RNA extraction could be repeated or DNase treatment of the existing cDNA sample could be performed to eradicate modest DNA contamination.

2.7.8 Confirmation of PCR products

While a detected DNA product may appear to be the correct size, this does not necessarily mean that the correct DNA product has been amplified. Therefore, in order to be assured that this was the case, direct sequencing of the product was performed. The DNA product was run on a TAE gel (as detailed in Section 2.7.6.1), and excised under UV light using a sterile blade. The DNA was extracted from the gel fragment using a QIAquick Gel Extraction kit (Qiagen) as per the manufacturer's instructions, and the purified DNA product was dispatched to Eurofins MWG Operon MWG (Ebersberg, Germany), for sequencing to confirm the identity of the excised DNA product.

2.8 Detection of pro-inflammatory cytokines by ELISA

Enzyme-linked immunosorbent assay (ELISA) is a widely used and sensitive method to detect and quantify relatively low concentrations of substances for example, proteins, antibodies and cytokines. The target antigen is immobilised to a solid surface, typically a 96-well plate and then complexed with an enzyme-linked antibody. Detection and quantification of the target antigen is performed by measuring the conjugated enzyme activity via incubation with a substrate that produces a colorimetric product. A Rat IL-1 beta Tissue Culture ELISA Ready-SET-Go![®] kit (eBioscience, Hatfield, UK) was used as per the manufacturer's protocol to monitor IL-1 β secretion from cells that had been subjected to various treatment combinations. Cells were seeded in complete appropriate culture medium at 2.5×10^5 cells/well 18 h before the required treatment incubation

period. The medium was aspirated and replaced with relevant reagents for the designated incubation period. One day prior to performing the ELISA, the 96-well plate was coated with capture Ab/coating buffer solution and incubated overnight at 4 °C. The following morning, wells were washed for 1 min 5x with 275 µl wash buffer (Table 2.1), then blocked with 200 µl/well 1x assay diluent for 1 h at RT, followed by a wash step. Interleukin-1β standards (39-2500 pg/ml) were prepared using 2-fold serial dilutions of 1x assay diluent and 1µg/ml recombinant rat IL-1β cytokine, and 100 µl/well were added in duplicate, in repeats of 6 along with 100 µl/well of samples. The plate was sealed and incubated overnight at 4 °C. The following morning, wells were washed and then 100 µl of detection Ab/assay diluent solution was applied to each well, the plate re-sealed and allowed to incubate at RT for 1 h. Another wash step was followed by the application of 100 µl/well of Avidin-HRP/assay diluent solution; the plate was sealed and incubated at RT for 30 min. A final set of 7x 2 min wash steps was performed, then 100 µl/well of substrate solution added and the plate was incubated at RT for 15 min. The final step was the addition of 50 µl/well of stop solution (Table 2.1). The absorbance of the samples/standards was read at 450 nm using a Tecan GENios microplate reader. The unknown IL-1β concentration of samples was estimated by comparing their absorbance to that of the known IL-1β standard concentrations (Fig. 2.7).

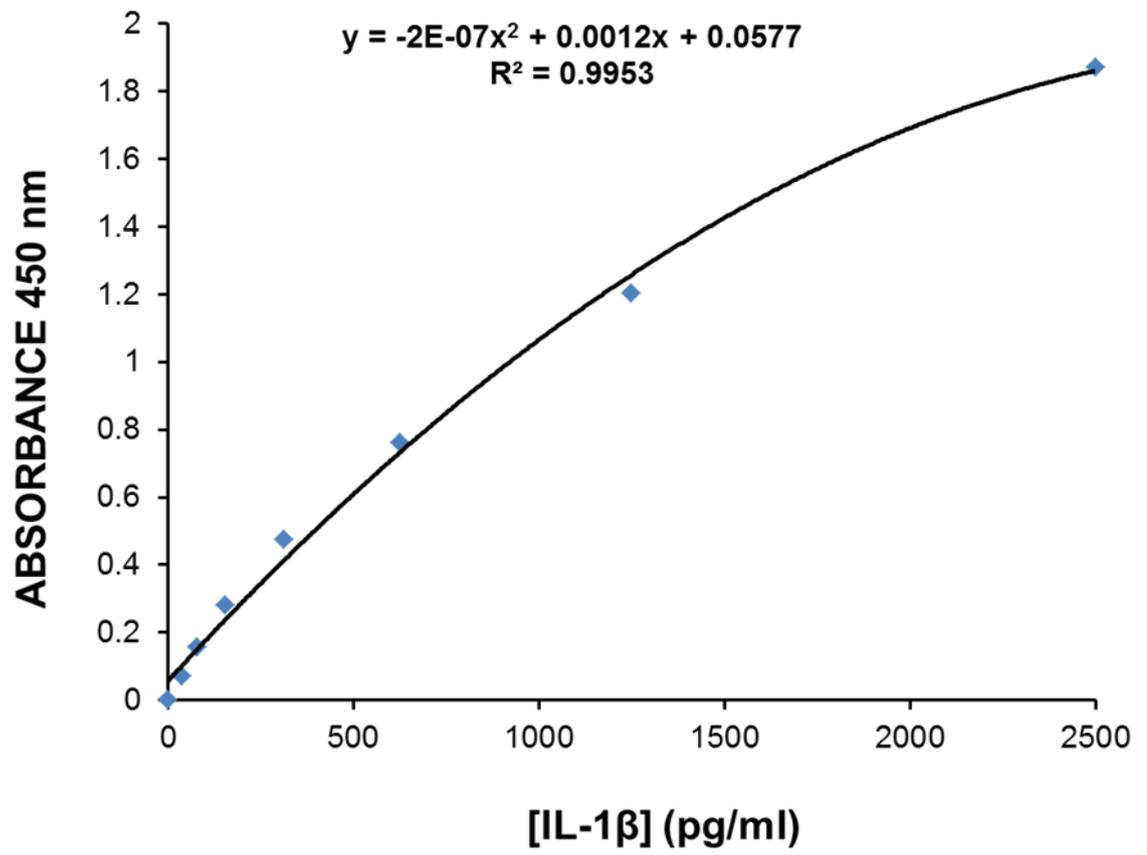


Fig. 2.7 ELISA standard curve for calculating IL-1 β concentration

2.9 Detection of cytosolic inflammasome protein components by immunocytochemistry (ICC)

Immunocytochemistry is a commonly used technique that allows the detection and visualisation of individual proteins expressed within cultured adherent cells, using antigen-specific antibodies. Thirteen millimetre glass coverslips were heat-sterilised at 140° C for 2 h and allowed to cool, then coated with a 5 mg/11.5 ml poly-D-lysine/dH₂O solution, allowed to dry overnight and kept sterile until used. Poly-D-lysine is a synthetic positively-charged amino acid chain, which promotes the adhesion of cells to the coverslips.

Cells were seeded onto coverslips placed into individual wells of a 24 well plate, at a density of 5 × 10³ cells/15 µl culture medium. Cells were incubated for 2 h to allow them to fully adhere to the coverslips, then each well was topped up to 500 µl of appropriate complete culture medium and left overnight, before treatments were conducted over various time periods.

Following incubation, the culture medium was aspirated and cells were fixed at RT with 400 µl/well 4% paraformaldehyde (Table 2.1) for 15 min, followed by two washes using 1 ml PBS. Paraformaldehyde is a cross-linking reagent that typically forms intermolecular bridges via free amino groups, resulting in a network of linked antigens that preserve cell structure. Paraformaldehyde-fixed cells were blocked and permeabilised to allow penetration of antibodies to intracellular target proteins, using 50 µl/CS 0.2% Triton X-100 antibody dilution solution (ADST) (Table 2.1) for 30 min at RT. The blocking solution was tipped off, and cells were probed using appropriate primary antibodies diluted in ADS overnight at 4 °C (Table 2.2). Cells were washed six times in PBS, and then

incubated for 30 min at RT using appropriate Alexa Fluor® conjugated secondary antibodies with DAPI to stain the nucleus of the cells. Cells were again washed six times in PBS, then mounted cell-side down on glass microscope slides using a small drop of Citifluor™ AF1 anti-fadent glycerol/PBS solution (Citifluor Ltd, London). The coverslips were then secured and sealed carefully around the edge to prevent movement and evaporation using clear nail varnish. Antibodies were visualised using a Nikon Eclipse 90i fluorescence microscope using DAPI and FITC channels, and micrographs were taken using NIS Elements software.

2.10 Data imaging and analysis

All experiments were performed in at least triplicate for each treatment group and repeated on at least three separate occasions. Results were expressed as mean \pm SEM; significance levels were calculated using Minitab General Linear Model ANOVA and were regarded as significant when $p < 0.05$. Densitometry was performed using analysis by Quantity One software, where optical density readings were normalised against loading controls in each well to eliminate variability in total protein loading.

Chapter 3 The role of PKR in pancreatic β -cell apoptosis

3.1 Introduction

Chronically elevated FFA concentrations as seen in obesity are proposed to exert a deleterious effect on pancreatic β -cell survival in a mechanism termed lipotoxicity. Although the exact mechanisms underlying this process are not well understood, lipotoxicity is suggested to underlie the progression of insulin resistance and insulin deficiency, and to drive a long-term reduction in β -cell mass, features that can be commonly seen in T2D (Cunha *et al.*, 2008; Butler *et al.*, 2003; Cnop *et al.*, 2005). The *in vitro* culture of pancreatic β -cell lines has provided many key observations regarding the molecular mechanisms that may cause lipotoxicity. Many studies have demonstrated that the degree of toxicity or protection afforded by a FA species during chronic incubation with pancreatic β -cells, can markedly vary according to the carbon chain length, degree of saturation or configuration of the individual FA species being studied (Newsholme *et al.*, 2007; Welters *et al.*, 2004; Eitel *et al.*, 2003; Dhayal *et al.*, 2008; Karaskov *et al.*, 2006; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Morgan *et al.*, 2008). Long-chain-SFAs ($\geq C16:0$) exert a detrimental effect on β -cell viability, whereas medium and shorter-chain SFAs ($\leq C14:0$) or LC-MUFAs ($\geq C16:1$) are well tolerated by β -cells and do not exert a detrimental effect on cell viability (Newsholme *et al.*, 2007; Welters *et al.*, 2004; Eitel *et al.*, 2003; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Morgan *et al.*, 2008). An additional important finding is that the *in vitro* co-incubation of LC-MUFAs with LC-SFAs, attenuates the cytotoxic effects observed in β -cells when they are incubated solely with LC-SFAs (Karaskov *et al.*, 2006; Welters *et al.*, 2004; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Morgan *et al.*, 2008). This protective mechanism exerted by LC-MUFA during co-incubation with toxic SFAs, is also

seen when rodent pancreatic β -cells are subjected to other detrimental stimuli, such as pro-inflammatory cytokines or serum withdrawal (Welters *et al.*, 2004).

The exact molecular mechanisms that mediate proapoptotic or protective actions of FFAs in pancreatic β -cells have yet to be fully elucidated, but recently, strong evidence has pointed towards a role for ER stress when associated with lipotoxicity (Qu *et al.*, 2009; Breckenridge *et al.*, 2003; Laybutt *et al.*, 2007). A lipotoxic environment is suggested to cause perturbations in normal ER balance and trigger an ERS (Eizirik *et al.*, 2008; Özcan *et al.*, 2004; Kharroubi *et al.*, 2004; Scheuner and Kaufman, 2008; Karaskov *et al.*, 2006; Cnop *et al.*, 2007). An ERS initiates the induction of four adaptive signal transduction responses termed the UPR, a predominantly protective response that attempts to restore ER homeostasis. However, prolonged or persistent ER stress may eventually lead to β -cell apoptosis (Qu *et al.*, 2009; Fonseca *et al.*, 2009). Apoptotic progression of pancreatic β -cells under ER stress conditions has been well established involving the PERK-dependent arm of the ERS (Morgan *et al.*, 2008; Diakogiannaki *et al.*, 2008; Cnop *et al.*, 2008; Diakogiannaki and Morgan, 2008; Scheuner and Kaufman, 2008; Laybutt *et al.*, 2007; Song *et al.*, 2007; Lee *et al.*, 2007).

Research has recently questioned whether PERK is the sole apoptotic-inducing factor within ER stress conditions. Results from studies suggest that PERK may have a less significant role in ER stress-mediated apoptosis than previously thought (Lee *et al.*, 2007; Lai *et al.*, 2008; Morgan, 2009). Williams (1999) first suggested a link between PKR and cellular stress mechanisms. However, in 2007, Lee and colleagues showed that PKR is simultaneously but independently

activated alongside PERK under lipotoxic conditions. In these circumstances it has been proposed that PERK may take on a predominantly cell survival role, whereas PKR may assume a predominantly proapoptotic role (Lee *et al.*, 2007). This was the first time an association had been made between PKR and nutrient overload.

Therefore these studies aimed to more fully investigate the role of PKR in rodent pancreatic β -cells exposed to FA species. Specifically, the aim was to explore whether the PKR protein is responsible for mediating proapoptotic responses when β -cells are subjected to lipotoxic conditions. Manipulation of PKR expression using a small molecule inhibitor, a dominant negative adenoviral vector system and RNAi methods were adopted, to examine whether alterations to the level of PKR activation in β -cells *in vitro*, had an impact on cell viability within a lipotoxic environment.

3.2 Materials and methods

3.2.1 Cell culture and FA treatment conditions

The culture of all rodent pancreatic β -cell lines was performed as described in Section 2.2. The use of PERK and PKR dominant negative adenoviral vectors were conducted as cited in Sections 2.6.1 and 2.6.2. The stable BRIN-BD11 PKR KD clonal cell lines (BRIN-BD11 PKR KD 333, BRIN-BD11 PKR KD 335, BRIN-BD11 PKR SV) were generated and cultured as described in Sections 2.2 and 2.6.6. Fatty acid solutions were prepared and experiments conducted using serum free culture medium (Section 2.3). Cell viability was assessed using vital dye staining (Section 2.4.1) or using PI staining via flow cytometry (Section 2.4.2), and this was used to calculate the proportion of dead cells which were expressed

as a percentage of the total for each treatment group. Western blotting was performed as described in Section 2.5.

3.2.2 Compound-16 treatment conditions

A 25 mM stock solution of Compound-16 (Fig. 3.1) was prepared in DMSO. All stocks were stored at -80 °C until required. Lower molar solutions of Compound-16 were prepared by diluting in serum free culture medium to the required concentration, and cells were pre-treated for 1 h before the addition of FA treatments.

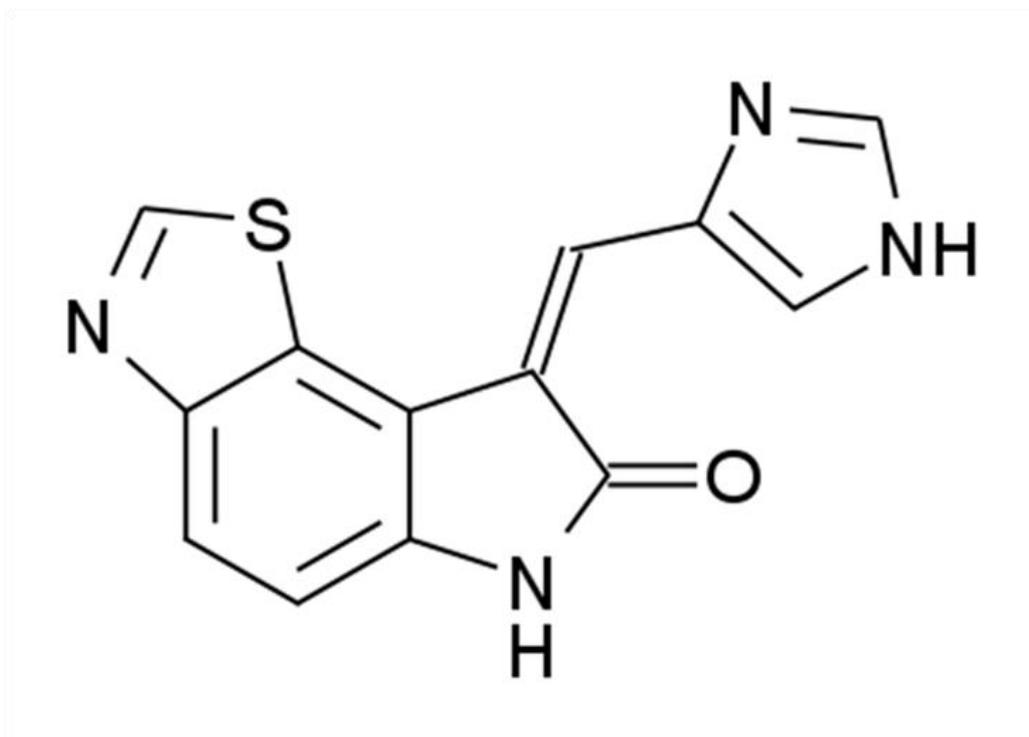


Fig. 3.1 A putative selective PKR inhibitor: Compound-16 structure

Imidazo-oxindole (Compound-16) is reported to be a selective and potent inhibitor of PKR. Its inhibitory effect on PKR is ATP-binding site directed. Compound-16 is stated to specifically inhibit the apoptotic PKR/eIF2 α /ATF4/CHOP10 pathway without stimulating the proliferative mTOR/p70S6K signalling pathway (Sigma-Aldrich Co., 2012).

3.3 Results

3.3.1 SFAs are cytotoxic to rodent pancreatic β -cells

Many earlier studies have established that chronic incubation with LC-SFAs (\geq C16:0) such as palmitate is cytotoxic to rodent pancreatic β -cells (BRIN-BD11, MIN6, RIN and INS-1) and isolated human pancreatic β -cells (Diakogiannaki *et al.*, 2007; Biden *et al.*, 2004; Eitel *et al.*, 2003; Eitel *et al.*, 2002; El-Assaad *et al.*, 2003; Kharroubi *et al.*, 2004). Our initial experiments were conducted to verify that previous results from our group were reproducible using laboratory-specific protocols and cell lines. There have been inconsistencies in the extent of SFA-induced toxicity reported in different rodent pancreatic β -cells *in vitro* (Lai *et al.*, 2008; Busch *et al.*, 2005).

Therefore, the first set of experiments employed the BRIN-BD11, INS-1 and MIN6 rodent pancreatic β -cells to compare the percentage cell death observed between the cell lines, when they were incubated with 250 μ M palmitate over a 24 h incubation period. This concentration of palmitate was adopted as it has been consistently used by our group and considered representative of FFA concentrations that may typically be observed in a physiologically hyperlipidaemic state (Welters *et al.*, 2004; Diakogiannaki *et al.*, 2007).

From the results obtained, it was apparent that the BRIN-BD11 cells were far more susceptible to palmitate-induced cell death than the INS-1 and MIN6 cell lines during this incubation period (Fig. 3.2). Nonetheless, all palmitate-treated cells exhibited a significant increase in percentage cell death compared to their control ($p < 0.001$). The percentage cell deaths observed from this experiment were as follows: BRIN-BD11 control (6.4%), BRIN-BD11 treated with palmitate

(93.1%); INS-1 control (5.2%), INS-1 treated with palmitate (37.1%); MIN6 control (4.2%), MIN6 treated with palmitate (22.3%).

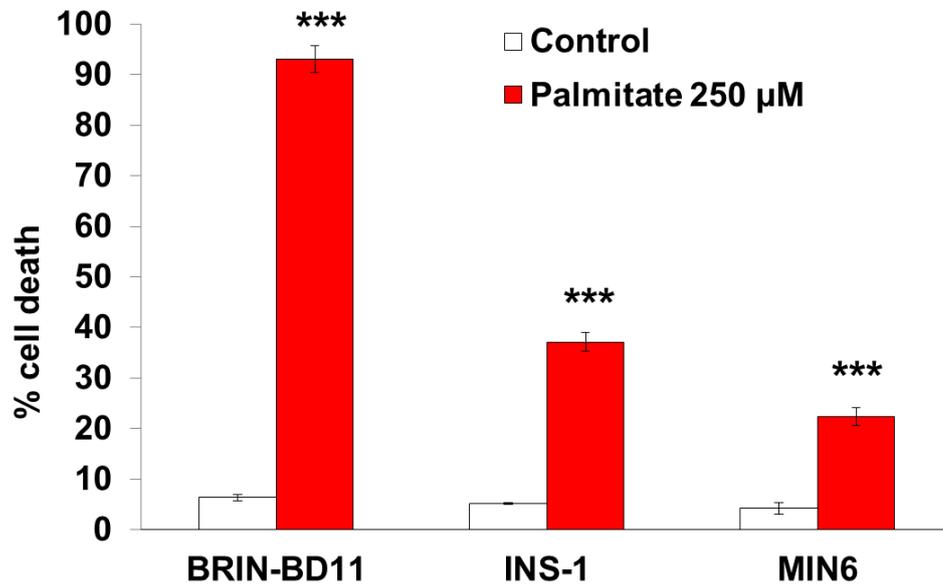


Fig. 3.2 The cytotoxic effect of palmitate on rodent β -cell death over a 24 h incubation period

The rodent β -cell lines BRIN-BD11, INS-1 and MIN6 were treated with 250 μ M palmitate over a 24 h incubation period. Cell death was assessed by trypan blue vital dye staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** $p < 0.001$ vs. control

These experiments were subsequently repeated to determine an optimal incubation period for each individual cell line, with the aim of observing a similar magnitude of percentage of cell death in each cell line for comparison purposes. The incubation period with palmitate was extended to 24 h for INS-1 cells and to 72 h for MIN6 cells. This resulted in a similar level of cell death seen over an 18 h incubation period using BRIN-BD11 cells (Fig. 3.3). Again, all palmitate-treated cells demonstrated a significant increase in percentage cell death compared to their control group ($p < 0.001$). To fully quantify these results, percentage cell deaths were as follows: BRIN-BD11 control (6.2%), BRIN-BD11 treated with palmitate (36.9%); INS-1 control (5.4%), INS-1 treated with palmitate (37.9%); MIN6 control (9.8%), MIN6 treated with palmitate (59%). Unless stated otherwise, the incubation periods for individual cell lines remained as detailed here for the rest of the studies in this section.

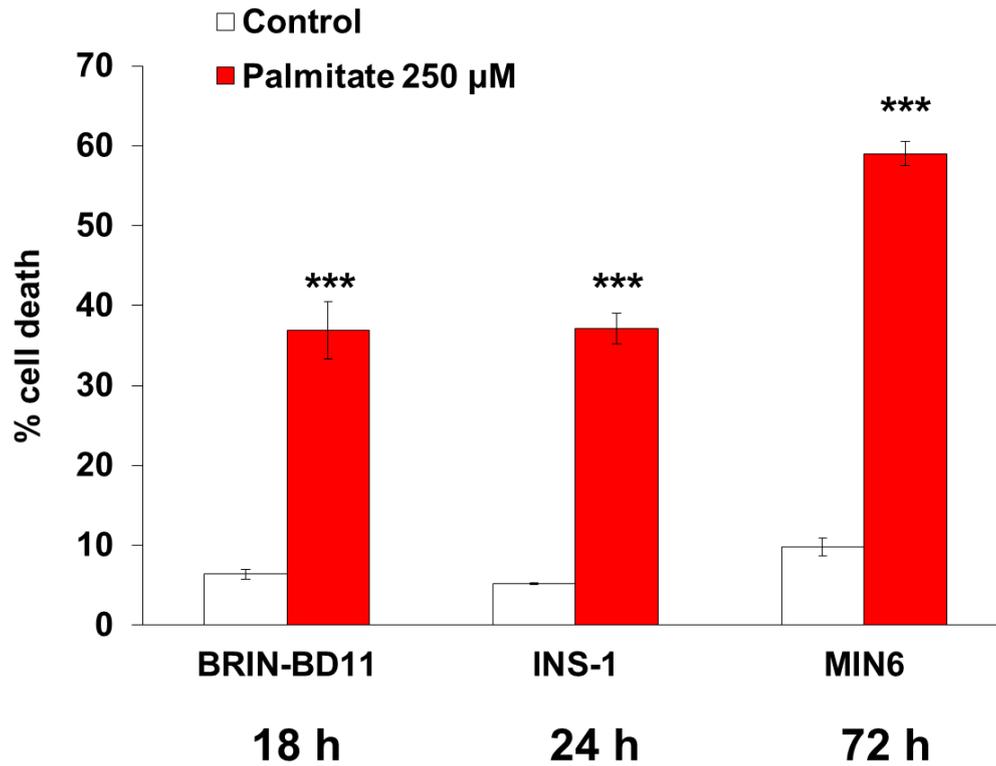


Fig. 3.3 The cytotoxic effect of palmitate on rodent β -cell death: optimal incubation times

Rodent β -cells were treated with 250 μ M palmitate: BRIN-BD11 cells were incubated for 18 h, INS-1 cells were incubated for 24 h and MIN6 cells were incubated for 72 h. Cell death was assessed by trypan blue vital dye staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** $p < 0.001$ vs. control

Although the exact mechanism whereby palmitate causes β -cell apoptosis is yet to be fully elucidated, there has been much recent evidence to suggest that ER stress plays a role in this process. Western blotting was used to examine the effect of palmitate on rodent pancreatic β -cells, to investigate any changes in the phosphorylation of the widely-accepted ER stress marker eIF2 α (denoted as phospho-eIF2 α) (Fig. 3.4). Results for the BRIN-BD11 cells (Fig. 3.4a) showed a 3.9-fold increase in phospho-eIF2 α expression in the 250 μ M palmitate group compared to the control group ($p < 0.001$). The INS-1 cells (Fig. 3.4b) showed a 4.3-fold increase in phospho-eIF2 α expression in the 250 μ M palmitate group compared to the control ($p < 0.001$). Finally, the MIN6 cells (Fig. 3.4c) showed a 1.3-fold increase in phospho-eIF2 α expression in the 250 μ M palmitate group compared with the control ($p < 0.01$).

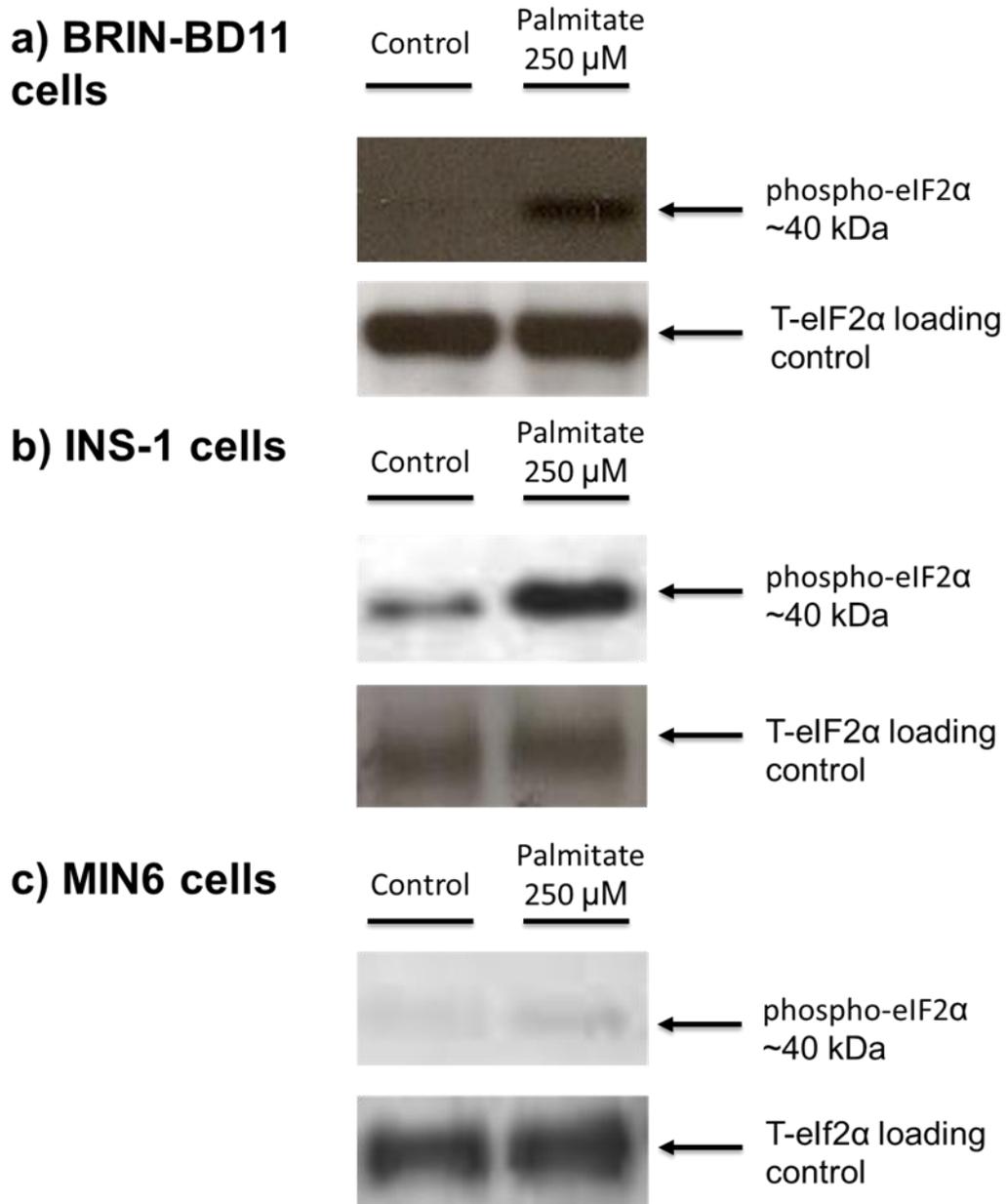


Fig. 3.4 The effect of palmitate on ER stress in rodent β -cells

Rodent β -cells were treated with 250 μ M palmitate: BRIN-BD11 cells were incubated for 18 h, INS-1 cells were incubated for 24 h and MIN6 cells were incubated for 72 h. After incubation, whole cell lysates were probed using phospho-eIF2 α antibody. Results represent blots from experiments performed in triplicate (N=3).

Our group has previously reported that the cytotoxic effect observed when BRIN-BD11 β -cells are incubated with palmitate is dose-dependent (Welters *et al.*, 2004; Welters *et al.*, 2006, Diakogiannaki *et al.*, 2007). To corroborate these findings and to compare variability between cell lines, we conducted experiments using BRIN-BD11, INS-1 and MIN6 cells incubated with palmitate concentrations that ranged from 10 μ M to 250 μ M (Fig. 3.5). The results showed that palmitate clearly increased percentage cell death in a dose-dependent manner in all three cell lines tested. To quantify these results, percentage cell deaths in the BRIN-BD11 cells (Fig. 3.5a) were as follows: control (15.7%); 10 μ M palmitate (20.2%); 25 μ M palmitate (35.6%, $p < 0.01$); 50 μ M palmitate (45.6%, $p < 0.001$); 100 μ M palmitate (66.4%, $p < 0.001$) and 250 μ M palmitate (81.6%, $p < 0.001$). The EC_{50} of this dose-response curve for the BRIN-BD11 cells was ~ 20 μ M. Percentage cell deaths in the INS-1 cells (Fig. 3.5b) were: control (7.9%); 10 μ M palmitate (6.5%); 25 μ M palmitate (10.6%); 50 μ M palmitate (19.2%, $p < 0.05$); 100 μ M palmitate (31.1%, $p < 0.001$) and 250 μ M palmitate (70.3%, $p < 0.001$). The EC_{50} in the INS-1 cells was ~ 80 μ M. Finally, the percentage cell deaths in the MIN6 cells (Fig. 3.5c) were: control (13.2%); 15 μ M palmitate (27.7%, $p < 0.001$); 25 μ M palmitate (39.8%, $p < 0.001$); 50 μ M palmitate (56.8%, $p < 0.001$); 100 μ M palmitate (61.5%, $p < 0.001$) and 250 μ M palmitate (72.2%, $p < 0.001$). The EC_{50} in the MIN6 cells was ~ 10 μ M.

Furthermore, the increased percentage cell deaths observed in all cell lines incubated with 250 μ M palmitate, correlated with the increased phosphorylation status of eIF2 α under the same experimental conditions as demonstrated by our previous study.

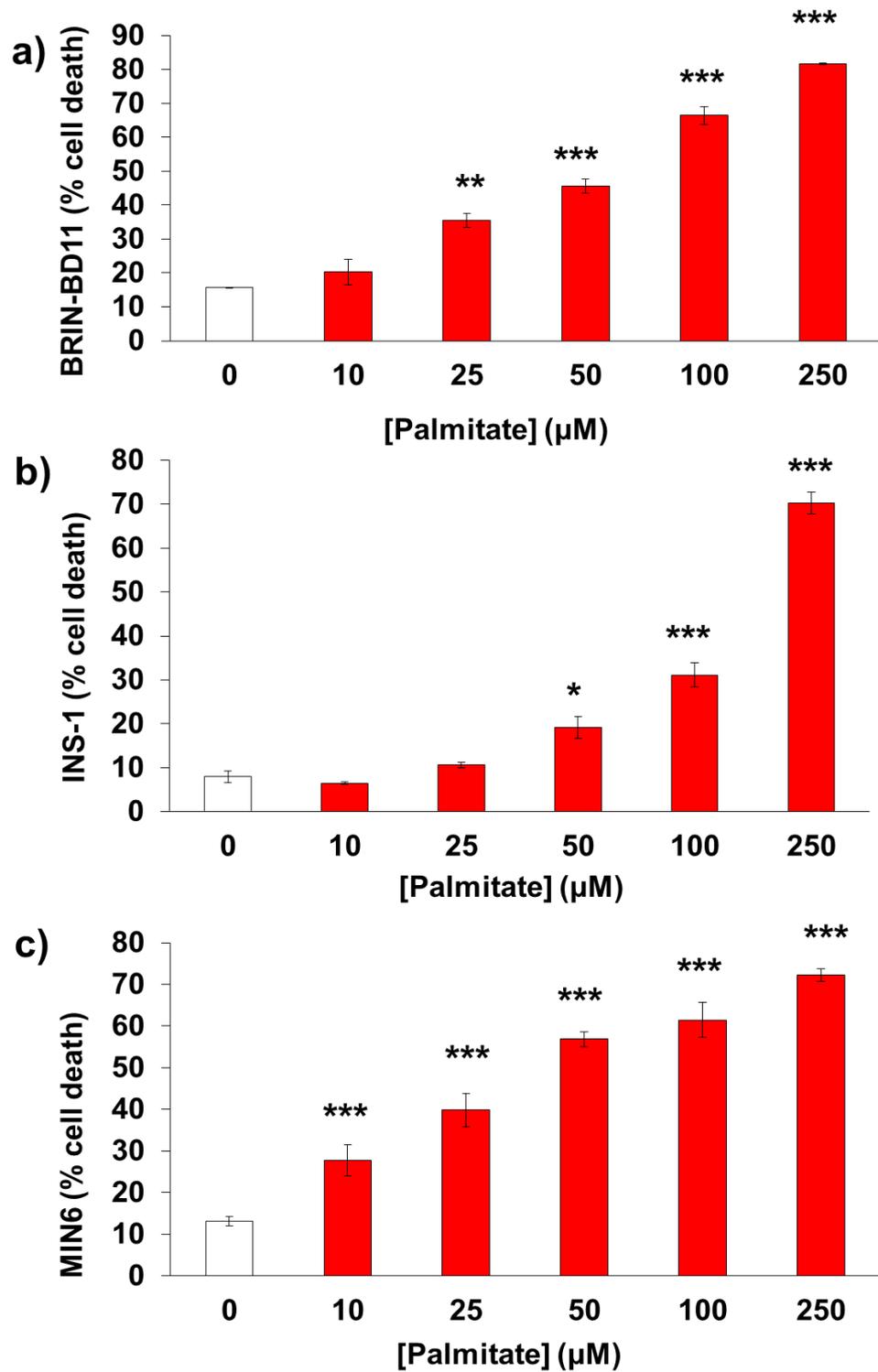


Fig. 3.5 The cytotoxic effect of palmitate on rodent β -cell death is dose-dependent

Rodent β -cells were treated with increasing palmitate concentrations: a) BRIN-BD11 incubated for 18 h, b) INS-1 incubated for 24 h, c) MIN6 incubated for 72 h. Cell death was assessed by trypan blue vital dye staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control

3.3.2 MUFAs are cytoprotective to rodent pancreatic β -cells

The loss of viability observed in pancreatic β -cells when they are incubated with the LC-SFA palmitate is ameliorated during co-incubation with the LC-MUFA palmitoleate. When cultured alone with pancreatic β -cells, palmitoleate is shown to be well-tolerated and does not affect cell death. Our group and others have also demonstrated that palmitoleate exerts a protective effect against proapoptotic stimuli such as serum withdrawal (Welters *et al.*, 2004; Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2007; Diakogiannaki and Morgan, 2008; Diakogiannaki *et al.*, 2008; Eitel *et al.*, 2003; Eitel *et al.*, 2002; Maedler *et al.*, 2003). Therefore, the cytoprotective action of palmitoleate was investigated in BRIN-BD11, INS-1 and MIN6 cells (Fig. 3.6). Incubation with 250 μ M palmitate resulted in a significant increase in percentage cell death in all cell lines when compared with control groups ($p < 0.001$) and this is in line with our previous results. Palmitoleate at 250 μ M was well-tolerated in all three cell lines, as no increase in percentage cell death was observed when compared to the control. More critically however, was the observation that co-incubation of 250 μ M palmitoleate with 250 μ M palmitate completely ameliorated the increased percentage cell death observed when cells were incubated with 250 μ M palmitate alone. To fully quantify these results, percentage cell deaths in the BRIN-BD11 cells (Fig. 3.6a) were as follows: control (6.4%); 250 μ M palmitate (36.9%); 250 μ M palmitoleate (5.8%) and both palmitate and palmitoleate (5.2%, $p < 0.001$ vs 250 μ M palmitate). The percentage cell deaths in the INS-1 cells (Fig. 3.6b) were: control (5.2%); 250 μ M palmitate (37.1%); 250 μ M palmitoleate (4.2%) and both palmitate and palmitoleate (5.8%, $p < 0.001$ vs 250 μ M palmitate). Finally, the percentage cell deaths in the MIN6 cells (Fig. 3.6c) were: control (13.2%); 250

μM palmitate (61.2%); 250 μM palmitoleate (9.5%) and both palmitate and palmitoleate (11.5%, $p < 0.001$ vs 250 μM palmitate).

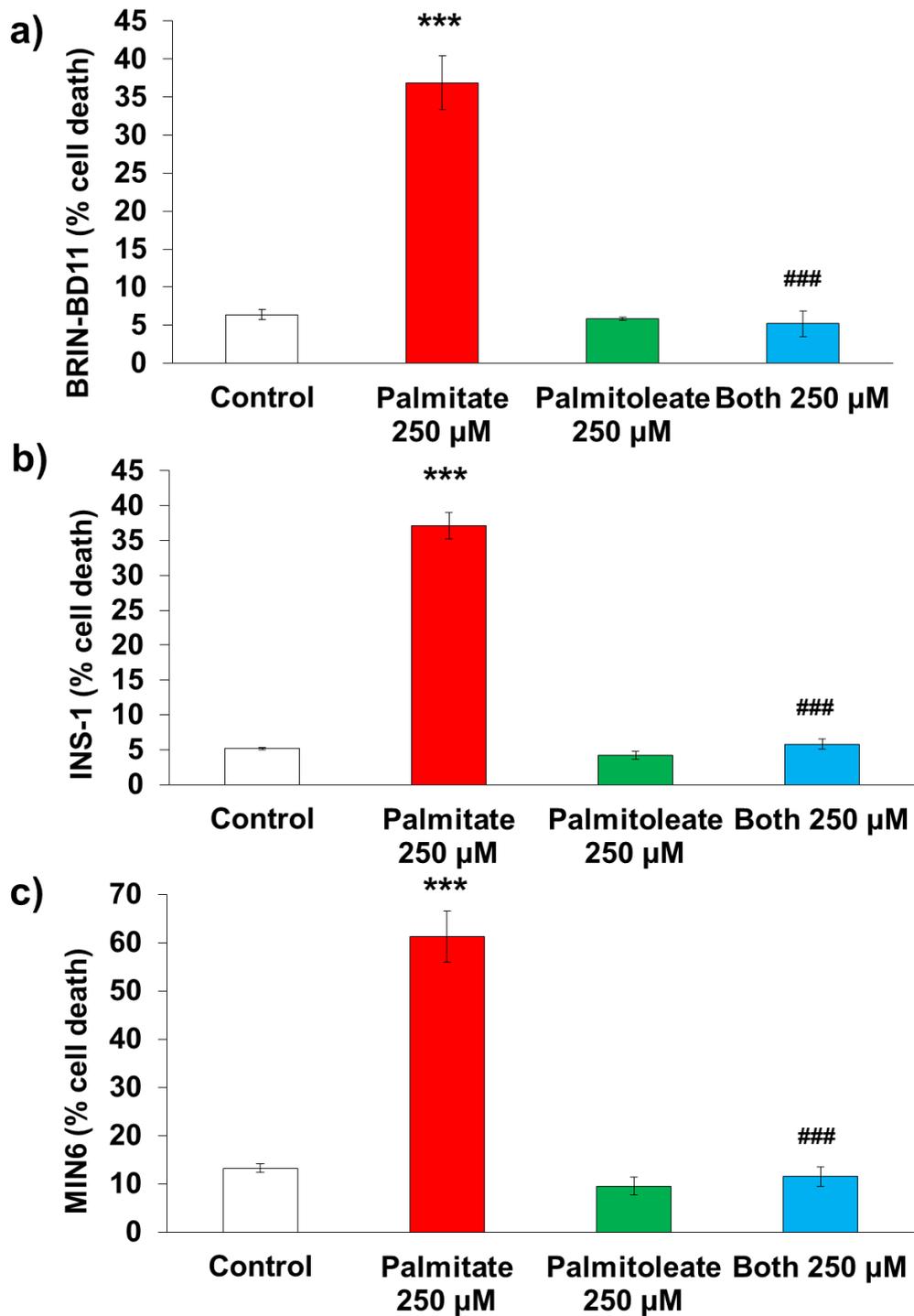


Fig. 3.6 The cytoprotective effect of palmitoleate on rodent β -cell death

Rodent β -cells were treated with 250 μ M palmitate, 250 μ M palmitoleate or a combination of both FA: a) BRIN-BD11 incubated for 18 h, b) INS-1 for 24 h and c) MIN6 incubated for 72 h. Cell death was assessed by trypan blue vital dye staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. palmitate

Western blotting was used to investigate the effect of these LC-FAs on the phosphorylation status of eIF2 α in BRIN-BD11 pancreatic β -cells (Fig. 3.7). Results showed a 7.1-fold increase in eIF2 α phosphorylation compared to the control when BRIN-BD11 cells were incubated with 250 μ M palmitate ($p<0.001$), and this correlated with significantly increased percentage cell death as shown in the previous study. The phosphorylation of eIF2 α when BRIN-BD11 cells were incubated with palmitoleate alone showed a 3.2-fold increase compared to the control and although this was significant ($p<0.01$), upregulation of phospho-eIF2 α under these treatment conditions did not affect cell viability as no increase in percentage cell death was observed. Finally, phosphorylation of eIF2 α when BRIN-BD11 cells were co-incubated with palmitate and palmitoleate showed a 6.7-fold increase compared to the control and again, although this was significant ($p<0.001$), upregulation of phospho-eIF2 α under these experimental conditions did not result in increased percentage cell death.

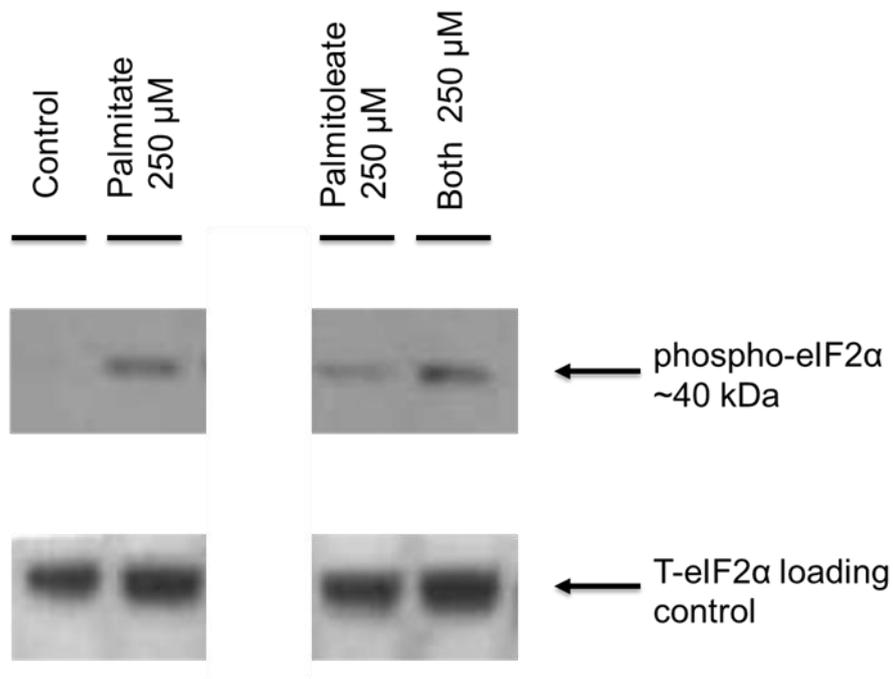


Fig. 3.7 Palmitoleate can ameliorate ER stress in BRIN-BD11 cells

BRIN-BD11 β -cells were incubated for 18 h with 250 μ M palmitate or 250 μ M palmitoleate alone, or co-incubated with both 250 μ M palmitate and palmitoleate, compared to control. Following incubation, whole cell lysates were probed using phospho-eIF2 α antibody. Results represent blots from experiments performed in triplicate (N=3).

In contrast to the dose-dependent cytotoxic effect observed when incubating rodent cells with palmitate, palmitoleate has been reported to exert a dose-dependent cytoprotective effect in BRIN-BD11 and INS-1 cells. This cytoprotective effect was investigated here in MIN6 cells, and BRIN-BD11 and INS-1 cells by co-incubating them with 250 μM palmitate, and adding palmitoleate in concentrations ranging from 10 μM to 250 μM . Results from these studies clearly demonstrated that the co-incubation of palmitoleate with palmitate exerts a significant cytoprotective effect in all cell lines (Fig. 3.8). Results were most striking in the BRIN-BD11 cells and INS-1 cells, and showed that the addition of even the lowest concentration of palmitoleate (10 μM), significantly reduced the extent of cell death ($\text{EC}_{50} \sim 15 \mu\text{M}$). This cytoprotective effect was slightly less efficacious in MIN6 cells, but nevertheless still showed a significant protective action ($\text{EC}_{50} \sim 45 \mu\text{M}$). The percentage cell deaths observed in the BRIN-BD11 cells (Fig. 3.8a) were as follows: control (250 μM palmitate), 75.2%; 250 μM palmitate and 10 μM palmitoleate, 47.8% ($p < 0.001$); 250 μM palmitate and 25 μM palmitoleate, 27.9% ($p < 0.001$); 250 μM palmitate and 50 μM palmitoleate, 7.6% ($p < 0.001$); 250 μM palmitate and 100 μM palmitoleate, 2.6% ($p < 0.001$) and 250 μM palmitate and 250 μM palmitoleate, 1.6% ($p < 0.001$). The percentage cell deaths observed in the INS-1 cells (Fig. 3.8b) were: control (250 μM palmitate), 64.3%; 250 μM palmitate and 10 μM palmitoleate, 40.7% ($p < 0.01$); 250 μM palmitate and 25 μM palmitoleate, 16.7% ($p < 0.001$); 250 μM palmitate and 50 μM palmitoleate, 13.7% ($p < 0.001$); 250 μM palmitate and 100 μM palmitoleate, 6.4% ($p < 0.001$) and 250 μM palmitate and 250 μM palmitoleate, 4.8% ($p < 0.001$). Finally, percentage cell deaths observed in the MIN6 cells (Fig. 3.8c) were: control (250 μM palmitate), 60.1%; 250 μM palmitate and 10 μM palmitoleate, 58.7%; 250 μM palmitate and 25 μM palmitoleate, 41.1%

($p < 0.001$); 250 μM palmitate and 50 μM palmitoleate, 27.5% ($p < 0.001$); 250 μM palmitate and 100 μM palmitoleate, 11.6% ($p < 0.001$) and 250 μM palmitate and 250 μM palmitoleate, 11.9% ($p < 0.001$).

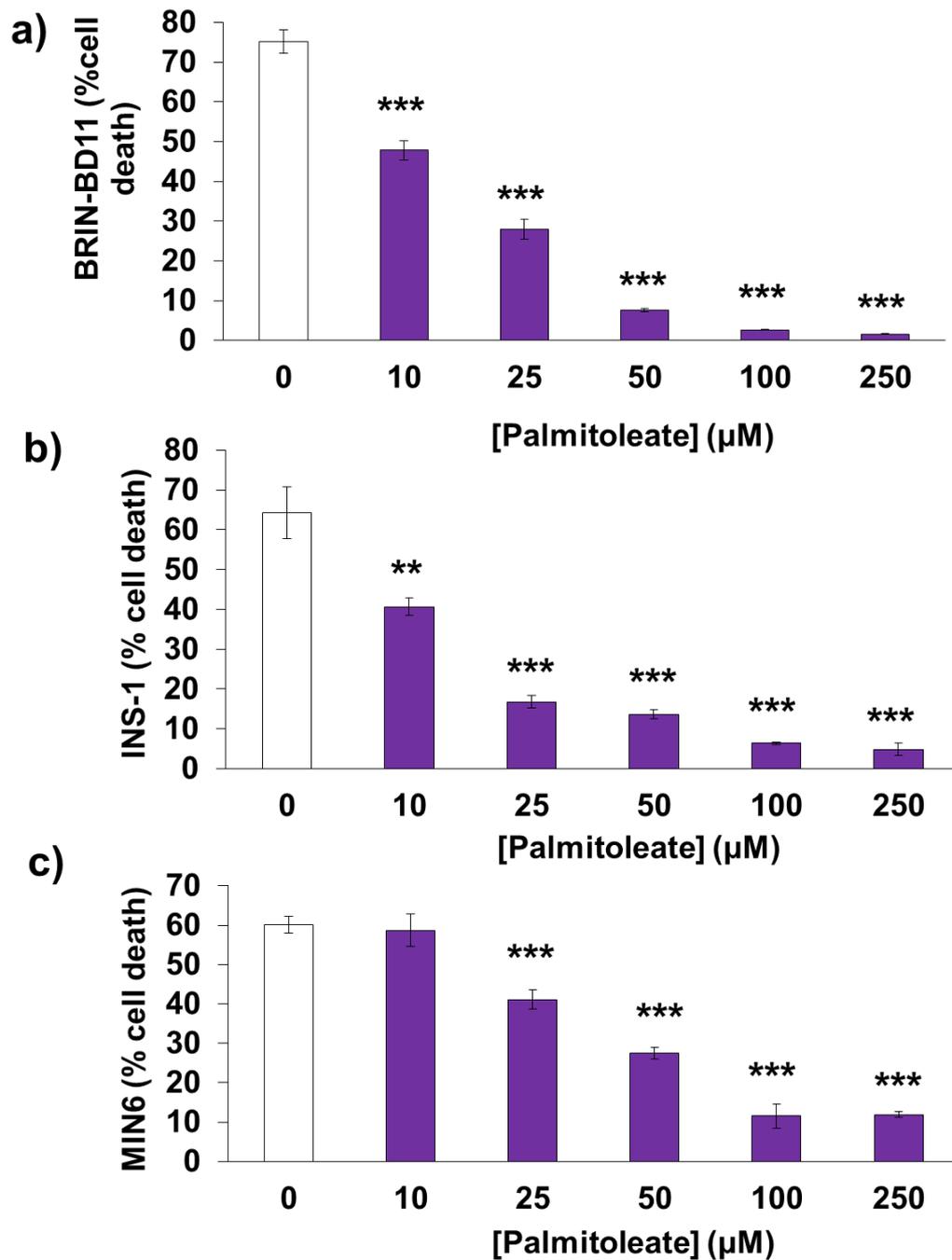


Fig. 3.8 The cytoprotective effect of palmitoleate on rodent β -cell death is dose-dependent

Rodent β -cells were treated with 250 μ M palmitate and increasing concentrations of palmitoleate: a) BRIN-BD11 incubated for 18 h, b) INS-1 for 24 h, c) MIN6 cells for 72 h. Cell death was assessed by trypan blue vital dye staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** $p < 0.001$, ** $p < 0.01$ vs. control

3.3.3 Compound-16 is cytoprotective to BRIN-BD11 rodent pancreatic β -cells under lipotoxic stress

The molecular mechanism underlying the cytotoxic action of palmitate in rodent β -cells is not well understood. The involvement of the stress-sensing PKR protein has been recently suggested to be a potential key player in mediating the proapoptotic responses observed in rodent β -cells during lipotoxic conditions. Therefore, the availability of a reputedly specific small molecule PKR inhibitor (Compound-16), offered a potential method to investigate whether inhibition of this protein had an impact on cytotoxicity induced in rodent β -cells upon exposure to palmitate. Initial experiments were conducted to assess the effect of Compound 16 on the viability of BRIN-BD11 cells when co-incubated with palmitate. BRIN-BD11 cells were incubated with Compound-16 using various concentrations ranging from 1 μ M to 25 μ M. A second group was pre-incubated for 1 h with Compound-16, but then co-incubated with 250 μ M palmitate for the remaining incubation period.

Our results have demonstrated here for the first time in BRIN-BD11 pancreatic β -cells, that Compound-16 did not detrimentally affect viability when cultured alone at any concentration (Fig. 3.9). Furthermore, this novel data also shows that between 5 μ M and 25 μ M concentrations, Compound-16 afforded significant cytoprotection against palmitate-induced cell death ($p < 0.001$) when compared to the control group ($IC_{50} \sim 5 \mu$ M). To fully quantify these findings, percentage cell deaths in the control group (no palmitate) were as follows: control, 16.7%; 5 μ M Compound-16, 16.4%; 10 μ M Compound-16, 16.3% and 25 μ M Compound-16, 15.6%. Percentage cell deaths in the group co-incubated with 250 μ M palmitate

and Compound-16 were: control (250 μ M palmitate), 59.8%; 5 μ M Compound-16, 31.3%; 10 μ M Compound-16, 30.6% and 25 μ M Compound-16, 36.4%.

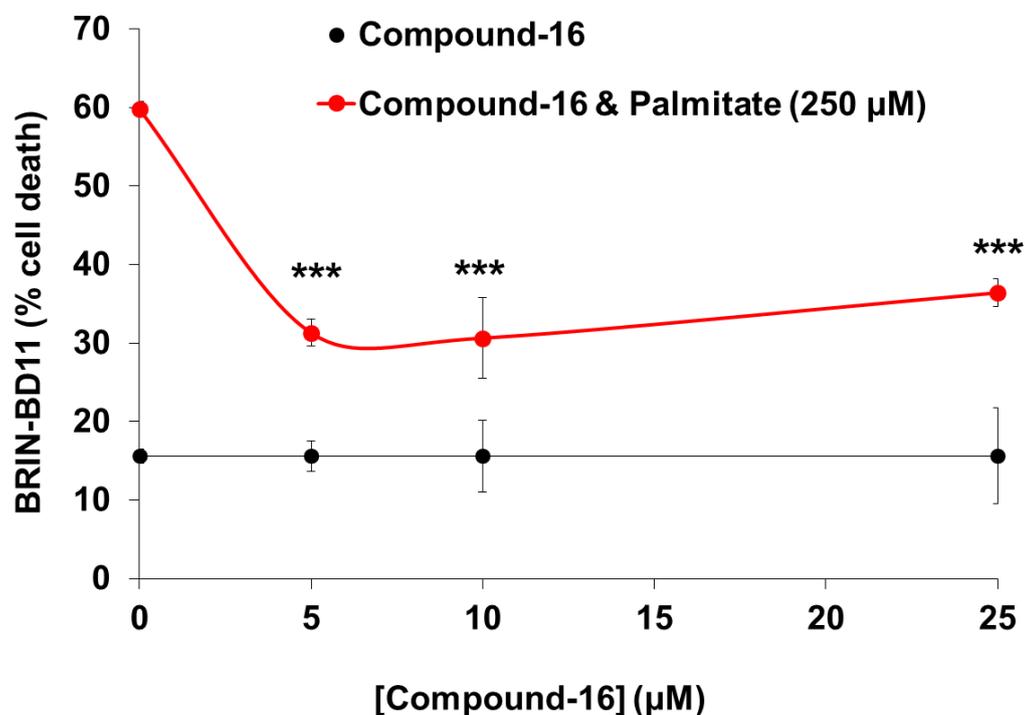


Fig. 3.9 The cytoprotective effect of Compound-16 on BRIN-BD11 β -cell death when incubated with palmitate

BRIN-BD11 rodent β -cells were treated with various concentrations of Compound-16. A second treatment group of BRIN-BD11 cells were pre-incubated with the same concentrations of Compound-16 for 1 h, then incubated for a further 17 h with 250 μ M palmitate. Cell death was assessed by trypan blue vital dye. Results represent mean \pm SEM from experiments in triplicate (N=3). *** $p < 0.001$ vs. control

Recent studies have identified PKR as a protein central to nutrient sensing (Nakamura *et al.*, 2010). Therefore, we sought to investigate in the BRIN-BD11 cell model whether PKR may be activated in response to nutrient deprivation as well as nutrient overload as shown previously in mouse models (Nakamura *et al.*, 2010). We conducted experiments to examine whether incubation with Compound-16 might rescue BRIN-BD11 cells from death induced by serum-starvation. Cells were cultured in serum-free conditions, and incubated with various concentrations of Compound-16 over increasing time intervals. Results from these studies demonstrate for the first time that at 10, 15 and 25 μM concentrations, Compound-16 was able to significantly protect BRIN-BD11 cells against death induced by serum-starvation ($p < 0.001$). Fully quantified percentage cell deaths for these studies were: complete culture medium control, 12.1%; serum-free, 64.2%; serum-free and 10 μM Compound-16, 41.5%; serum-free and 15 μM Compound-16, 38.3%; serum-free and 25 μM Compound-16, 46.7%. Furthermore, through repeated time-course experiments, it was established that this cytoprotective action was effective for up to a 30 h incubation period before significant protection by Compound-16 was lost (Fig. 3.10).

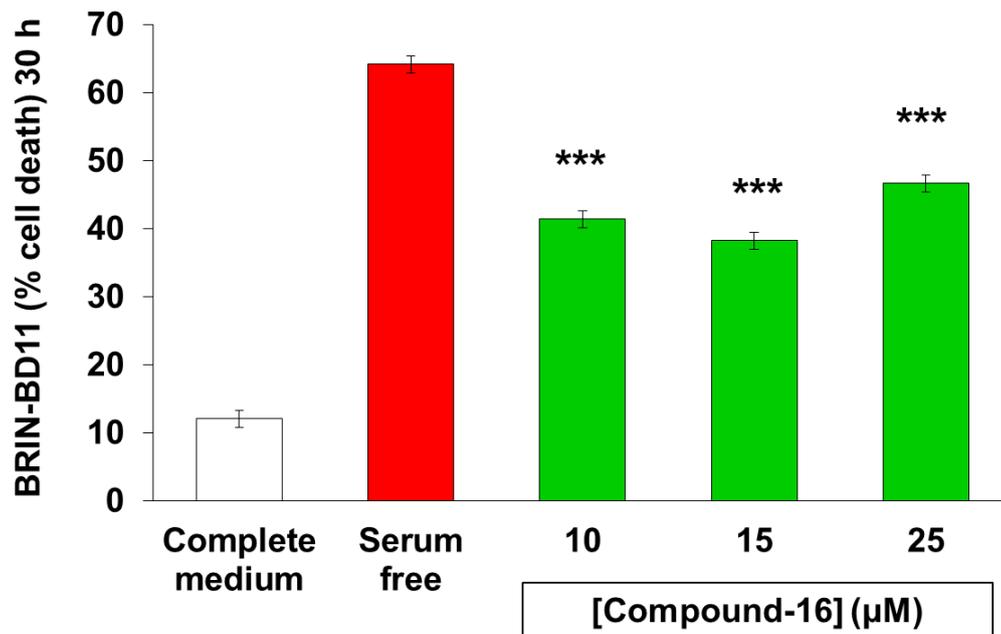


Fig. 3.10 The cytoprotective effect of Compound-16 on BRIN-BD11 β -cell death during serum starvation

BRIN-BD11 rodent β -cells were cultured in serum-free conditions, with varying concentrations of Compound-16 for up to a 30 h incubation period. Cell death was assessed by trypan blue vital dye. Results represent mean \pm SEM from experiments (N=6). *** $p < 0.001$ vs. control

3.3.4 Compound-16 improves BRIN-BD11 viability but upregulates the ER stress marker phospho-eIF2 α under lipotoxic stress

The results from our previous experiments clearly demonstrate that incubating cells with Compound-16 significantly reduced palmitate-induced cytotoxicity in BRIN-BD11 cells. Western blotting was conducted using the ER stress marker eIF2 α , to investigate whether any changes in the phosphorylation status of this protein correlated with the improved cell viability seen. BRIN-BD11 cells were incubated with 250 μ M palmitate, 5 μ M Compound-16 (the previously established IC₅₀), and co-incubated with 5 μ M Compound-16 and 250 μ M palmitate (Fig. 3.11). Whole cell lysates were probed for phospho-eIF2 α as described in Section 2.5. There was a 7.5-fold increase in phospho-eIF2 α ($p < 0.001$) compared to the control. Somewhat surprisingly, there was an even greater 8.9-fold upregulation of phospho-eIF2 α observed in BRIN-BD11 cells when they were incubated with Compound-16 compared to untreated control cells ($p < 0.001$), despite the reduction in percentage cell death as shown in our previous results. There was a 7.9-fold increase in phospho-eIF2 α upregulation when cells were co-incubated with 250 μ M palmitate and Compound-16 ($p < 0.001$), and this was 1.1-fold less than when cells were incubated with 250 μ M palmitate alone.

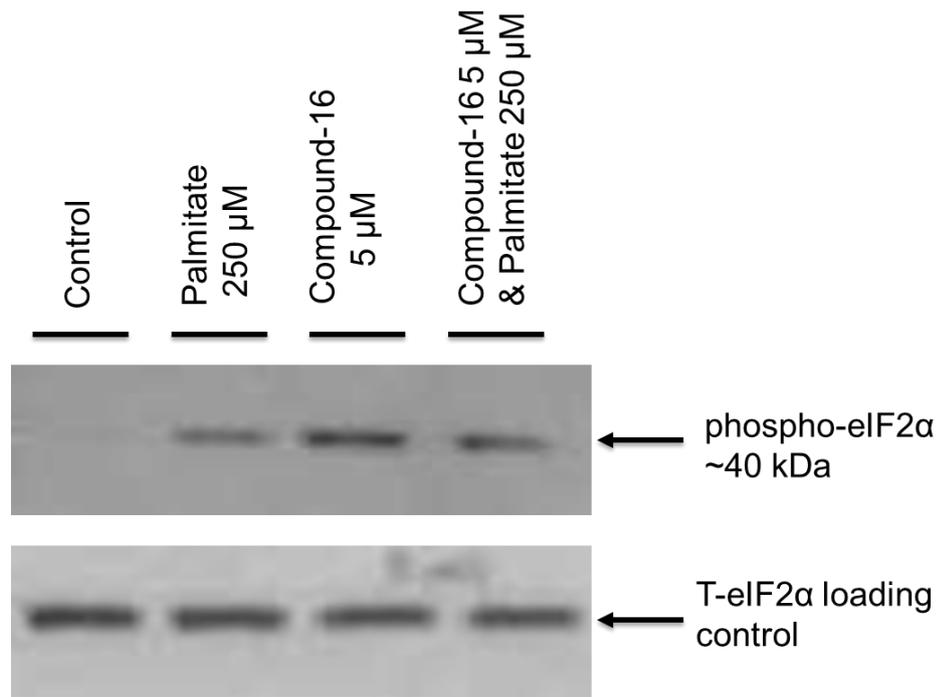


Fig. 3.11 The effect of Compound-16 on the ER stress marker phospho-eIF2α in BRIN-BD11 cells

BRIN-BD11 β -cells were incubated for 18 h with various combinations of 250 μ M palmitate, 5 μ M Compound-16 or 250 μ M palmitoleate. Following incubation, whole cell lysates were probed using phospho-eIF2 α antibody. Results represent blots from experiments performed in triplicate (N=3).

3.3.5 Compound-16 is toxic to INS-1 cells

The cytoprotection afforded by Compound-16 in BRIN-BD11 cells was investigated here for the first time to our knowledge in the INS-1 cell model. These cells were incubated following the protocol as detailed previously in Section 3.3.3. Cell viability was assessed by trypan blue vital dye. Surprisingly, despite multiple replications to test a wide range of Compound-16 concentrations, it was clear that this compound was poorly tolerated by the INS-1 cells (Fig. 3.12). Incubation with Compound-16 alone resulted in significant cell death at concentrations of 5 μ M or above ($p < 0.001$). The percentage cell deaths for this palmitate-free control group were as follows: control, 14.4%; 1 μ M Compound-16, 8.5%; 2.5 μ M Compound-16, 11.8% and 5 μ M Compound-16, 71%. Furthermore, in contrast to our results presented in the BRIN-BD11 cells, co-incubation of Compound-16 and 250 μ M palmitate did not afford significant protection against palmitate-induced cell death at any concentration tested. Indeed, there was a significant increase in percentage cell death seen when 5 μ M Compound-16 was co-incubated with 250 μ M palmitate compared to the 250 μ M palmitate control in this group ($p < 0.001$). The percentage cell deaths for this co-incubation group were: control (250 μ M palmitate), 64.7%; 250 μ M palmitate and 1 μ M Compound-16, 55.3%; 250 μ M palmitate and 2.5 μ M Compound-16, 52.7% and finally, 250 μ M palmitate and 5 μ M Compound-16, 82.4%.

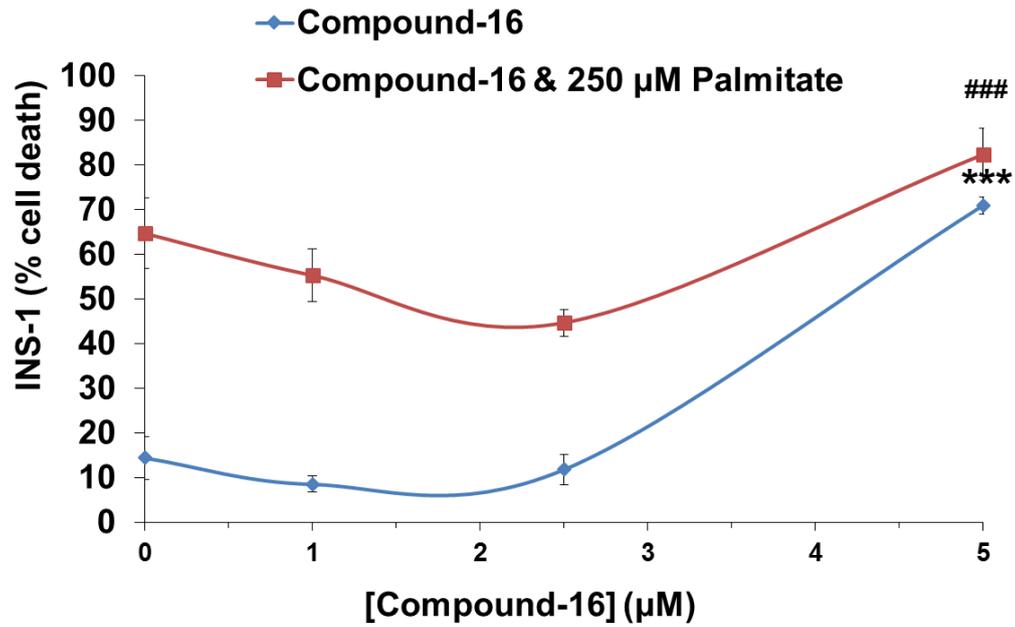


Fig. 3.12 The cytotoxic effect of Compound-16 on INS-1 cell viability

INS-1 rodent β -cells were incubated with increasing concentrations of Compound-16 and incubated for 24 h. A second treatment group were pre-incubated with the identical concentrations of Compound-16 for 1 h, then incubated for a further 23 h with 250 μ M palmitate. Cell death was assessed by trypan blue vital dye. Results represent mean \pm SEM from experiments (N=8). *** $p < 0.001$ vs. control, ### $p < 0.001$ vs. 250 μ M palmitate control

3.3.6 Compound-16 is toxic to MIN6 cells

The effect of Compound-16 cells was investigated in MIN6, again for the first time to our knowledge. We undertook repeated incubations testing a range of Compound-16 concentrations, following the protocol detailed previously in Section 3.3.3. Our results showed that Compound-16 was potently toxic to MIN6 cells (Fig. 3.13). The incubation of Compound-16 alone with the MIN6 cells was highly toxic at all concentrations tested and statistically significant at all concentrations ($p < 0.001$). The percentage cell deaths in this group were: control, 16.1%; 5 μM Compound-16, 82.9%; 10 μM Compound-16, 97.1%; 15 μM Compound-16, 98.1%; 20 μM Compound-16, 99.1% and 25 μM Compound-16, 99.5%. Likewise, the co-incubation of Compound-16 with 250 μM palmitate afforded no significant protection against palmitate-induced cell death at all concentrations tested. In fact, there was significant increase in percentage cell death seen when all concentrations of Compound-16 were co-incubated with 250 μM palmitate compared to the 250 μM palmitate control group ($p < 0.001$). The percentage cell deaths when MIN6 cells were co-incubated with 250 μM palmitate and Compound-16 were: control (250 μM palmitate), 63.3%; 250 μM palmitate and 5 μM Compound-16, 88.9%; 250 μM palmitate and 10 μM Compound-16, 98.2%; 250 μM palmitate and 15 μM Compound-16, 99.7%; 250 μM palmitate and 20 μM Compound-16, 99.3% and finally, 250 μM palmitate and 25 μM Compound-16, 98.9%.

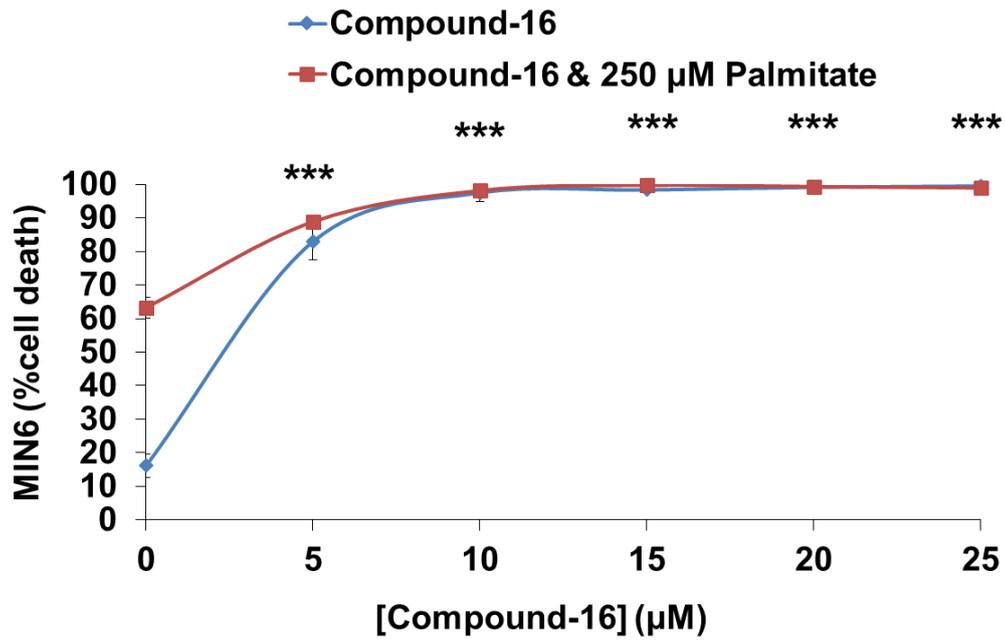


Fig. 3.13 The cytotoxic effect of Compound-16 on MIN6 cell viability

MIN6 rodent β -cells were treated with increasing concentrations of Compound-16 and incubated for 72 h. A second treatment group of MIN6 cells were pre-incubated with identical concentrations of Compound-16 for 1 h, then incubated for a further 72 h with 250 μ M palmitate. Cell death was assessed by trypan blue vital dye. Results represent mean \pm SEM from experiments (N=6). *** $p < 0.001$ vs. control in both groups

3.3.7 The role of PERK and PKR in ER stress in BRIN-BD11 cells: use of adenoviral vectors

Lee and colleagues (2007) were one of the first groups to propose a less major role for PERK in apoptotic pathways, and suggested that this role may be fulfilled by PKR. Following on from the targeted small molecule inhibition of PKR using Compound-16, we used an alternative investigative technique employing dominant negative (DN) adenoviral vectors to transiently reduce protein function of both PERK and PKR. The aim here was to study in parallel the individual and the combined activity of PERK and PKR kinases when BRIN-BD11 cells were incubated with 250 μ M palmitate.

Culture conditions and the use of DN adenoviral vectors in BRIN-BD11 cells were carried out as described in Sections 2.6.1 and 2.6.2. All groups were treated with 250 μ M palmitate and transfected with DN vectors individually or co-transfected with both vectors. Whole cell lysates were extracted for Western blotting and probed using the ER stress marker eIF2 α to investigate alterations to its phosphorylation status (Fig. 3.14).

There was no statistically significant difference between the expression of phospho-eIF2 α in parental BRIN-BD11 cells with normal kinase activity treated with 250 μ M palmitate, and the expression of phospho-eIF2 α in cells transfected with the Ad/GV16 empty vector incubated with 250 μ M palmitate. By contrast, there was a 3.8-fold decrease in phospho-eIF2 α expression in the cells transfected with the Ad-PERK Δ C vector incubated with 250 μ M palmitate, compared with the 250 μ M palmitate-treated control cells ($p < 0.001$). However, a 5.4-fold decrease in phospho-eIF2 α expression was also detected in the cells

transfected with the Ad- Δ 6PKR vector incubated with 250 μ M palmitate, when compared with the 250 μ M palmitate treated control cells ($p < 0.001$). Finally, a 4.6-fold decrease in phospho-eIF2 α expression was detected in the cells transfected with both Ad-PERK Δ C and Ad- Δ 6PKR vectors treated with 250 μ M palmitate, compared with the 250 μ M palmitate-treated control cells ($p < 0.001$).

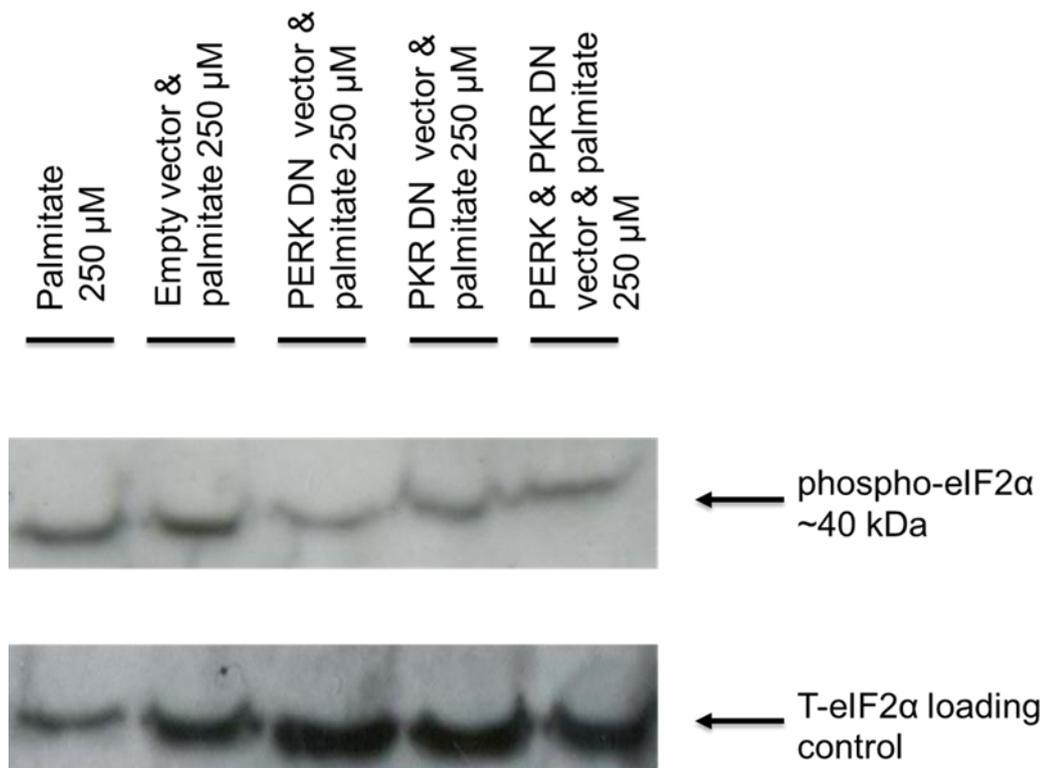


Fig. 3.14 The effect of PERK and PKR DN vectors on the ER stress marker phospho-eIF2 α in BRIN-BD11 β -cells

BRIN-BD11 cells were transfected with empty vector or dominant negative vectors (1 μ l/25 cm² flask) and cultured for 16 h in complete medium. This was replaced with serum-free culture medium and 250 μ M palmitate before a further 18 h incubation period. Following incubation, whole cell lysates were probed using phospho-eIF2 α antibody. Results represent blots from experiments performed in triplicate (N=3).

3.3.8 Quantification and selection of BRIN-BD11 PKR KD clonal cells

To supplement our existing data, a different approach to investigate the role of PKR was employed. By using RNAi techniques to specifically target PKR, clonal BRIN-BD11 PKR knockdown (KD) cells were created to manipulate PKR expression as detailed previously in Sections 2.6.3 – 2.6.7. Initially, whole cell lysates from the various PKR KD clones created were subjected to Western blotting and probed with a PKR antibody, to determine the extent of PKR KD (Fig. 3.15). This enabled the selection of suitable clones for experiments.

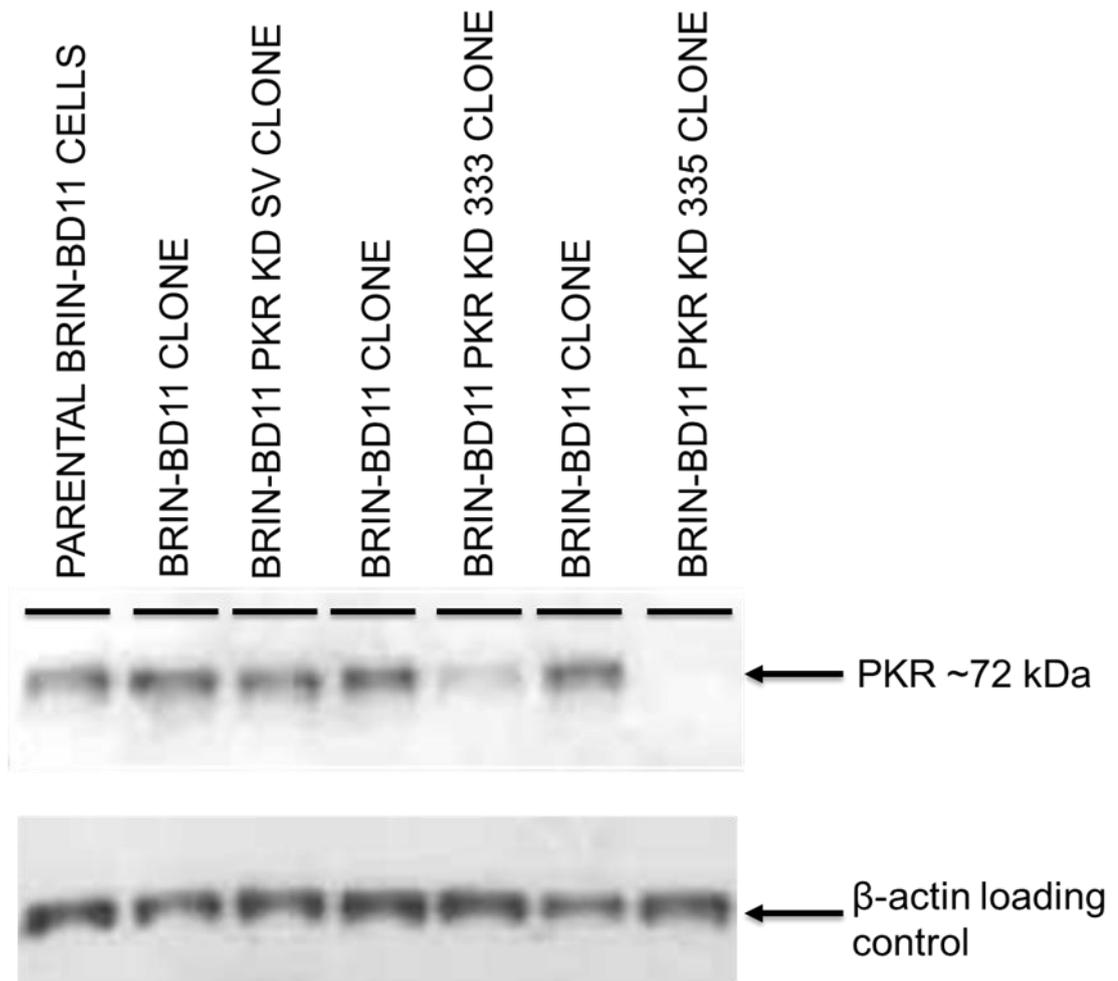


Fig. 3.15 Western blot of BRIN-BD11 PKR KD clones

BRIN-BD11 cells were transfected with rat PKR shRNA plasmid DNA with a puromycin-resistant marker. Cells were subsequently incubated with puromycin positive culture medium so only cells with the puromycin resistance survived. Individual colonies of transfected cells were picked and further cultured and grown. Once adequate cell growth had been achieved, whole cell lysates were probed using PKR antibody. Results represent blots from experiments performed in triplicate (N=3).

Two clones were selected along with a PKR scrambled vector (SV) as control cells. There was no statistically significant difference between the expression of PKR in the parental BRIN-BD11 cells or the SV PKR clone. There was approximately 70% KD in the BRIN-BD11 PKR KD 333 clonal cells and approximately 95% KD in the BRIN-BD11 PKR KD 335 clonal cells when compared to the level of PKR expression in the parental control cells ($p < 0.001$).

3.3.9 BRIN-BD11 PKR KD clonal cell responses to palmitate

The selected PKR clones were used to investigate whether KD of the PKR protein affected cell viability in response to incubation with different FA species. It was important however, to initially establish that there was no observable difference in responses to FA species between the parental BRIN-BD11 cells and the control BRIN-BD11 PKR SV cells.

Our results from these novel experiments demonstrated that when both cell lines (parental BRIN-BD11 cells and BRIN-BD11 PKR SV cells), were incubated with increasing concentrations of palmitate, a dose-dependent increase in cell death was observed in line with our previous findings presented in Section 3.3.1. More critically however, there was no significant difference in the amount of percentage cell death seen between the parental BRIN-BD11 cells and the BRIN-BD11 PKR SV cells (Fig 3.16).

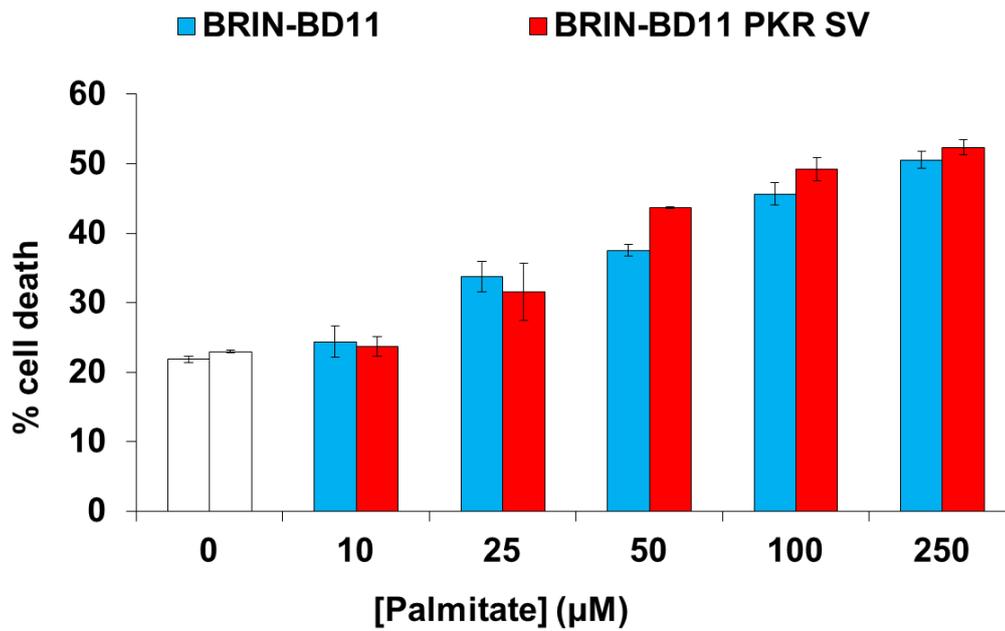


Fig. 3.16 The cytotoxic effect of palmitate on BRIN-BD11 PKR SV β -cell death is the same as parental BRIN-BD11 β -cells

BRIN-BD11 β -cells and BRIN-BD11 PKR SV cells were treated with increasing palmitate concentrations and incubated for 18 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments in triplicate (N=3).

The next set of studies aimed to assess the level of cell death observed in the individual PKR KD clonal cell lines compared to the BRIN-BD11 SV control cells during the same experimental conditions. Our results here showed that palmitate induced a dose-dependent cytotoxic effect on the BRIN-BD11 PKR KD 333 cells (Fig. 3.17). These findings correlated with other rodent cell models presented in Section 3.3.1. We observed a 1.2-fold increase in cell death in the 10 μ M palmitate-treated group compared to the BRIN-BD11 PKR KD 333 control. Although showing a trend towards increasing cell death, this was not statistically significant. There was a 1.9-fold increase in cell death in the 25 μ M palmitate-treatment group compared to the BRIN-BD11 PKR KD 333 control ($p<0.01$). We observed a 2.7-fold increase in cell death in the 50 μ M palmitate-treatment group compared to the BRIN-BD11 PKR KD 333 control ($p<0.001$). There was a 4-fold increase in cell death in the 100 μ M palmitate-treatment group compared to the BRIN-BD11 PKR KD 333 control ($p<0.001$), and finally we saw a 4.6-fold increase in cell death in the 250 μ M palmitate-treatment group compared to the BRIN-BD11 PKR KD 333 control ($p<0.001$).

Furthermore, when comparing the amount of cell death between the two cell lines, there was a 3.2-fold decrease in cell death observed in the BRIN-BD11 PKR KD 333 control cells when compared to the BRIN-BD11 PKR SV control cells ($p<0.001$). A 3-fold decrease in cell death was seen when comparing the BRIN-BD11 PKR KD 333 cells treated with 10 μ M palmitate, with the BRIN-BD11 PKR SV cells treated with 10 μ M palmitate ($p<0.001$). A 2.8-fold decrease in cell death was seen when comparing the BRIN-BD11 PKR KD 333 cells treated with 25 μ M palmitate, with the BRIN-BD11 PKR SV cells treated with 25 μ M palmitate ($p<0.001$). A 2.7-fold decrease in cell death was seen between the BRIN-BD11

PKR KD 333 cells treated with 50 μM palmitate and the BRIN-BD11 PKR SV cells treated with 50 μM palmitate ($p<0.001$). Finally, a 1.7-fold decrease was observed when comparing the BRIN-BD11 PKR KD 333 cells treated with 100 μM palmitate, with the BRIN-BD11 PKR SV cells treated with 100 μM palmitate ($p<0.01$). No statistical difference was seen between either cell line treated with 250 μM palmitate.

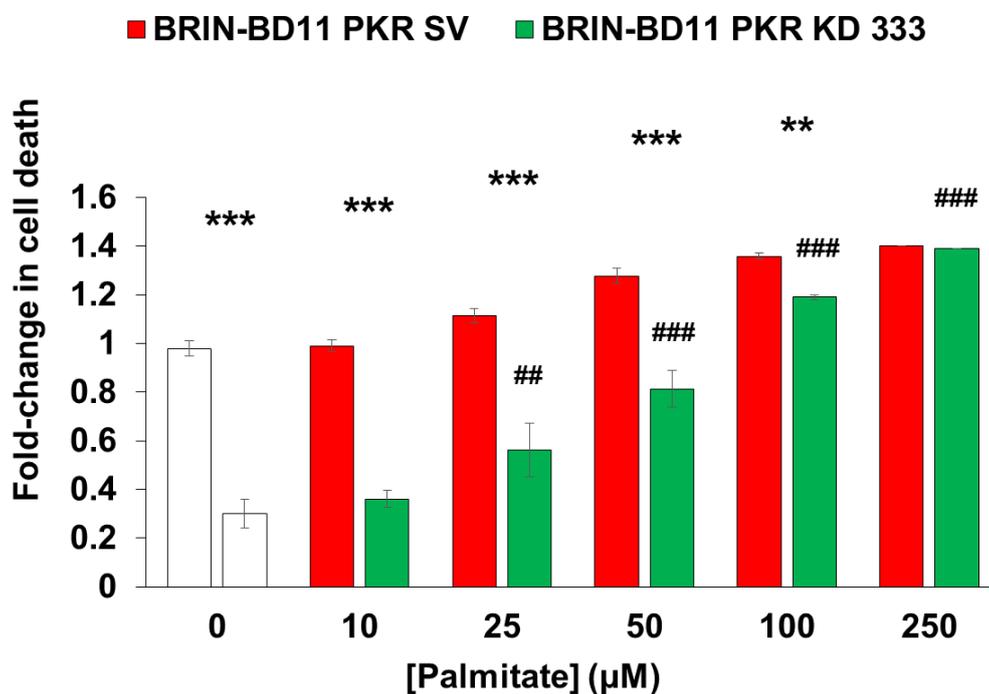


Fig. 3.17 The cytotoxic effect of palmitate on BRIN-BD11 PKR KD 333 β -cell death is less than in BRIN-BD11 PKR SV β -cells

BRIN-BD11 PKR SV β -cells and BRIN-BD11 PKR KD 333 clonal cells were treated with increasing palmitate concentrations and incubated for 18 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean normalised data \pm SEM from experiments in triplicate (N=3). *** $p<0.001$, ** $p<0.01$ vs. BRIN-BD11 PKR SV control cells; ### $p<0.001$, ## $p<0.01$ vs. BRIN-BD11 333 control

Our next results are data presented from the BRIN-BD11 PKR KD 335 cells. Again, in line with our other rodent data, these show that palmitate induced a dose-dependent cytotoxic effect in the BRIN-BD11 PKR KD 335 cell line (Fig. 3.18). There was a modest 1.1-fold and 1.3-fold increase in cell death seen in the 10 and 25 μM palmitate-treatment groups respectively, compared to the BRIN-BD11 PKR KD 335 control. Although demonstrating a trend towards increasing cell death, these were not statistically significant. There was a 1.6-fold increase in cell death in the 50 μM palmitate-treatment group compared to the BRIN-BD11 PKR KD 335 control ($p < 0.05$). We observed a 2.8-fold increase in cell death in the 100 μM palmitate-treatment group compared to the BRIN-BD11 PKR KD 335 control ($p < 0.001$). Finally, we saw a 4-fold increase in cell death in the 250 μM palmitate-treatment group compared to the BRIN-BD11 PKR KD 335 control ($p < 0.001$).

Upon comparison of the BRIN-BD11 PKR KD 335 cells with the BRIN-BD11 PKR SV cells, there was a 3.3-fold decrease in the amount of cell death seen when comparing the PKR KD 335 control cells with the PKR SV control cells ($p < 0.001$). A 3-fold decrease in cell death was seen when comparing the BRIN-BD11 PKR KD 335 cells treated with 10 μM palmitate, with the BRIN-BD11 PKR SV cells treated with 10 μM palmitate ($p < 0.001$). A 2.8-fold decrease in cell death was seen when comparing the BRIN-BD11 PKR KD 335 cells treated with 25 μM palmitate, with the BRIN-BD11 PKR SV cells treated with 25 μM palmitate ($p < 0.001$). A 2.7-fold decrease in cell death was seen between the BRIN-BD11 PKR KD 333 cells treated with 50 μM palmitate and the BRIN-BD11 PKR SV cells treated with 50 μM palmitate ($p < 0.001$). A 1.7-fold decrease was observed when comparing the BRIN-BD11 PKR KD 335 cells treated with 100 μM palmitate,

with the BRIN-BD11 PKR SV cells treated with 100 μM palmitate ($p < 0.001$). Finally, a 1.3-fold decrease in cell death was seen between the BRIN-BD11 PKR KD 335 cells treated with 250 μM palmitate and the BRIN-BD11 PKR SV cells treated with 250 μM palmitate ($p < 0.01$).

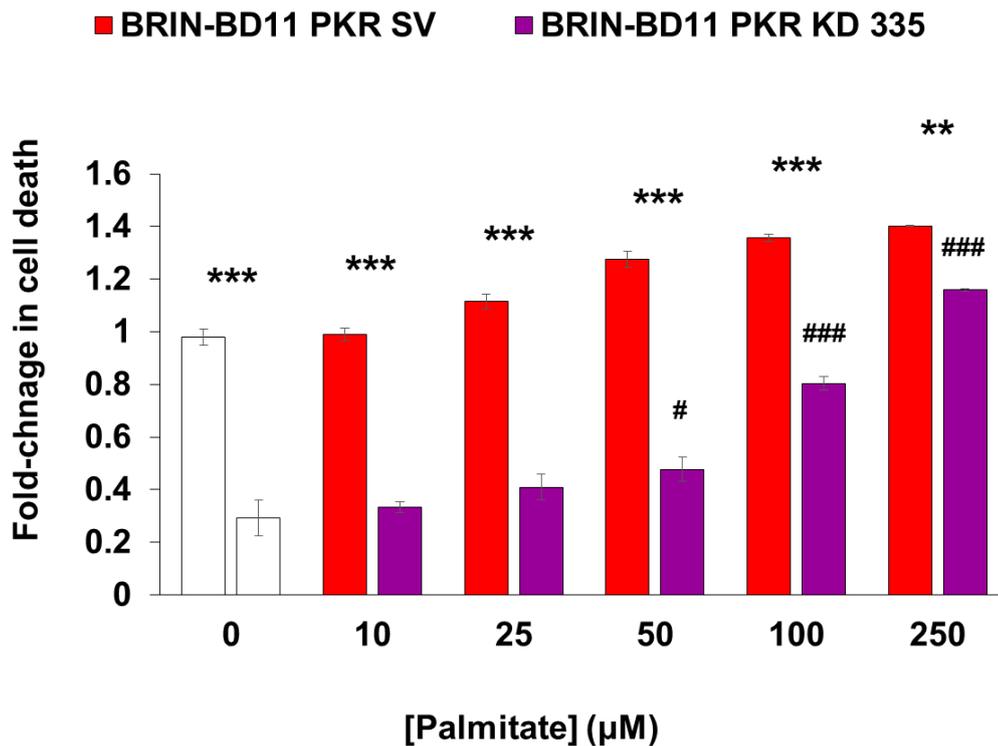


Fig. 3.18 The cytotoxic effect of palmitate on BRIN-BD11 PKR KD 335 β -cell death is less than in BRIN-BD11 PKR SV β -cells

BRIN-BD11 PKR SV control β -cells and BRIN-BD11 PKR KD 335 clonal cells were treated with increasing palmitate concentrations and incubated for 18 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean normalised data \pm SEM from experiments in triplicate (N=3). *** $p < 0.001$, ** $p < 0.01$ vs. BRIN-BD11 PKR SV control cells, ### $p < 0.001$, # $p < 0.05$

In these last results for this section, we report that the BRIN-BD11 PKR KD 335 clonal cells were more resistant to palmitate-induced cell death than the BRIN-BD11 PKR KD 333 clonal cells (Fig. 3.19). There was no significant difference in percentage cell death seen between the controls (no palmitate), 10 or 25 μM palmitate-treatment groups of either cell line, although there was a trend of decreased percentage cell death in the BRIN-BD11 PKR KD 335 cells. However, there was a 1.7-fold decrease in percentage cell death observed in the BRIN-BD11 PKR KD 335 cells treated with 50 μM palmitate, when compared to the BRIN-BD11 PKR KD 333 cells treated with 50 μM palmitate ($p < 0.001$). A 1.5-fold decrease in percentage cell death was observed when comparing the BRIN-BD11 PKR KD 335 cells treated with 100 μM palmitate, with the BRIN-BD11 PKR KD 333 cells treated with 100 μM palmitate ($p < 0.001$). Finally, we observed a 1.2-fold decrease in percentage cell death in the BRIN-BD11 PKR KD 335 cells treated with 250 μM palmitate, compared to the BRIN-BD11 PKR KD 333 cells treated with 250 μM palmitate ($p < 0.01$).

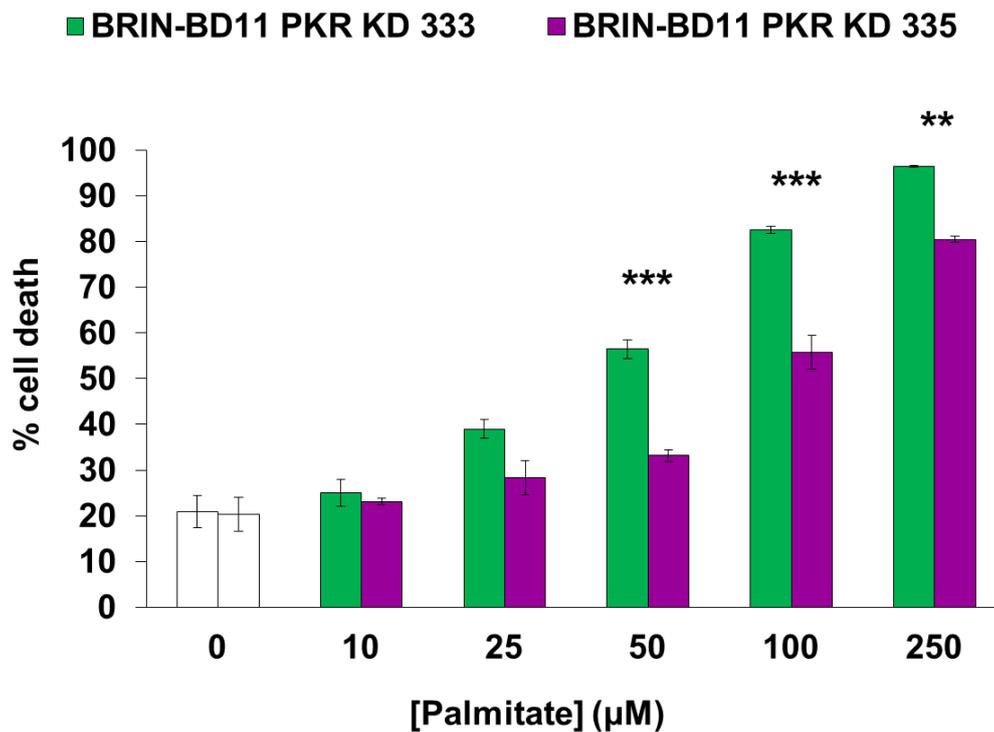


Fig. 3.19 The cytotoxic effect of palmitate is less in BRIN-BD11 PKR KD 335 β -cells than BRIN-BD11 PKR KD 333 β -cells

BRIN-BD11 PKR KD 333 clonal β -cells and BRIN-BD11 PKR KD 335 clonal β -cells were treated with increasing palmitate concentrations and incubated for 18 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** $p < 0.001$, ** $p < 0.01$ vs. BRIN-BD11 PKR KD 333 cells

3.3.10 BRIN-BD11 PKR KD clonal cell responses to palmitoleate

As the final set of experiments in this section, we aimed to investigate the responses of the PKR KD β -cells to incubation with the LC-MUFA palmitoleate. The treatment of both cell lines with 250 μ M palmitate resulted in a significant increase in percentage cell death compared to the controls ($p < 0.001$). This was in line with our previous findings. Incubation with 250 μ M palmitoleate alone was well-tolerated in both cell lines, with an approximate 3-fold reduction in percentage cell death seen in the 250 μ M palmitoleate-treatment groups compared to the 250 μ M palmitate-treatment groups ($p < 0.001$). Likewise, percentage cell death was significantly reduced when cells were co-incubated with both palmitate and palmitoleate, compared to the palmitate treated groups ($p < 0.001$). Importantly, there was no significant difference in the amount of percentage cell death observed between the parental BRIN-BD11 cells or the BRIN-BD11 PKR SV cells (Fig. 3.20). To fully quantify these findings, percentage cell deaths observed in the parental BRIN-BD11 cells were as follows: control, 20.2%; 250 μ M palmitate, 63.6%; 250 μ M palmitoleate, 9.8% and both FA species, 10.5%. The percentage cell deaths seen in the BRIN-BD11 PKR SV cells were: control, 20.8%; 250 μ M palmitate 66.2%; 250 μ M palmitoleate 11% and finally, both FA species, 11.4%.

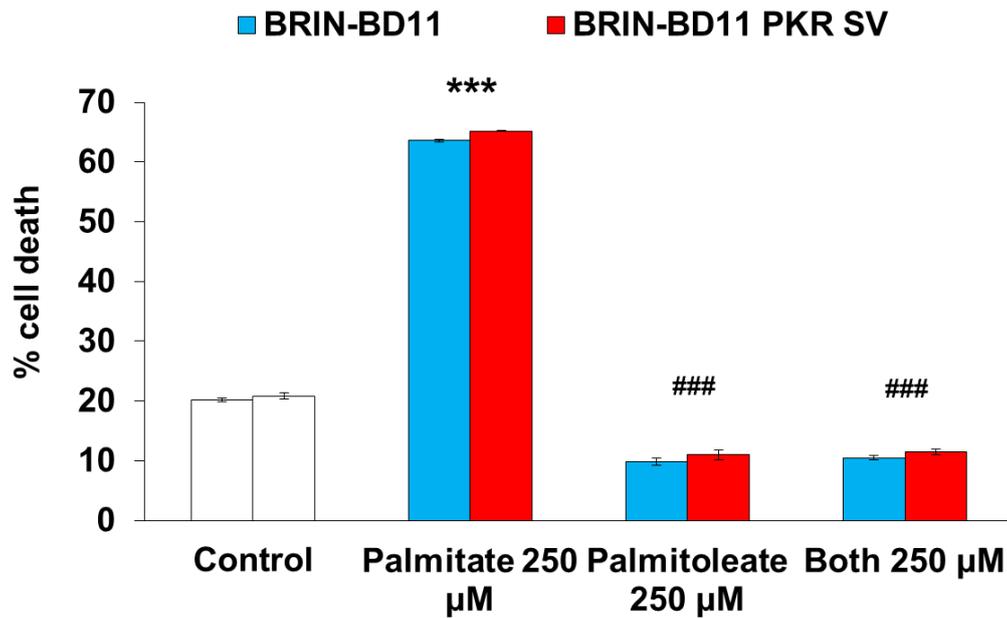


Fig. 3.20 The cytoprotective effect of palmitoleate on BRIN-BD11 PKR SV β -cell viability is the same as parental BRIN-BD11 β -cells

Parental BRIN-BD11 β -cells and BRIN-BD11 PKR SV cells were treated 250 μ M palmitate, 250 μ M palmitoleate or a combination of both FA species and incubated for 18 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** p < 0.001 vs. controls; ### p < 0.001 vs. palmitate-treated cells

The cytoprotective effect of PKR KD was greater in the BRIN-BD11 PKR KD 333 clonal β -cells than in the BRIN-BD11 PKR SV cells (Fig. 3.21). There was a significant 1.5-fold decrease in percentage cell death seen between the control group of the BRIN-BD11 PKR KD 333 cells, compared to the control group of the BRIN-BD11 PKR SV cells ($p<0.01$). Furthermore, there was a 1.6-fold decrease in percentage cell death seen in the BRIN-BD11 PKR KD 333 cells treated with 250 μ M palmitate compared to the palmitate-treatment group of the BRIN-BD11 PKR SV cells ($p<0.001$). There was no statistical difference between either cell line when comparing the percentage cell deaths observed in the 250 μ M palmitoleate-treatment groups, or the groups co-incubated with both FA species. To fully quantify these results, percentage cell deaths in the BRIN-BD11 PKR 333 cell line, percentage cell deaths were as follows: control, 13.6%; 250 μ M palmitate, 40.8%; 250 μ M palmitoleate, 10.2% and both FA species, 8.7%.

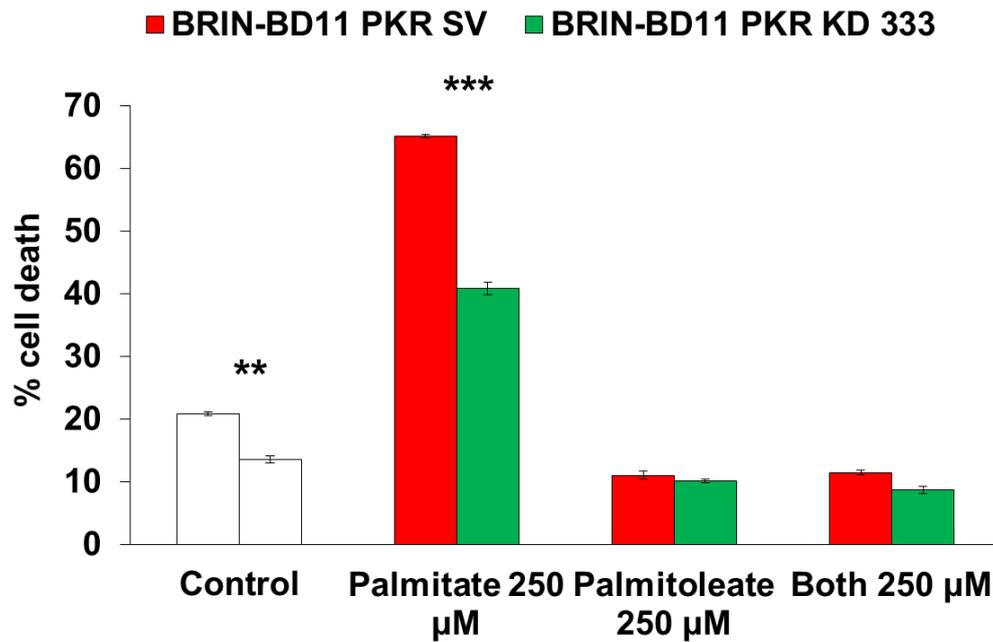


Fig. 3.21 The cytoprotective effect of palmitoleate on BRIN-BD11 PKR KD 333 β -cells is greater than in parental BRIN-BD11 β -cells

Parental BRIN-BD11 β -cells and BRIN-BD11 PKR KD 333 cells were treated 250 μ M palmitate, 250 μ M palmitoleate or a combination of both FA species and incubated for 18 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** p =<0.001, ** p = 0.01 vs. BRIN-BD11 PKR SV cells

The cytoprotective effect of PKR KD was significantly greater in the BRIN-BD11 PKR KD 335 clonal β -cells compared to the BRIN-BD11 PKR SV cells when treated alone or when co-incubated with both FA species (Fig. 3.22). There was a significant 3.9-fold decrease in percentage cell death observed in the control group of the BRIN-BD11 PKR KD 335 cells compared to the BRIN-BD11 PKR SV control group ($p<0.001$). A 2.2-fold decrease in percentage cell death was seen in the 250 μ M palmitate-treatment group of the BRIN-BD11 PKR KD 335 cells compared to the BRIN-BD11 PKR SV cells treated with 250 μ M palmitate ($p<0.001$). There was a 2.8 fold reduction in percentage cell death observed in the BRIN-BD11 PKR KD 335 cells treated with 250 μ M palmitoleate, compared to the BRIN-BD11 PKR SV group treated with 250 μ M palmitoleate ($p<0.001$). Finally, there was a 2.9-fold decrease in percentage cell death observed in the BRIN-BD11 PKR KD 335 cells treated with both FA species, compared to the BRIN-BD11 PKR SV group treated with both FA species ($p<0.001$). Full quantification of percentage cell death results in the BRIN-BD11 PKR KD 335 cells are as follows: control, 5.4%; 250 μ M palmitate, 29.5%; 250 μ M palmitoleate, 4.1% and both FA species, 4.1%.

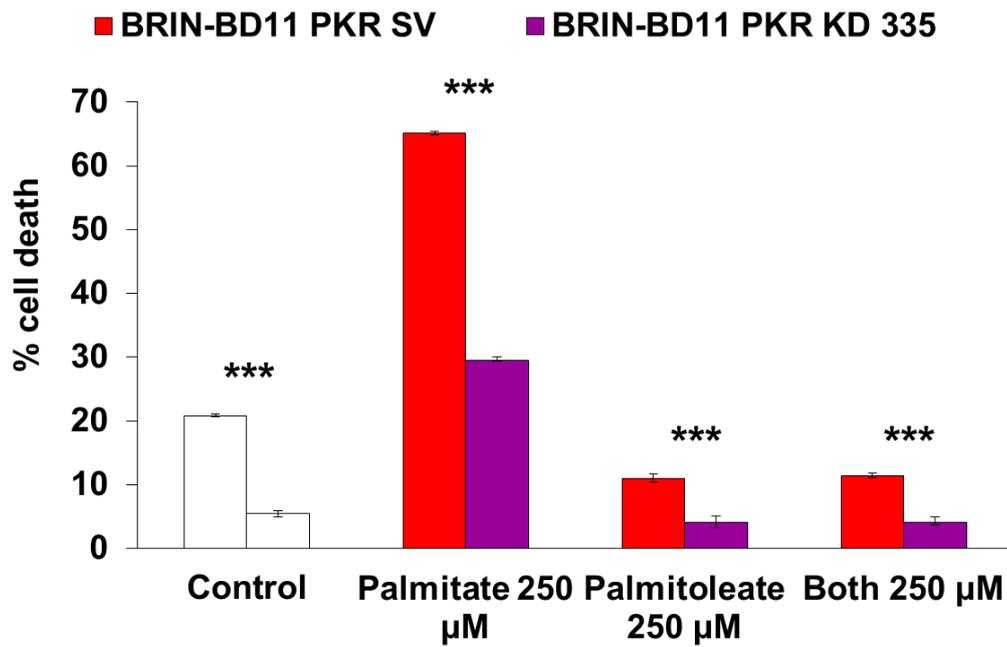


Fig. 3.22 The cytoprotective effect of palmitoleate on BRIN-BD11 PKR KD 335 β -cells is greater than in parental BRIN-BD11 β -cells

Parental BRIN-BD11 β -cells and BRIN-BD11 PKR KD 335 cells were treated 250 μ M palmitate, 250 μ M palmitoleate or a combination of both FA species and incubated for 18 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** p < 0.001 vs. BRIN-BD11 cells

A final comparison was made between the two PKR KD clonal cell lines (Fig. 3.23). From this comparison, we saw that there was significantly less percentage cell death seen in the BRIN-BD11 PKR KD 335 clonal cells in all treatment groups compared to the BRIN-BD11 PKR KD 333 cells. There was a 2.5-fold reduction in percentage cell death seen in the BRIN-BD11 PKR KD 335 control group compared to the control of the BRIN-BD11 PKR KD 333 cells ($p<0.001$). We observed a 1.4-fold reduction in percentage cell death in the BRIN-BD11 PKR KD 335 cells treated 250 μ M palmitate compared to the 250 μ M palmitate-treatment group in the BRIN-BD11 PKR KD 333 cells ($p<0.01$). There was a 2.6-fold decrease in percentage cell death seen in the BRIN-BD11 PKR KD 335 cells treated with 250 μ M palmitoleate, compared to the 250 μ M palmitoleate-treatment group in the BRIN-BD11 PKR KD 333 cells ($p<0.01$). Finally, we saw a 2.2-fold decrease in percentage cell death seen in the BRIN-BD11 PKR KD 335 cells treated with both FA species, compared to the co-incubation of FA species in the BRIN-BD11 PKR KD 333 cells ($p<0.01$).

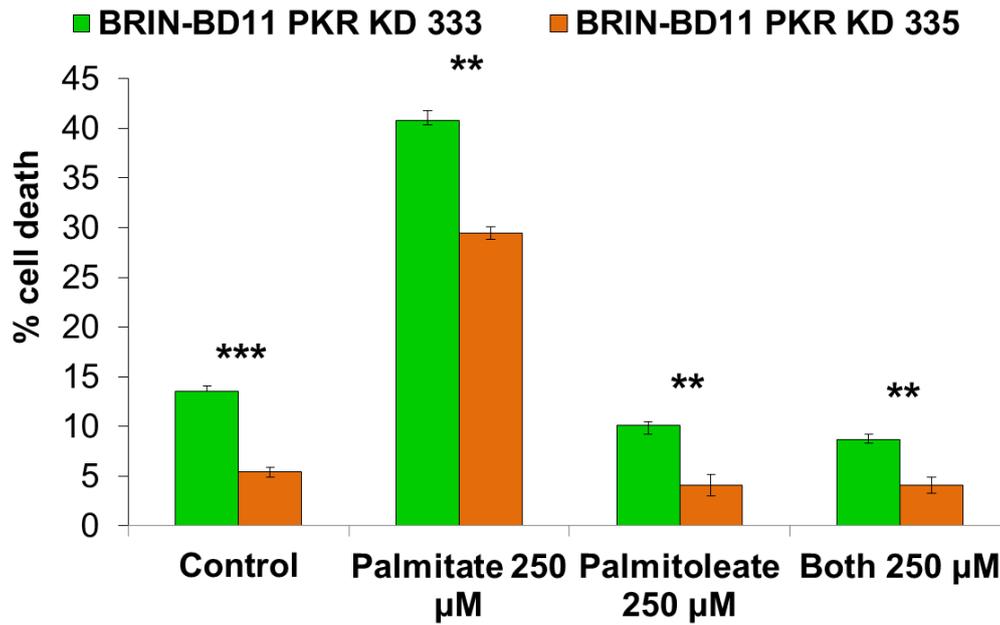


Fig. 3.23 The cytoprotective effect of palmitoleate on BRIN-BD11 PKR KD 335 β -cells is greater than on BRIN-BD11 PKR KD 333 β -cells

BRIN-BD11 PKR KD 333 β -cells and BRIN-BD11 PKR KD 335 cells were treated 250 μ M palmitate, 250 μ M palmitoleate or a combination of both FA species and incubated for 18 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments in triplicate (N=3). *** p <0.001, ** p <0.01 vs. BRIN-BD11 PKR KD 333 cells

3.4 Summary of novel results

- A transient KD approach using Compound-16, a putative small molecule inhibitor of PKR, showed that between concentrations of 5 – 25 μ M, Compound-16 significantly protected BRIN-BD11 cells ($p < 0.001$) against palmitate-induced cell death compared to control cells treated with palmitate (Fig. 3.9).
- Compound-16 significantly protected BRIN-BD11 cells against serum-starvation ($p < 0.001$). This was effective for up to a 30 h incubation period before significant protection by Compound-16 was lost (Fig. 3.10).
- Western blotting of lysates from BRIN-BD11 cells incubated with 5 μ M Compound-16 showed a significant increase ($p < 0.001$) in the ER stress marker phospho-eIF2 α (Fig. 3.11), despite improving cell viability during chronic palmitate exposure.
- Compound-16 is significantly cytotoxic to INS-1 cells (Fig. 3.12) at ≥ 5 μ M concentrations ($p < 0.001$). Moreover, Compound-16 is potentially cytotoxic to MIN6 cells (Fig. 3.13) at all concentrations tested (0.5 – 25 μ M, $p < 0.001$).
- The use of PERK and PKR DN adenoviral vectors demonstrated that transient KD of these kinases individually, or together, significantly reduced eIF2 α phosphorylation ($p < 0.001$) (Fig. 3.14).
- The use of shRNA to create stable PKR KD clones in BRIN-BD11 cells, demonstrated that KD of PKR significantly protected cells against palmitate-induced cell death ($p < 0.001$), when compared to the PKR SV control clone. The 95% KD clone afforded greater protection than the 70% KD clone ($p < 0.001$). Furthermore, PKR KD significantly potentiated the cytoprotective action of palmitoleate ($p < 0.001$) (Fig. 3.16 – Fig. 3.23).

3.4 Discussion

The structural requirements for the cytotoxic or cytoprotective action of different FA species when incubated with rodent pancreatic β -cells have been well established in previous studies. However, there are inconsistencies within the literature with regard to the potency of cytotoxicity or cytoprotection seen between the various FA species (Welters *et al.*, 2004; Eitel *et al.*, 2003; Dhayal *et al.*, 2008, Newsholme *et al.*, 2007; Wei *et al.*, 2006; El-Assaad *et al.*, 2003; Moffitt *et al.*, 2005; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Morgan *et al.*, 2008). This is likely attributable to a varied approach to experimental design between studies. For example, different cell models and passage number have been adopted; varying incubation periods and glucose concentrations, and different ratios of FA species to BSA are all notable examples of experimental variability that have been documented throughout the literature.

For our studies, we sought to investigate the most appropriate cell model to investigate the role of the PKR protein in response to LC-SFA and LC-MUFA species in conditions of lipotoxicity. To do this, we first needed to validate and standardise our experimental conditions between the cell models used. We used chronic palmitate incubation conditions to evoke an ER stress response and induce cell death within our pancreatic β -cell models. Our first experiment demonstrated that indeed, palmitate was cytotoxic to our cells over a chronic 24 h incubation period. Importantly however, our data served to emphasise that the extent of percentage cell death induced by palmitate was very different between the cell lines.

MIN6 cells are reported to express significantly higher levels of ER chaperone proteins, such as GRP78, than INS-1 and BRIN-BD11 cells and as such, it is noted these cells are more resistant to apoptosis and ER stress induced by palmitate incubation (Lai *et al.*, 2008; Diakogiannaki *et al.*, 2008). MIN-6 β -cells are also reported to express high levels of stearoyl-CoA desaturase 1 (SCD1), an enzyme that desaturates FA and favours TG synthesis, a capacity that may confer protection by making these pancreatic β -cells less susceptible to lipotoxicity (Busch *et al.*, 2005). This may account for the higher resistance to ER stress and subsequently lower levels of palmitate-induced cell death seen in MIN6 cells during lipotoxic incubation conditions. This may also explain why conversely the protective effects of palmitoleate in MIN6 cells are not so pronounced, and correlates with the reduced upregulation state of phospho-eIF2 α observed in MIN6 cells when they were incubated with palmitate in these studies.

Our results showed a 4.2 fold-decrease in percentage cell death between the BRIN-BD11 cells and the MIN6 β -cells, and a 2.5-fold decrease in percentage cell death between the BRIN-BD11 cells and the INS-1 β -cells, indicating that the BRIN-BD11 cells are more susceptible to palmitate-induced cell death. This data correlates with results shown previously by our group (Welters *et al.*, 2004; Dhayal *et al.*, 2008, Newsholme *et al.*, 2007; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Morgan *et al.*, 2008). However, given this *in vitro* β -cell variability in response to chronic FA incubation, for meaningful comparisons to be made between cell models, we needed to determine an optimal incubation period for each cell line. Time-course experiments allowed us to define these cell-model specific incubation periods.

Having achieved this, we demonstrated comparable percentage cell deaths between the cell models in dose-curve studies. The LC-SFA palmitate exerts a dose-dependent cytotoxic effect on pancreatic β -cells, and the LC-MUFA palmitate induces a dose-dependent cytoprotective effect on the pancreatic β -cells. Furthermore, the *in vitro* co-incubation of palmitate with palmitoleate was able to attenuate the cytotoxic effects observed when pancreatic β -cells are incubated with palmitate. In addition, we showed that the incubation of β -cells with palmitate led to an upregulation of the ER stress marker phospho-eIF2 α , a protein integral to cell survival pathways in cells subjected to persistent ER stress. This outcome was consistently observed in all three rodent pancreatic β -cell lines tested under the same experimental conditions. This preliminary set of experiments was felt to be a critical standardisation step, and to confirm that previous results were not specific to a particular cell model.

Notwithstanding the previous validation steps, these results do not shed light on specific molecular mechanisms that may underlie these cytotoxic or cytoprotective actions. It has been suggested that lipotoxicity initiates ER stress, which if persistent drives β -cell apoptosis. This mechanism is thought to underlie the progression of T2D, but the exact molecular processes that mediate these proapoptotic effects in response to FA are unclear (Qu *et al.*, 2009; Breckenridge *et al.*, 2003; Laybutt *et al.*, 2007; Eizirik *et al.*, 2008; Özcan *et al.*, 2004; Kharroubi *et al.*, 2004; Scheuner and Kaufman, 2008; Karaskov *et al.*, 2006; Cnop *et al.*, 2007). However, pancreatic β -cell apoptosis involving the PERK-dependent arm of the ER stress pathway as a result of lipotoxicity has been well established (Morgan *et al.*, 2008; Diakogiannaki *et al.*, 2008; Cnop *et al.*, 2008; Diakogiannaki and Morgan, 2008; Scheuner and Kaufman, 2008; Laybutt *et al.*, 2007; Song *et*

al., 2007; Lee *et al.*, 2007). This suggests a pathway by which lipotoxicity-induced β -cell death may be targeted.

Interestingly, a more recent study suggested that the PERK protein may have a less significant role in ER stress-mediated apoptosis than formerly thought. Lee and colleagues (2007) proposed that PERK may have a predominant cell-survival role under ER stress conditions. By contrast, the PKR protein was found to be simultaneously but independently activated alongside PERK under lipotoxic conditions and as such, was suggested to assume a greater proapoptotic role than previously thought (Lee *et al.*, 2007; Lai *et al.*, 2008; Morgan, 2009). Adopting the use of suitable cell models to conduct more in-depth investigations into the functional properties of PKR may be instrumental in understanding the exact role of this protein in lipotoxic mechanisms, and furthermore, may indicate whether PKR could be a potential viable target for pharmacological intervention in the treatment of T2D.

There are currently two models of PKR-null mice, but results suggest that these models elicit a normal response to IFN and furthermore, they respond almost normally to viral infection. These results are atypical, because by knocking out PKR, one would expect an effect on the anti-viral, anti-tumoral and apoptotic pathways where PKR is an integral protein, but this is not the case. These aberrant responses have been explained by the possible activation of redundant pathways involving other eIF2- α kinases, suggested to compensate for the loss of functional PKR in these mice (Abraham *et al.*, 1999; Yang *et al.*, 1995; Jammi *et al.*, 2003). Notwithstanding this, these findings highlight the unsuitability of

these particular mouse models for PKR-specific studies, and emphasise the need for different approaches to enable exclusive manipulation of PKR activity.

A small molecule inhibitor of PKR could potentially provide a solution to this redundancy problem, because the induced inhibition of translation could be blocked precisely with PKR as a sole and specific target, if indeed PKR is the upstream mediator that phosphorylates eIF2 α . 2-Aminopurine (2-AP) was heralded as one such small molecule, and has been historically widely used to disrupt protein synthesis following viral infection (Huang and Schneider, 1990; Frémont *et al.*, 2006; Jammi *et al.*, 2003). However, 2-AP was demonstrated to inhibit other protein kinases so was clearly not specific to PKR, which questions the validity of any such results (Posti *et al.*, 1999). Jammi and colleagues (2003) screened a library of different ATP-binding site-directed inhibitors of PKR and found a high-affinity PKR inhibitor named Compound-16. This molecule was demonstrated to effectively inhibit RNA-induced PKR autophosphorylation ($IC_{50} = 210 \pm 0.04 \mu\text{M}$) and rescued PKR-dependent translation block to 50% (vs. control) at a concentration of 10 μM .

This brings us to the main investigative aim of this chapter. Preliminary experiments by our group suggested that Compound-16 may exert an inhibitory effect on palmitate-mediated toxicity in BRIN-BD11 cells. This compound was therefore adopted in these studies to selectively inhibit autophosphorylation and activation of the PKR protein in rodent β -cell lines incubated with different FA species. Following dose-curve experiments, our results here confirm that indeed, Compound-16 is capable of significantly rescuing BRIN-BD11 cells from palmitate-induced cell death with an effective range between 5 μM – 25 μM in

line with our previous findings. Furthermore, results established that the BRIN-BD11 cells were not detrimentally affected by sole incubation with Compound-16 at the concentrations tested.

It has been generally accepted that the GCN2 kinase is activated in response to nutrient deprivation (Wek *et al.*, 2006), although a newly identified role for PKR within nutrient sensing pathway suggests that there is a level of redundancy within this kinase family (Nakamura *et al.*, 2010). It is important to consider that a role for PKR involvement in nutrient sensing may include nutrient deprivation as well as nutrient overload. Therefore, the effect of Compound-16 under conditions of serum starvation was also investigated in BRIN-BD11 cells. Our results here for the first time, show a significant reduction in cell death when cells were incubated with 10, 15 and 25 μ M concentrations of Compound-16. Furthermore, our results demonstrate that the cytoprotective effect of Compound-16 was able to significantly ameliorate cell death induced by serum withdrawal, and this cytoprotection was significant for up to a 30 h incubation period. This suggests that the inhibition of PKR phosphorylation during lipotoxic conditions can improve cell viability. However, these results alone do not prove that the inhibition of the PKR protein is solely responsible for this outcome.

The eIF2 α protein has been extensively used to demonstrate that cells incubated with palmitate exhibit ER stress, as seen by upregulation of the phosphorylation status of this protein. Elevated phospho-eIF2 α is consistently shown to correlate with an *in vitro* increase in percentage cell death in cell models incubated with SFA species (Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Cnop *et al.*, 2007). By contrast, sole or co-incubation of pancreatic β -

cells with palmitoleate confers protection against palmitate-induced toxicity. This is shown by a decrease in percentage cell death (Welters *et al.*, 2004) and a reduction in the level of phospho-eIF2 α seen (Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Cnop *et al.*, 2008). Western blotting to investigate the phosphorylation of eIF2 α was conducted on cells incubated with Compound-16. It was expected that due to the improved cell viability observed, a reduction in the phosphorylation status of eIF2 α would also be seen.

However, our results showed that despite improved cell viability, the phosphorylation status of eIF2 α was consistently higher when cells were co-incubated with Compound-16 and palmitate, than when cells were incubated with palmitate alone. This suggests that Compound-16 may exert its cytoprotective effects via a different signalling pathway, not involving eIF2 α . This may be a cell-specific phenomenon but interestingly, Chen *et al.*, (2008) also found that while Compound-16 conferred protection against death in a neuronal cell model they used, it did not reduce phosphorylation of eIF2 α . Therefore, our data appears to support this phenomenon. Furthermore, Shimazawa and Hara (2006) reported that Compound-16 successfully inhibited PKR following tunicamycin-induced ER stress and improved cell viability in a different neuronal cell model. However, a limitation of the Shimazawa and Hara (2006) study was that improved cell viability was not correlated with phospho-PKR or phospho-eIF2 α quantification; therefore it is difficult to make any conclusions regarding PKR or eIF2 α involvement from this.

Nonetheless, we decided to extend our studies to test the efficacy of Compound-16 in INS-1 cells and MIN6 cells, as to our knowledge, PKR inhibition in these

cell lines using this compound has not yet been reported. Surprisingly, Compound-16 was potently cytotoxic when incubated alone or when co-incubated with palmitate. Our results demonstrated a huge increase in percentage cell death compared to the control cells in both cell models. Cytotoxicity was greater in the MIN6 cells compared to the INS-1 cells but nevertheless, it was clear that no protection was afforded by Compound-16 in either cell model. Section 4.3.4 covers additional Compound-16 studies conducted in a novel human pancreatic β -cell 1.1B4, but to prevent repetition, has been collectively discussed below.

Initially, it was considered that the DMSO concentration used to dissolve Compound-16 may have been responsible for such a dramatic loss of cell viability. DMSO-related toxicity in cell culture models has been suggested within the literature (Hanslick *et al.*, 2009), although this appears to be more commonly associated with high concentrations used for cryopreservation purposes (Zambelli *et al.*, 1998). However, we conducted extensive dose-dependent experiments using DMSO in the BRIN-BD11, INS-1, MIN6 and 1.1B4 cell lines (data not shown) and these results demonstrated that DMSO ($\leq 10\%$) was well tolerated in chronic incubation conditions (≤ 24 h) before exerting any significant detrimental effect on cell viability. The experimental conditions we followed under which DMSO was used as a vehicle to introduce Compound-16 into the cell models, dictated that we essentially used DMSO at very low concentrations ($<0.01\%$). Moreover, in light of the clear cytoprotective effect in the BRIN-BD11 cells using Compound-16 dissolved in DMSO in identical culture conditions, when taken together, we considered it unlikely that DMSO-toxicity was the cause for such extensive cell death.

Furthermore, during the course of testing DMSO for cytotoxicity in the cell models, it became evident that additional factors were involved. In contrast to our previously reported findings, the incubation of Compound-16 with the BRIN-BD11 cells unexpectedly resulted in extreme toxicity to this cell-line, whereas previously, it had afforded consistent cytoprotection against palmitate-induced cell death. The MSDS sheet recommended storing the diluted Compound-16 stock solution at -20 °C. However, after a storage period of about 3 – 4 weeks, it became apparent that the stock solution degraded at -20 °C and became cytotoxic in a manner not typically observed when fresh stocks were used in the initial BRIN-BD11 cell experiments. As a consequence of this previously undocumented observation, and following extensive supervisory discussion, a new stock of Compound-16 was purchased. It was pre-diluted in an identical manner to the original stock solution, but was stored at -80 °C until required. This successfully prevented the unexpected degradation-related toxicity observed in the BRIN-BD11 cells when using the compound after it had been stored at -20 °C.

In light of this, we conducted further dose-dependent testing of Compound-16 but it was clear that this compound remained potently toxic to the INS-1, MIN6 and 1.1B4 cells. It is not clear why this was the case when it was significantly cytoprotective in BRIN-BD11 cells under conditions of palmitate toxicity and serum starvation. One possibility is that Compound-16 caused 100% PKR KD resulting in complete loss of cell viability *in vitro*. However, given that PKR-null mice are viable this scenario is unlikely (Abraham *et al.*, 1999; Yang *et al.*, 1995).

Another possibility is that Compound-16 is not solely PKR-specific, and may target other e-IF2 α kinases within this family, perhaps in a manner suggested to occur with 2-AP (Jammi *et al.*, 2003). This may prevent the activation of redundant pathways in response to PKR KD, thus preventing cell rescue from loss of functional PKR as mentioned earlier with regard to PKR-null mice aberrant responses.

Jammi and colleagues (2003) also report that Compound-16 is an inhibitor of RNA-induced PKR autophosphorylation, that is, RNA-*dependent* activatory mechanisms. However, it has been established that PKR can be activated by RNA-*independent* mechanisms such as lipotoxicity. This suggests Compound-16 may only effectively inhibit PKR when RNA-induced activation initiates PKR autophosphorylation, thus the compound may not be able to inhibit PKR-autophosphorylation in response to lipotoxic RNA-independent activatory mechanisms.

It is possible that the cytoprotective effects observed in the BRIN-BD11 cells are an *in vitro* cell model-specific phenomenon. Clearly, whatever nutrient sensing pathway that allows Compound-16 to rescue BRIN-BD11 cells during persistent stress conditions, is not active in the INS-1, MIN6 cells, or 1.1B4 cells. Further studies are necessary to elucidate the exact targets of this Compound in different cell models, before any conclusions as to its suitability as a specific inhibitor of PKR can be made.

Nonetheless, with specific regard to the studies in this chapter, the extreme toxicity of Compound-16 to INS-1 and MIN6 cells, and an established increased

resistance to palmitate-induced cell death in MIN6 cells, meant that these two cell lines were not considered to be appropriate models for further investigation. What can be concluded is that in BRIN-BD11 cells under our experimental conditions, Compound-16 was significantly cytoprotective against lipotoxicity at concentrations of 5 – 25 μM . Furthermore, Compound-16 was able to significantly confer cytoprotection for up to 30 h against serum withdrawal at concentrations of 10 – 25 μM in BRIN-BD11 rodent pancreatic β -cells.

To complement our Compound-16 data, we adopted the use of DN adenoviral vectors for transient protein inhibition. Given the overlapping role more recently assigned to PKR alongside PERK within ER stress-induced pathways, it was considered an important step to define the independent activation status of each protein during lipotoxic culture conditions. Hence, our aim was to use these vectors to transiently inhibit the PKR and PERK proteins so Western blotting could be performed, to ascertain their phosphorylation status for quantification and comparison purposes.

Extensive validation and titration experiments were conducted to establish a viral titre that did not result in potent cytotoxicity. Preliminary experiments using the PERK and PKR vectors resulted in 100% cell death, or caused significant visible and aberrant cell morphology, and atypical monolayer growth patterns in the BRIN-BD11 and INS-1 cells used for these studies. This suggested that the vectors triggered a potent toxicity-related stress response in the cells, thus Western blotting of ER stress markers under these experimental conditions would not have been useful. However, an appropriate viral titre and pre-dosing incubation period was established and confirmed through morphology studies,

and the absence of increased expression of phospho-eIF2 α was confirmed by Western blotting. Our results, shown for the first time using these vectors in the BRIN-BD11 cells under our unique experimental conditions, demonstrated that individual and combined transient KD of the PERK and PKR protein resulted in reduced phosphorylation of eIF2 α . Thus, our novel results in rodent pancreatic β -cells appear to correlate with findings by Lee and colleagues (2007), where they demonstrated that in HEK293 and HeLa cells, PKR KD resulted in decreased eIF2 α activity and increase cell viability.

The ability to target PKR for KD within cell culture models provides the opportunity to investigate the effect of PKR manipulation on both ER stress and cell viability during experimental conditions of lipotoxicity. This allows an insight into the functional responses of cells to various FA species, where PKR function has been specifically targeted. Therefore, we created stable PKR knock-down clones using shRNA against PKR in INS-1 and BRIN-BD11 cells. We ultimately selected two BRIN-BD11 clonal cell lines and a BRIN-BD11 SV clone for use as a control within these novel studies.

In the first instance, to ensure there were no off-target effects of the PKR construct, the effect of dose-dependent palmitate-induced cell death in parental BRIN-BD11 cells was compared against the PKR SV clone, and indeed, there was no significant difference in percentage cell death between either cell line. This was an important initial step to verify that the SV cells responded to our experimental conditions in the same way as the parental BRIN-BD11 cells. Furthermore, this suggested that no immediate off-targets effects due to the PKR vector were apparent.

Our two clones BRIN-BD11 PKR 333 and PKR 335, with PKR KD of approximately 70% and 95% respectively, were used to investigate functional responses to FA species under conditions of lipotoxicity. Our results from these studies clearly showed a significant reduction in percentage cell death in both PKR clones compared to the parental BRIN-BD11 cells. Furthermore, the most significant protection against palmitate-induced cell death was observed in the clone with the highest level of PKR KD.

It has been well cited-in the literature that the incubation of pancreatic β -cell models with palmitoleate can protect against palmitate-induced toxicity (Welters *et al.*, 2004; Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Cnop *et al.*, 2008; Eitel *et al.*, 2003; Morgan *et al.*, 2008). However, while the underlying molecular mechanisms of this protection are not yet fully elucidated, it has been suggested that due to the rapid initiation of protection, cytoprotection may be receptor-mediated (Morgan, 2009). Our results here for the first time demonstrate that PKR KD appears to mediate enhanced cytoprotection by palmitoleate, above the level of cytoprotection seen when incubating cells with palmitoleate alone or when cells are co-incubated with both LC-FA species. While not definitive, these results do suggest that this may involve the downstream eIF2 α /ATF4/CHOP10 apoptotic signalling pathway with a role for PKR as a direct upstream mediator.

In summary, our results clearly suggest that PKR does appear to be integral to nutrient sensing pathways within the pancreatic β -cell. Targeted inhibition of normal PKR function in BRIN-BD11 cells significantly improves cell viability, and

correlates with reduced upregulation of the ER stress marker phospho-eIF2 α in response to lipotoxicity.

It is not possible to fully conclude this section without identifying limitations of this work. Alterations to cell viability and to the phosphorylation status of eIF2 α , along with our PKR KD data, have provided valuable correlative information. Results from our studies, combined with evidence from others (Lee *et al.*, 2007; Nakamura *et al.*, 2010), strongly suggests that PKR mediates cytotoxic or cytoprotective mechanisms in pancreatic β -cells, in response to chronic incubation with FA species. However, we have also cited that redundancy from the activity of other kinases such as PERK, HRI and GCN2 has been reported (Wek *et al.*, 2006; Ron & Walter, 2007; Chen *et al.*, 2008). As such, defining the activity of the PKR protein itself is a logical step to more clearly understand the exact underlying molecular mechanisms within the ER stress pathways in response to lipotoxicity.

A predominant aim throughout these studies was to investigate how changes in β -cell viability in response to chronic incubation with LC-FAs impacted on alterations to PKR activity: specifically, looking at changes in the phosphorylation status of PKR. A known issue at the outset of the study was the absence of commercially available rat phospho-PKR antibodies. Nonetheless, there is relatively high amino acid sequence homology between human vs. rat, and mouse vs. rat phospho-PKR proteins (Fig. 3.24 and Fig. 3.25). Therefore, it was considered entirely feasible to use phospho-PKR antibodies raised in mouse and human, against the lysates we extracted from rodent models. Extensive validation experiments were performed using all current commercially available mouse and human phospho-PKR antibodies, including variations in epitope

specificities, to determine if any of these products were able to detect phospho-PKR in the BRIN-BD11 cell lysates.

Mouse vs. rat phospho-PKR amino acid sequence alignment

Similarity: 441/520 (84.8%)

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496 ilktlaewrnisekkrntc 515
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Fig. 3.25 Alignment of mouse vs. rat phospho-PKR amino acid sequence

A comparison of the mouse PKR amino acid sequence with the rat PKR amino acid sequence was made to determine percentage homology.

Initially, it was thought a band at the correct predicted molecular weight of phospho-PKR (~68 kDa) was detected. However, upon more-detailed examination, it was evident that a second band at a lower molecular weight (~40 kDa) was also routinely detected on each blot. This second erroneous band reflected the same pattern as the band we initially suspected to be phospho-PKR band. This suggested that these bands may have been non-specific, so further extensive validation experiments were performed using various secondary antibodies. In addition, after further literature reviews, we adopted the use of positive control lysates, blocking peptides and gel shift electrophoresis analysis in an attempt to identify the phospho-PKR band with certainty. Unfortunately, results from these studies were also inconclusive.

Nonetheless, alternative markers within the PKR signalling pathway were also investigated by Western blot. The PACT protein, a well-cited upstream activator of PKR, along with downstream proapoptotic markers ATF4, ATF3 and nuclear protein CHOP10 were examined. In the absence of being able to detect phospho-PKR, alterations to the activity and expression of distal and proximal proteins to PKR, would have provided important information regarding the signalling pathways involved in lipotoxic conditions. It was unfortunate that these antibodies exhibited poor specificity, thus results from these studies were inconclusive.

Chapter 4 Characterisation of the responses of 1.1B4 human pancreatic β -cells to FFA

4.1 Introduction

Various *in vitro* cell models are readily available and convenient for use in diabetes research (Skelin *et al.*, 2010). The last few decades has seen a range of β -cell lines established as models to investigate β -cell function and many of these models have been rodent cell lines (Asfari *et al.*, 1992; Gazdar *et al.*, 1980; Santerre *et al.*, 1981; McClenaghan *et al.*, 1996; Efrat *et al.*, 1988; Miyazaki *et al.*, 1990). While these models have yielded valuable information from functional and molecular studies, there are many differences between rodent and human β -cells. Any significant results found in rodent cell lines also need to be established in primary islets and due to their limited availability, a great deal of effort has been invested in developing human β -cell lines from human pancreatic sources (Hohmeier and Newgard, 2004).

The limitations with human and rodent cell lines have included low insulin secretion, poor insulin secretion capacity in response to secretagogues, or cells only capable of secreting insulin over limited passage. In the case of some adult β -cell lines, the supplementation of factors necessary to maintain and promote growth also initiates dedifferentiation (Dufayet de la Tour *et al.*, 2001; Demeterco *et al.*, 2002; Gueli *et al.*, 1987; Levine *et al.*, 1995; Beattie *et al.*, 1999; Russ *et al.*, 2008), or de-differentiation may occur over continuous passage. Cells may contain abnormal chromosome numbers or express gene mutations that may affect typical protein expression and metabolism; all these considerations may go some way towards explaining why many pancreatic β -cells lines have aberrant secretory responses (Ulrich *et al.*, 2002; Skelin *et al.*, 2010).

In 2011, a UK group described the development of a novel human insulin-secreting β -cell line designated as 1.1B4. The cells were created by the electrofusion of an immortal human pancreatic epithelial carcinoma cell line (PANC-1) with normal cultured human islets, creating a cell line that has been reported to be stable in culture and to display characteristics of typical pancreatic β -cells. For example, these cells were responsive to known insulin secretagogues, and expressed major genes involved in proinsulin processing and the insulin secretory pathway such as the GLUT-1 glucose transporter and glucokinase (McCluskey *et al.*, 2011).

There have been extensive studies into the molecular mechanisms underlying lipotoxicity, and the structural properties required of various FA species that elicit a cytotoxic or cytoprotective effect in rodent β -cells, but these have not been investigated in the 1.1B4 human pancreatic β -cell line. Therefore, for the first time, we used the 1.1B4 human pancreatic β -cells to investigate their *in vitro* responses to different FA species, to consider their suitability as an appropriate model for the study of β -cell dysfunction during lipotoxic conditions such as those seen in T2D.

4.2 Materials and methods

The human pancreatic β -cell line 1.1B4 was obtained from the Human Protection Agency Culture Collections. Cells were cultured in RPMI-1640 medium containing 11 mM glucose, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. All cells were grown in 75 cm² flasks at 37 °C and with 5% CO₂ at 100% humidity and were used in experiments when approximately 80% confluent; all experiments were performed on cells below

passage number 35. Cells were passaged as described in Section 2.2.1, and treatment with FA was conducted as described in Section 2.3. Cell death was assessed using PI staining and flow cytometry (Section 2.4.2).

4.3 Results

4.3.1 Medium-chain SFA responses in 1.1B4 human pancreatic β -cells

The effects of incubating different FA species with rodent pancreatic β -cells have already been well-established, so it was important to elucidate the differential responses of incubating various FA species with this novel insulin secreting human pancreatic β -cell line. Initial time course experiments were conducted (data not shown) to determine a suitable incubation period where a similar level of cell death was evoked by incubation with 250 μ M palmitate, as we saw in our previously used rodent models. This was determined to be 24 h. Increasing concentrations of two medium-chain-SFA, laurate (C12:0) (Fig. 4.1) and myristate (C14:0) (Fig. 4.2) were shown to be well-tolerated by the human pancreatic β -cells and exerted no significant detrimental effect on percentage cell death over the 24 h incubation period, when treatment groups of any concentration were compared to the control.

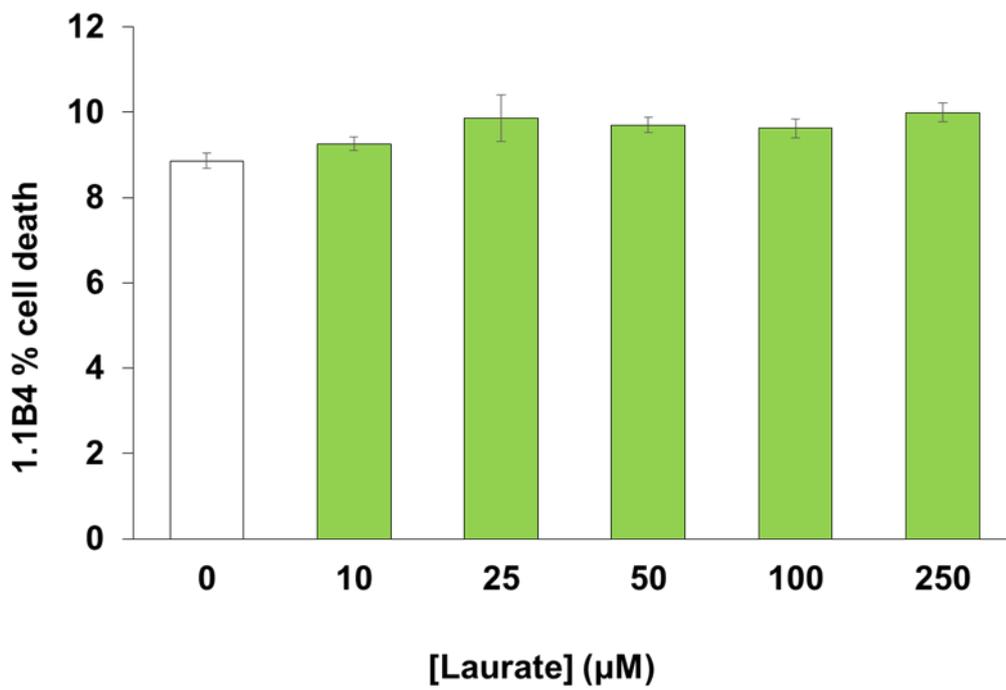


Fig. 4.1 The effect of laurate (C12:0) on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with increasing concentrations of laurate and incubated for 24 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in quadruplicate (N=4).

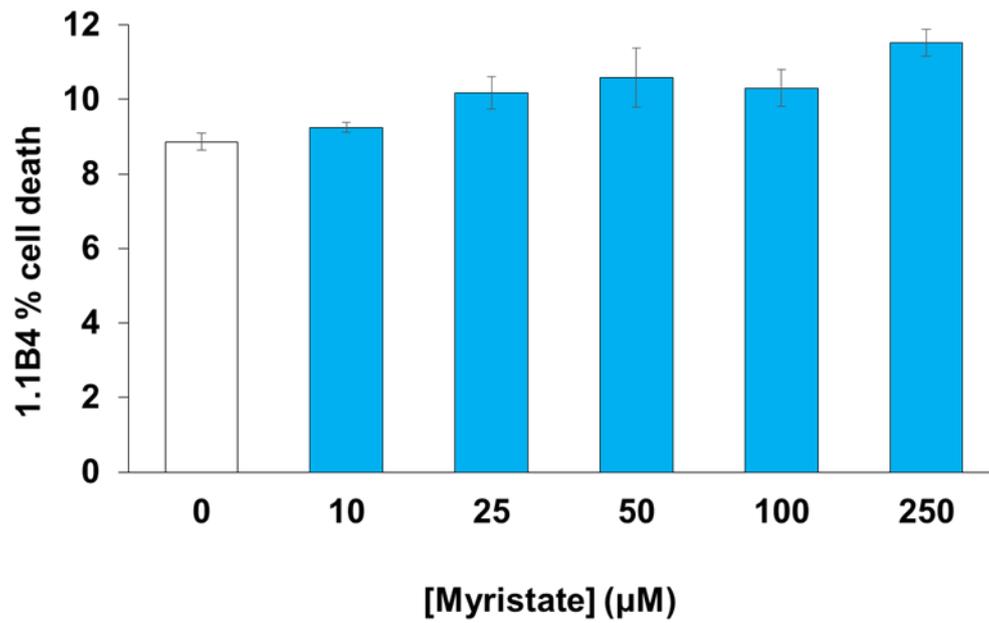


Fig. 4.2 The effect of myristate (C14:0) on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with increasing concentrations of myristate and incubated for 24 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in quadruplicate (N=4).

4.3.2 LC-SFA responses in 1.1B4 human pancreatic β -cells

The LC-SFA species palmitate (C16:0) (Fig. 4.3) and stearate (C18:0) (Fig. 4.4) were demonstrated to exert a cytotoxic effect on the 1.1B4 human pancreatic β -cells in a dose-dependent manner. Results for cells incubated with palmitate showed cell death to be: control (no palmitate) (8.9%); 10 μ M (10.9%); 25 μ M (9.6%); 50 μ M (10.6%); 100 μ M (17.4%) and 250 μ M (35.5%). These results demonstrated that palmitate at 100 and 250 μ M induced a significant increase in percentage cell death when compared to the control ($p < 0.01$ and $p < 0.001$ respectively). Results for stearate showed cell death to be: control (no stearate) (8.8%); 10 μ M (9%); 25 μ M (9.6%); 50 μ M (10.1%); 100 μ M (14.1%) and 250 μ M (18.6%). These results determined that stearate at 100 and 250 μ M also induced a significant increase in percentage cell death when compared to the control ($p < 0.01$ and $p < 0.001$ respectively). Figure 4.5 shows the combined effects of the four different SFA species used to induce lipotoxicity for comparison purposes. It is noteworthy from this figure that the cytotoxic effect of stearate (C18:0) is less than that observed with palmitate (C16:0).

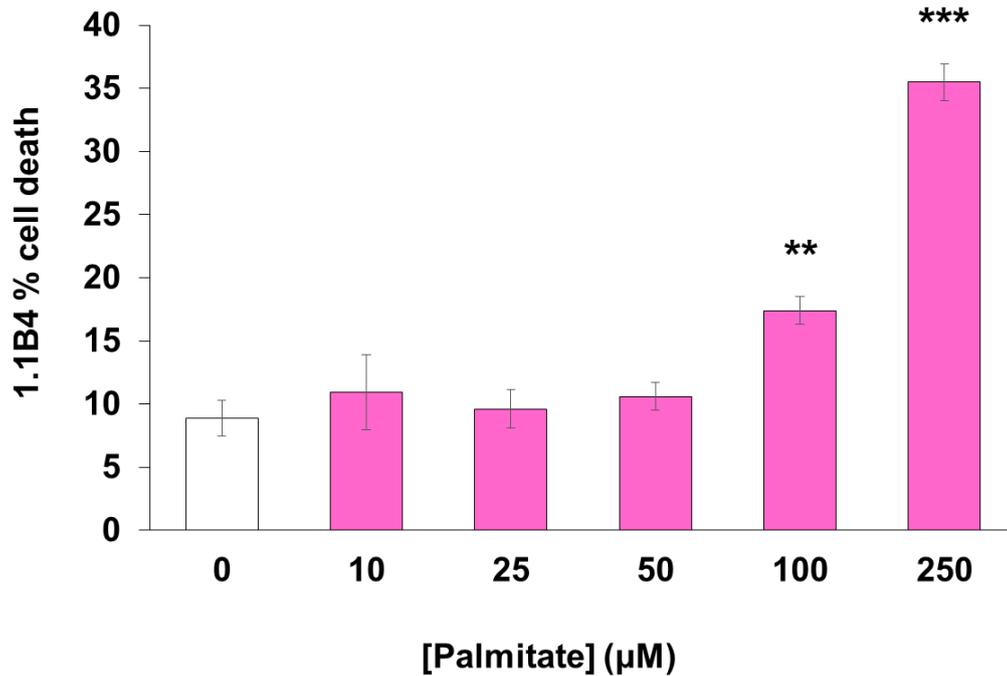


Fig. 4.3 The effect of palmitate (C16:0) on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with increasing concentrations of palmitate and incubated for 24 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in quadruplicate (N=4). *** $p < 0.001$, ** $p < 0.01$ vs. control

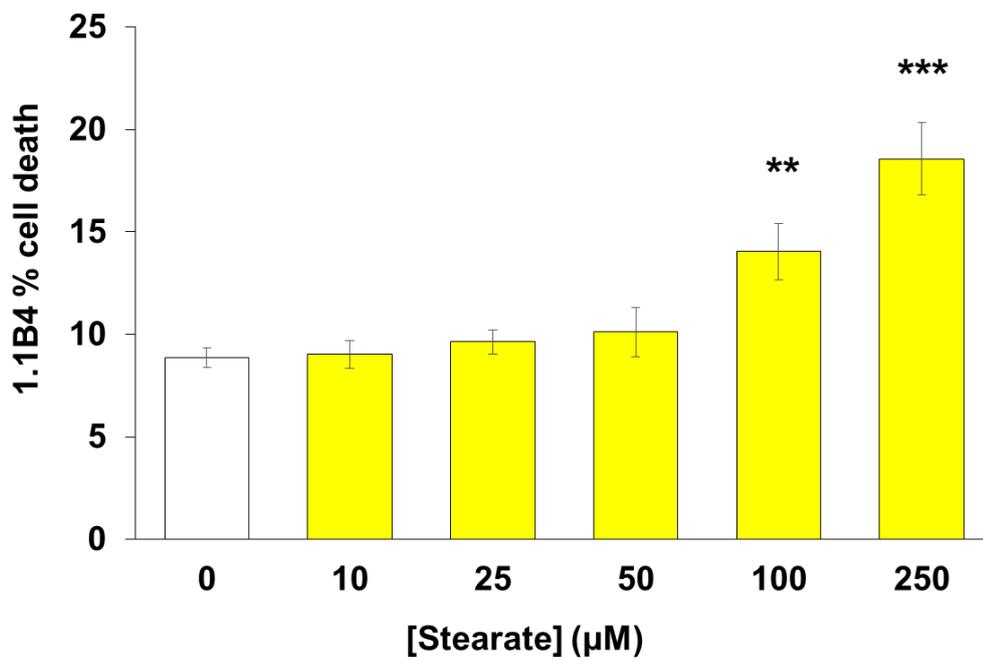


Fig. 4.4 The effect of stearate (C18:0) on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with increasing concentrations of stearate and incubated for 24 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in quadruplicate (N=4). *** $p < 0.001$, ** $p < 0.01$ vs. control

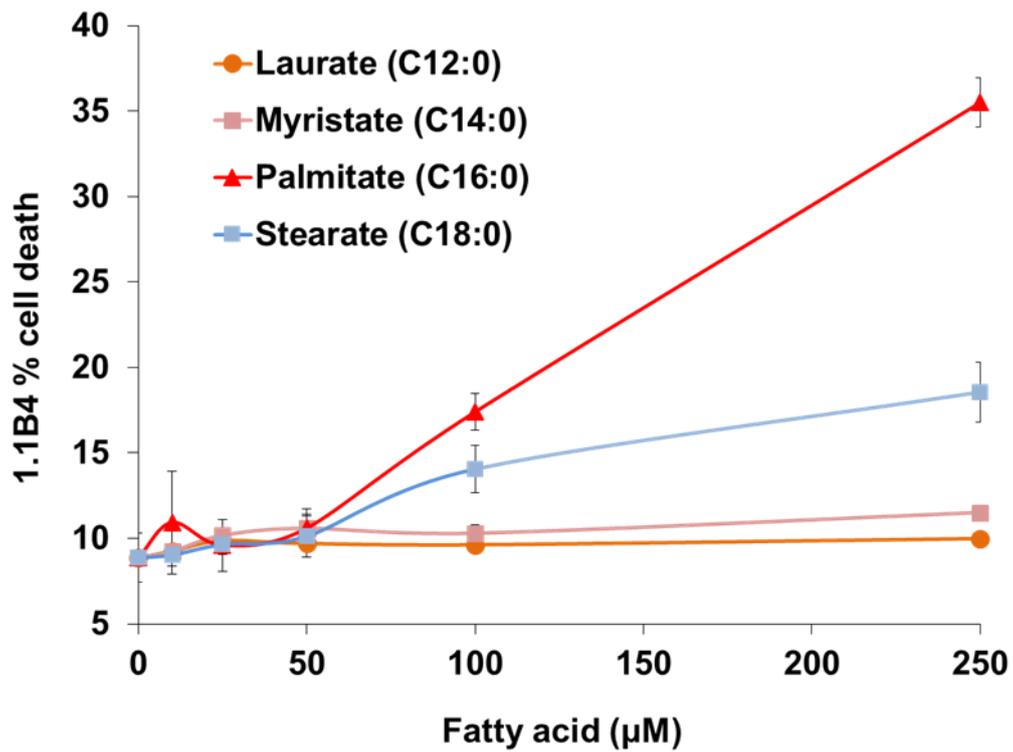


Fig. 4.5 Comparison of the effects of incubating SFA species on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with different length saturated fatty acids to assess their effect on cell viability over a 24 h incubation period. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in quadruplicate (N=4).

4.3.3 The cytoprotective effect of LC-MUFA on 1.1B4 human pancreatic β -cell death

It has been well-observed in rat pancreatic β -cells that the cytotoxic effects observed when incubating cells with LC-SFA is ameliorated when cells are co-incubated with both palmitate and the LC-MUFA palmitoleate (C16:1). It has also been established that palmitoleate is well tolerated when cultured alone with rat pancreatic β -cells. Indeed, palmitoleate has been shown to be cytoprotective against serum starvation and pro-inflammatory cytokines (Welters *et al.*, 2004). However, the cytoprotective effects of MUFA have not yet been reported in the 1.1 B4 human pancreatic β -cell line. Experiments were conducted to determine the effect of palmitoleate on percentage cell death in the 1.1B4 β -cells, where cells were incubated with each FA species alone, or co-incubated with both LC-FA species (palmitate *and* palmitoleate).

Results in this study (Fig. 4.6) were found to be: control (4.5%); 250 μ M palmitate (47.3%); 250 μ M palmitoleate (2.1%) and both FA species (2.8%). These results demonstrate that palmitate induced a 10.5-fold increase in percentage cell death compared to the control ($p < 0.001$), and this is in line with our previous findings. Palmitoleate at 250 μ M was shown to be well-tolerated and had no effect on percentage cell death when compared to the control. Importantly, the co-incubation of palmitoleate and palmitate brought about a 17-fold decrease in percentage cell death, so was able to completely ameliorate the cytotoxic effect observed when cells were incubated with 250 μ M palmitate alone ($p < 0.001$).

The 1.1B4 human pancreatic β -cells were incubated with 250 μ M palmitate (considered the control group) and increasing concentrations of palmitoleate

(C16:1) (Fig. 4.7). The percentage cell deaths for this experiment were found to be: control (51.5%); 10 μM (32%); 25 μM (23.9%); 50 μM (12.4%); 100 μM (9.2%) and 250 μM (8.2%). Palmitoleate reduced percentage cell death in a dose-dependent manner; and all concentrations were significant when compared to the control ($p < 0.001$).

The effect of the LC-MUFA species oleate (C18:1) on percentage cell death in the 1.1B4 cells was investigated next (Fig. 4.8). Results from this experiment were: control (51.7%); 10 μM (45.5%); 25 μM (33%); 50 μM (25.2%); 100 μM (24.2%) and 250 μM (26.2%). Again, it was apparent that all concentrations of oleate were able to significantly reduce percentage cell death compared to the control ($p < 0.001$). Figure 4.9 shows the two different MUFA species for comparison purposes. It is again notable that the cytoprotective effect of oleate (C18:1) is less than that observed with palmitoleate (C16:1).

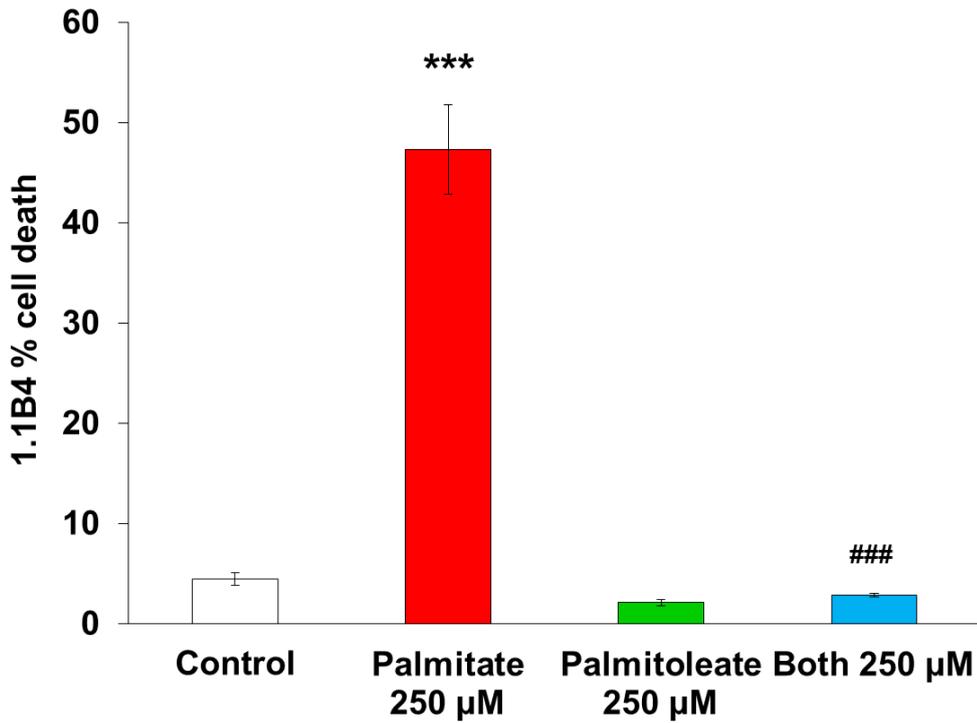


Fig. 4.6 The cytoprotective effect of palmitoleate (C16:1) on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with 250 μ M palmitate, 250 μ M palmitoleate or a combination of both FA and incubated for 24 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments (N=6). *** $p < 0.001$ vs. control, ### $p < 0.001$ vs. palmitate

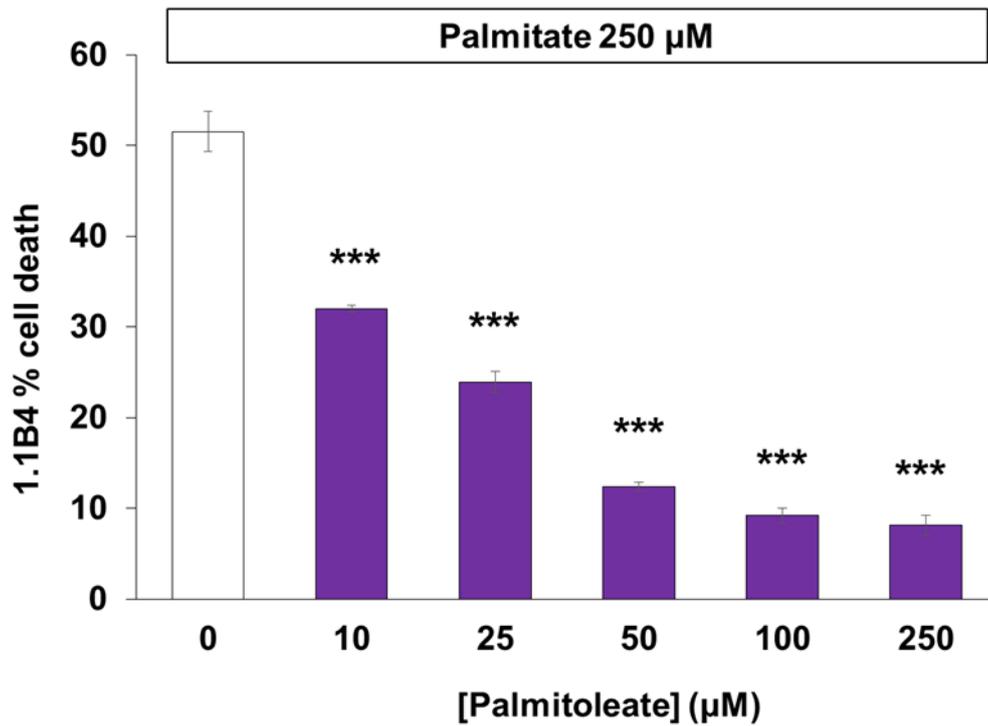


Fig. 4.7 The cytoprotective effect of palmitoleate on 1.1B4 human pancreatic β -cells is dose-dependent

1.1B4 human pancreatic β -cells were treated with 250 μ M palmitate and increasing concentrations of the monounsaturated fatty acid palmitoleate and incubated for 24 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments (N=5). *** $p < 0.001$ vs. control

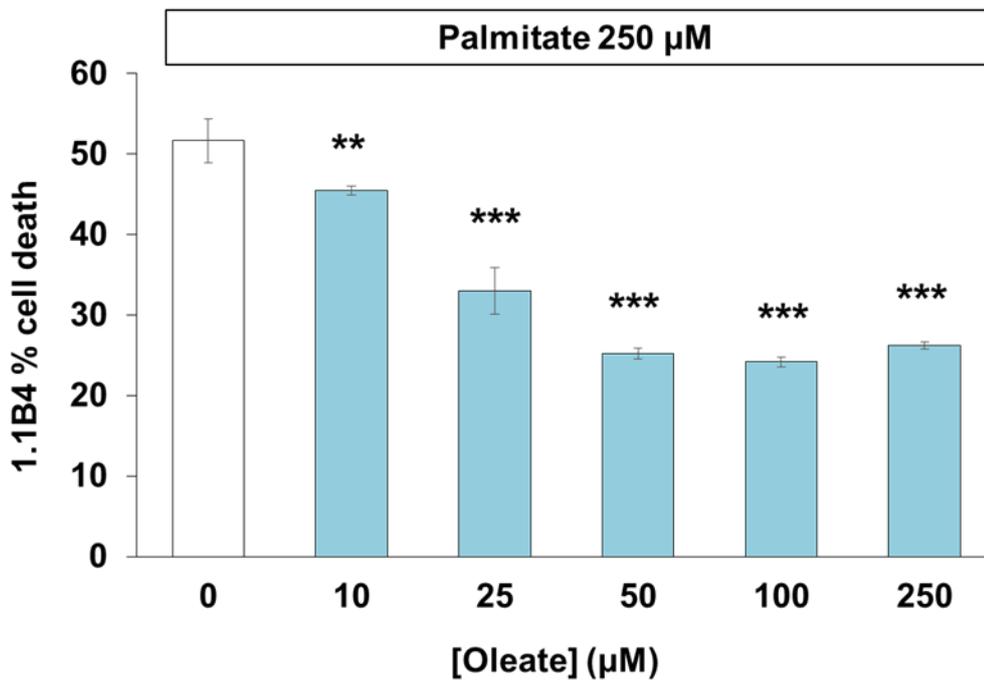


Fig. 4.8 The cytoprotective effect of oleate (C18:1) on 1.1B4 human pancreatic β -cells is dose-dependent

1.1B4 human pancreatic β -cells were treated with 250 μ M palmitate and increasing concentrations of the monounsaturated fatty acid oleate and incubated for 24 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments (N=5). *** $p < 0.001$, ** $p < 0.01$ vs. control

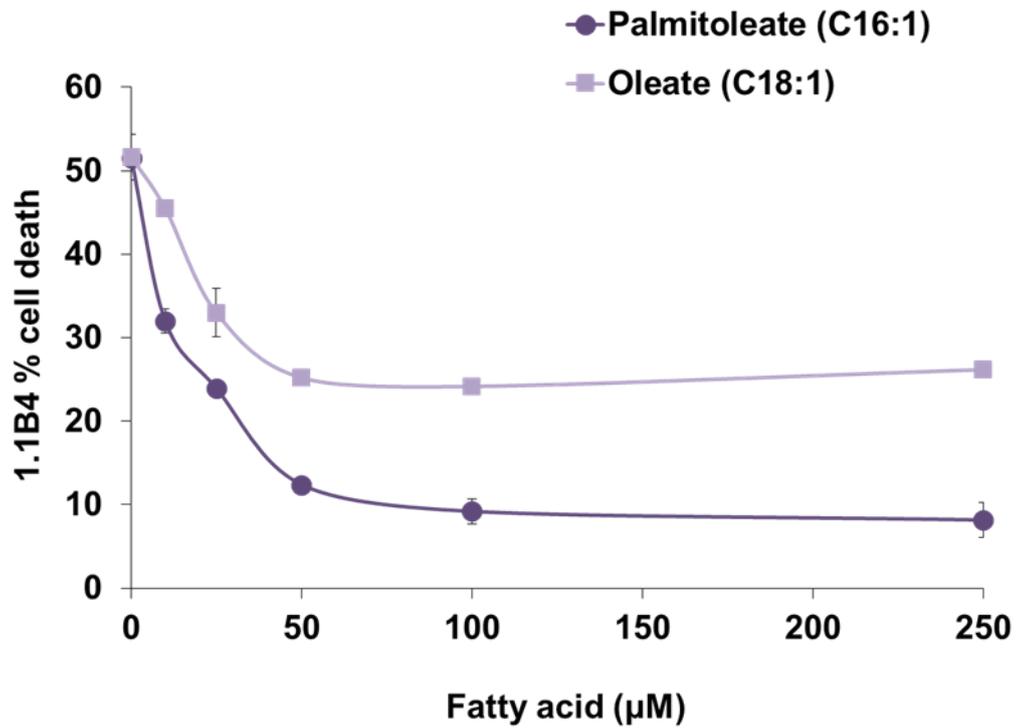


Fig. 4.9 Comparison of the effects of incubating MUFA species on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated 250 μ M palmitate and different length monounsaturated fatty acids to assess their effect on cell viability over a 24 h incubation period. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments (N=5).

4.3.4 Compound-16 is toxic to 1.1B4 cells

We have shown in Section 3.3.3 that Compound-16 exerts a cytoprotective effect in BRIN-BD11 cells during lipotoxic conditions, so we investigated here for the first time, whether Compound-16 was able to act as a cytoprotective agent in 1.1B4 human pancreatic β -cells. Cells were incubated for 24 h with Compound-16 using concentrations ranging from 1 μ M to 25 μ M. A second group of 1.1B4 cells were pre-incubated for 1 h with Compound-16, then they were additionally co-incubated with 250 μ M palmitate for the remaining 23 h incubation period (Fig. 4.10). Cell viability was assessed by flow cytometry using PI staining, and the proportion of dead cells was expressed as a percentage of the total for each treatment group.

Similar to the effects observed in both INS-1 and MIN6 rodent cells, incubation with Compound-16 alone proved to be toxic to the human pancreatic β -cells. The percentage cell death observed from cells incubated solely with Compound-16 were: control (24.7%); 1 μ M (60.9%); 5 μ M (59.2%); 10 μ M (53.6%); 15 μ M (48.2%), 20 μ M (55.4%) and 25 μ M (71.6%). These results demonstrated a significant increase in percentage cell death compared to the control at all concentrations tested ($p < 0.001$). The co-incubation of cells with 250 μ M palmitate and Compound-16 demonstrated that the addition of Compound-16 did not afford any protection against palmitate-induced cell death as was previously observed in the BRIN-BD11 cell line. Percentage cell death in this case was found to be: control (250 μ M palmitate) (65.4%); 1 μ M (87.4%); 5 μ M (85.9%); 10 μ M (87%); 15 μ M (91%), 20 μ M (92.8%) and 25 μ M (92.1%).

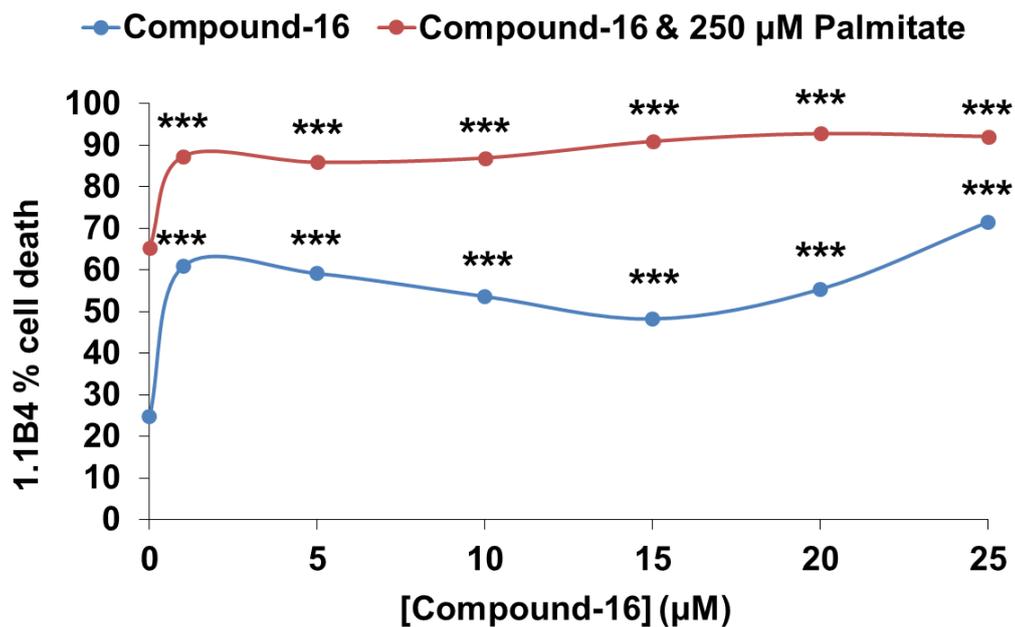


Fig. 4.10 The effect of Compound-16 on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with increasing concentrations of Compound-16. A second treatment group were pre-incubated with identical concentrations of Compound-16 for 1 h, then incubated for a further 23 h with 250 μ M palmitate. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. control

4.3.5 The effect of methyl FAs on 1.1B4 and BRIN-BD11 pancreatic β -cells

Methyl-fatty acids are alkylated derivatives of their FA counterparts, but the addition of a methyl group on the functional carboxyl group inhibits the carboxylate action, rendering the FA metabolically inert. It has been suggested that the presence of a functional carboxylate group is necessary for the cytoprotective or cytotoxic effects of the different FA species used in *in vitro* lipotoxicity studies in human islets and rodent β -cells (El-Assaad *et al.*, 2003; Hardy *et al.*, 2003). Therefore, experiments to investigate whether the addition of a methyl group can affect the cytotoxic or cytoprotective action of FA species in the 1.1B4 human pancreatic β -cells were conducted for the first time to our knowledge.

Cells were incubated for 24 h with increasing concentrations of methyl-palmitate (Fig. 4.11a)). The percentage cell death seen when cells were incubated with this methylated SFA were found to be: control (18.7%); 10 μ M (16%); 25 μ M (17.8%); 50 μ M (13.9%); 100 μ M (16.7%), and 250 μ M (14.6%). Although showing a slight downward trend, these results demonstrated that there was no significant effect on percentage cell death at any concentration compared to the control. This investigation was repeated in BRIN-BD11 cells for comparison purposes (Fig. 4.11b)), where again, although a slight downward trend was seen, there was no significant effect on percentage cell death at any concentration. Percentage cell death for the BRIN-BD11 cells were: control (19.2%); 10 μ M (18.2%); 25 μ M (17.3%); 50 μ M (16.4%); 100 μ M (17.5%), and 250 μ M (13.8%). Furthermore, no difference in the amount of percentage cell death was observed between each cell line.

The 1.1B4 human pancreatic β -cells were incubated with increasing concentrations of the C18:0 methyl-stearate FA species (Fig. 4.12a)). Results for the percentage cell death from this experiment were found to be: control (14.9%); 10 μ M (11.9%); 25 μ M (14.8%); 50 μ M (13.4%); 100 μ M (12.9%), and 250 μ M (9.9%). No detrimental effect on percentage cell death was observed at any concentration compared to the control, although results did follow a downward trend. For the BRIN-BD11 cells (Fig. 4.12b)), in contrast to the human pancreatic β -cells, a slight increasing trend in percentage cell death was observed, but these results were not significant when compared to the control. Results were found to be: control (22.4%); 10 μ M (23.6%); 25 μ M (23.3%); 50 μ M (25.9%); 100 μ M (28.1%), and 250 μ M (27.3%). Methyl-stearate exerted a greater cytotoxic effect on the BRIN-BD11 cells than the human β -cells at all concentrations tested ($p < 0.0001$). Fig. 4.13 shows the effect of the two methyl-SFA species in the 1.1B4 human pancreatic β -cells for comparison purposes.

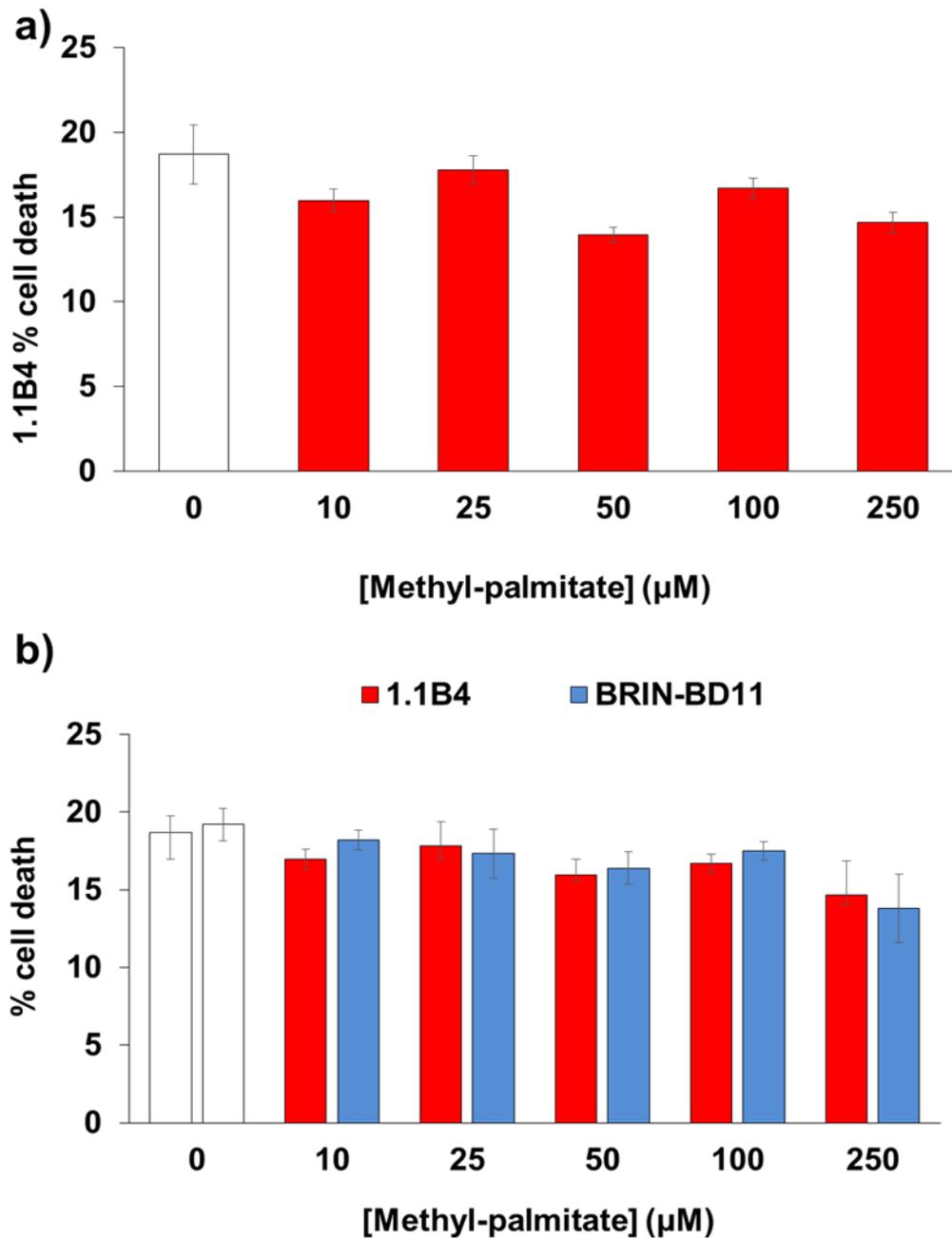


Fig. 4.11 The effect of methyl-palmitate on pancreatic β -cells

1.1B4 human pancreatic β -cells and BRIN-BD11 rat pancreatic β -cells were treated with increasing concentrations of methyl-palmitate: (a) 1.1B4 human β -cells were incubated for 24 h, (b) BRIN-BD11 cells were incubated for 18 h and effect on cell death was compared. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3).

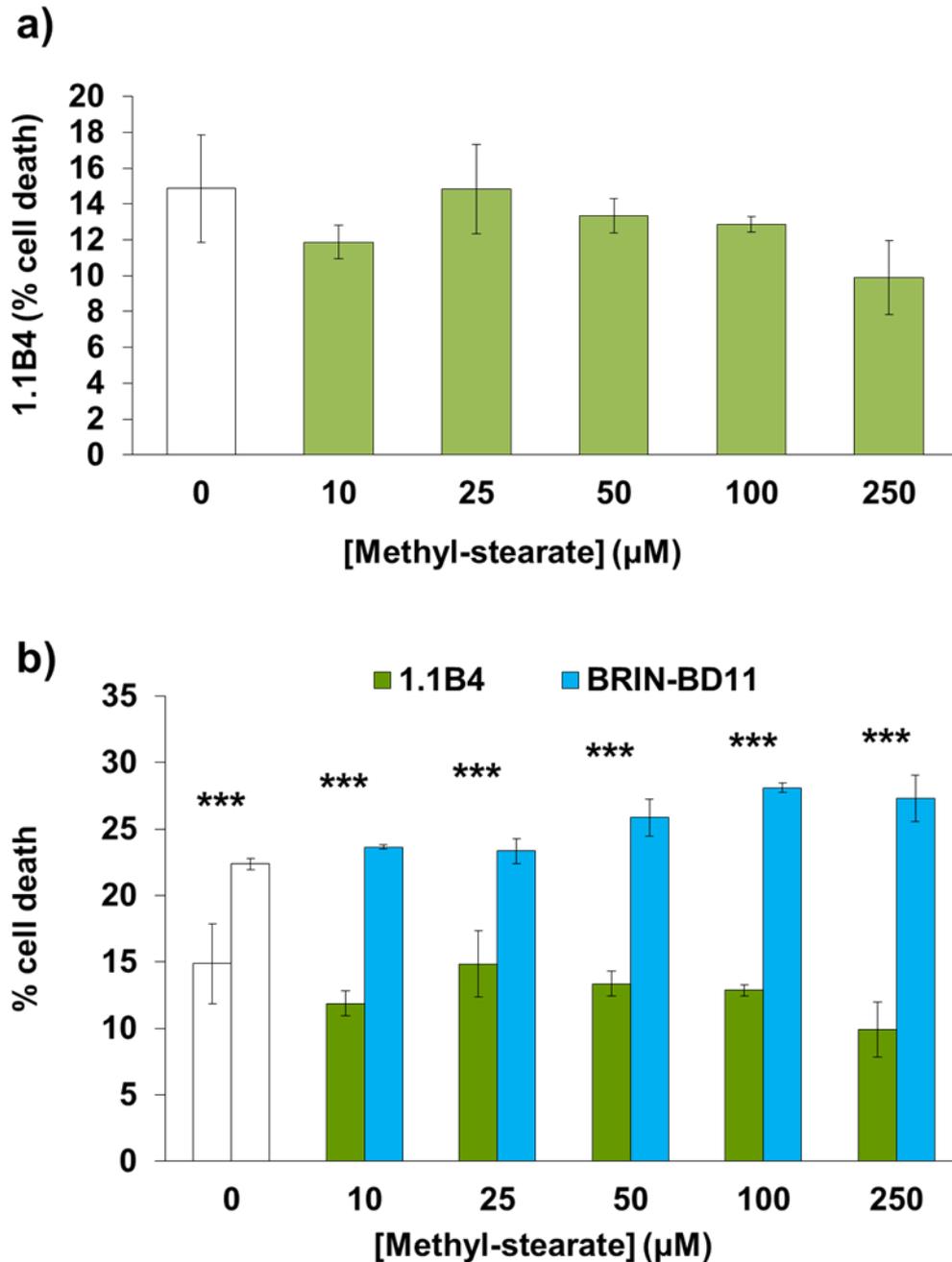


Fig. 4.12 The effect of methyl-stearate on pancreatic β -cells

1.1B4 human pancreatic β -cells and BRIN-BD11 rat pancreatic β -cells were treated with increasing concentrations of methyl-stearate: a) 1.1B4 human β -cells were incubated for 24 h, b) BRIN-BD11 cells were incubated for 18 h and effect on cell death was compared. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. 1.1B4 β -cells

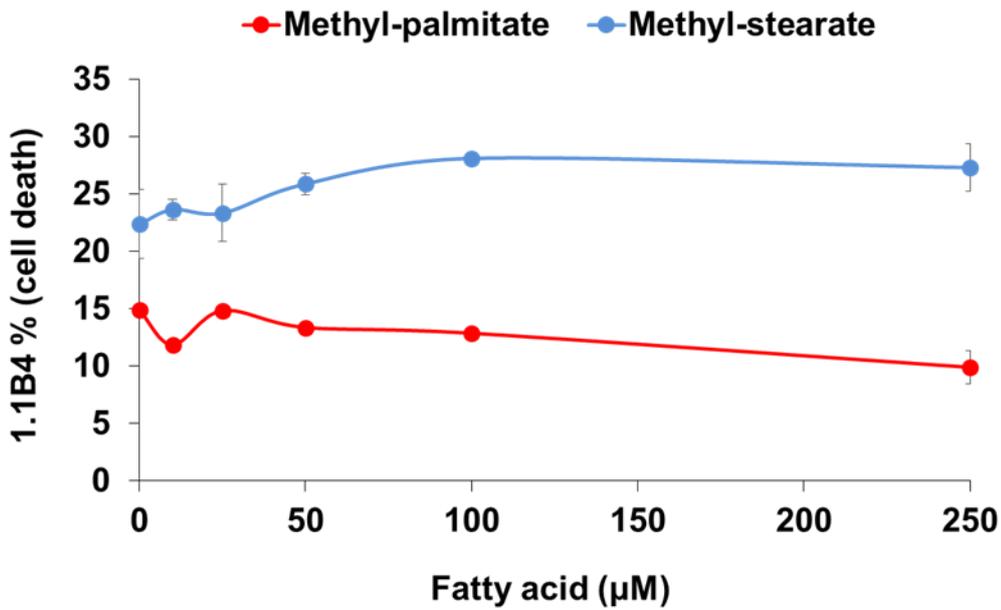


Fig. 4.13 Comparison of the effect of incubating methyl-SFA species on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with different length methyl-fatty acids to assess their effect on cell viability over a 24 h incubation period. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3).

The cytoprotective effect of methyl-palmitoleate on rat BRIN-BD11 cells has been reported previously by our group (Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2007), so this was investigated here in the 1.1B4 human pancreatic β -cells, where cells were incubated with 250 μ M palmitate (considered as the control group), and increasing concentrations of methyl-palmitoleate (Fig. 4.14a). Our results showed that the incubation of the human pancreatic β -cells with 100 and 250 μ M methyl-palmitoleate significantly decreased the percentage cell death seen compared to the control. Results were found to be: 250 μ M palmitate (control), 91.6%; 10 μ M methyl-palmitoleate, 90.8%; 25 μ M methyl-palmitoleate, 89.6%; 50 μ M methyl-palmitoleate, 88.1%; 100 μ M methyl-palmitoleate, 83.6%, ($p < 0.001$), and 250 μ M methyl-palmitoleate, 60.9%, ($p < 0.001$).

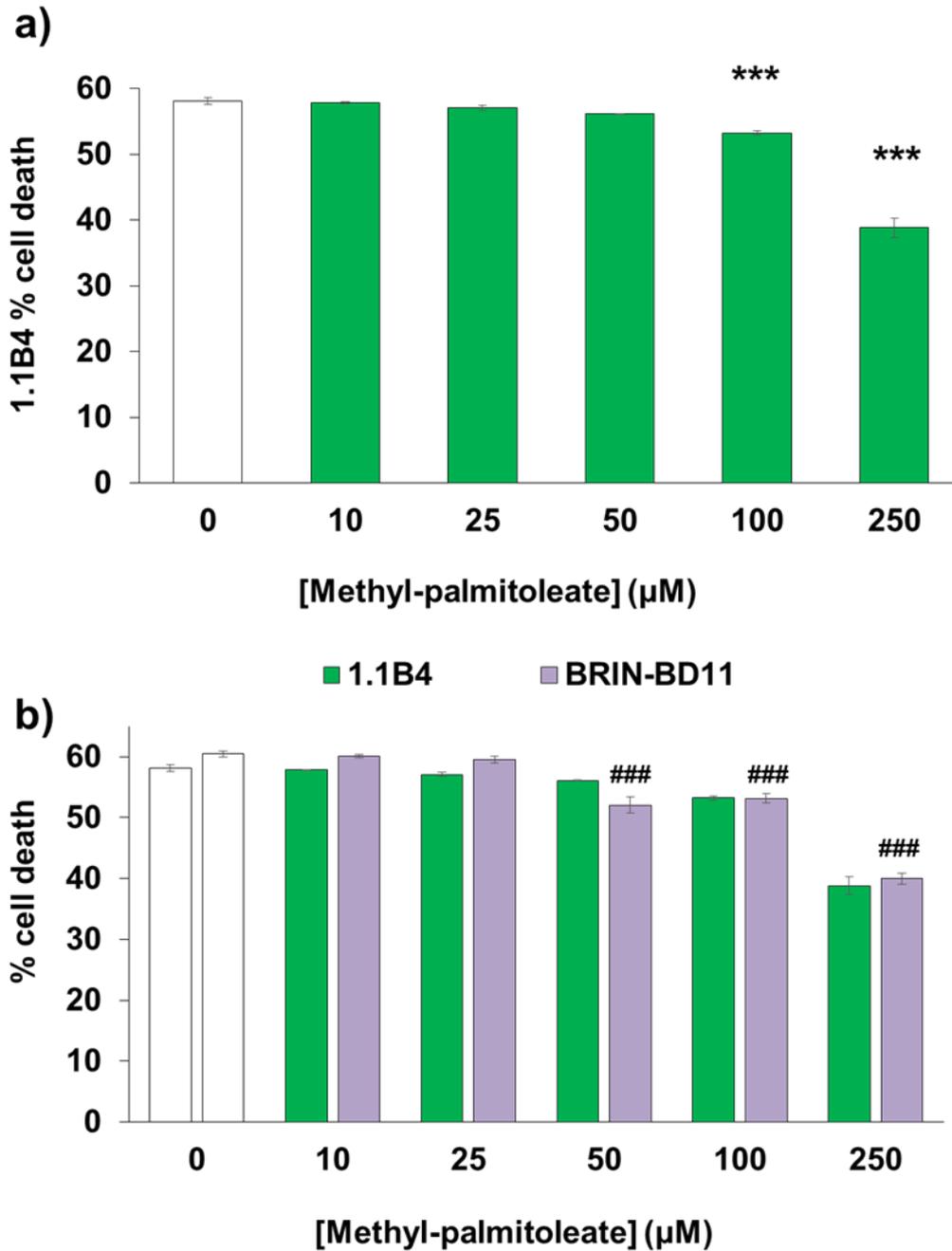


Fig. 4.14 The effect of methyl-palmitoleate on cultured pancreatic β -cells

1.1B4 human pancreatic β -cells and BRIN-BD11 rat pancreatic β -cells were treated with 250 μ M palmitate and increasing concentrations of methyl-palmitate: a) 1.1B4 human β -cells were incubated for 24 h, b) BRIN-BD11 cells were incubated for 18 h and effect on cell viability was compared. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed at least in triplicate (N=3). *** $p < 0.001$ vs. 1.1B4 control; ### $p < 0.001$ vs. BRIN-BD11 control

Methyl-palmitoleate was also co-incubated in BRIN-BD11 cells, and results demonstrated that at 50, 100 and 250 μM concentrations, incubation of cells with methyl-palmitoleate significantly decreased the percentage cell death observed compared to the control group (Fig. 4.14b)). The results for this cell line were as follows: control (93.1%); 10 μM (92.7%); 25 μM (91.7%); 50 μM (80.2%, $p < 0.001$); 100 μM (81.9%, $p < 0.001$) and 250 μM (61.6%, $p < 0.001$). Again, there was no difference in percentage cell death observed between either the human or rodent pancreatic β -cell lines.

In the final set of experiments for this section, 1.1B4 human pancreatic β -cells were incubated with 250 μM palmitate and increasing concentrations of the C18:1 methyl-MUFA oleate. Similar to the results observed with methyl-palmitoleate, 100 and 250 μM methyl-oleate significantly reduced the percentage cell death observed, so was protective against palmitate-induced cell death (Fig. 4.15a)). Results in this case were found to be: 250 μM palmitate (no methyl-oleate) considered as the control (91.6%); 10 μM methyl-palmitoleate (90.8%); 25 μM methyl-palmitoleate (89.6%); 50 μM methyl-palmitoleate (88.1%); 100 μM methyl-palmitoleate (83.6%, $p < 0.001$), and 250 μM methyl-palmitoleate (60.9%, $p < 0.001$).

BRIN-BD11 cells were incubated with methyl-oleate, where a significant decrease in percentage cell death was also observed at 100 and 250 μM concentrations (Fig. 4.15b)). Results here were shown to be: 250 μM palmitate (no methyl-oleate) considered as the control (50.9%); 10 μM methyl-oleate (50.6%); 25 μM methyl-palmitoleate (49.6%); 50 μM methyl-palmitoleate (48.5%); 100 μM methyl-palmitoleate (45.7%, $p < 0.01$), and 250 μM methyl-palmitoleate

(32%, $p < 0.001$). Fig. 4.16 shows the effect of the two methyl-MUFA species in the 1.1B4 human pancreatic β -cells for comparison purposes.

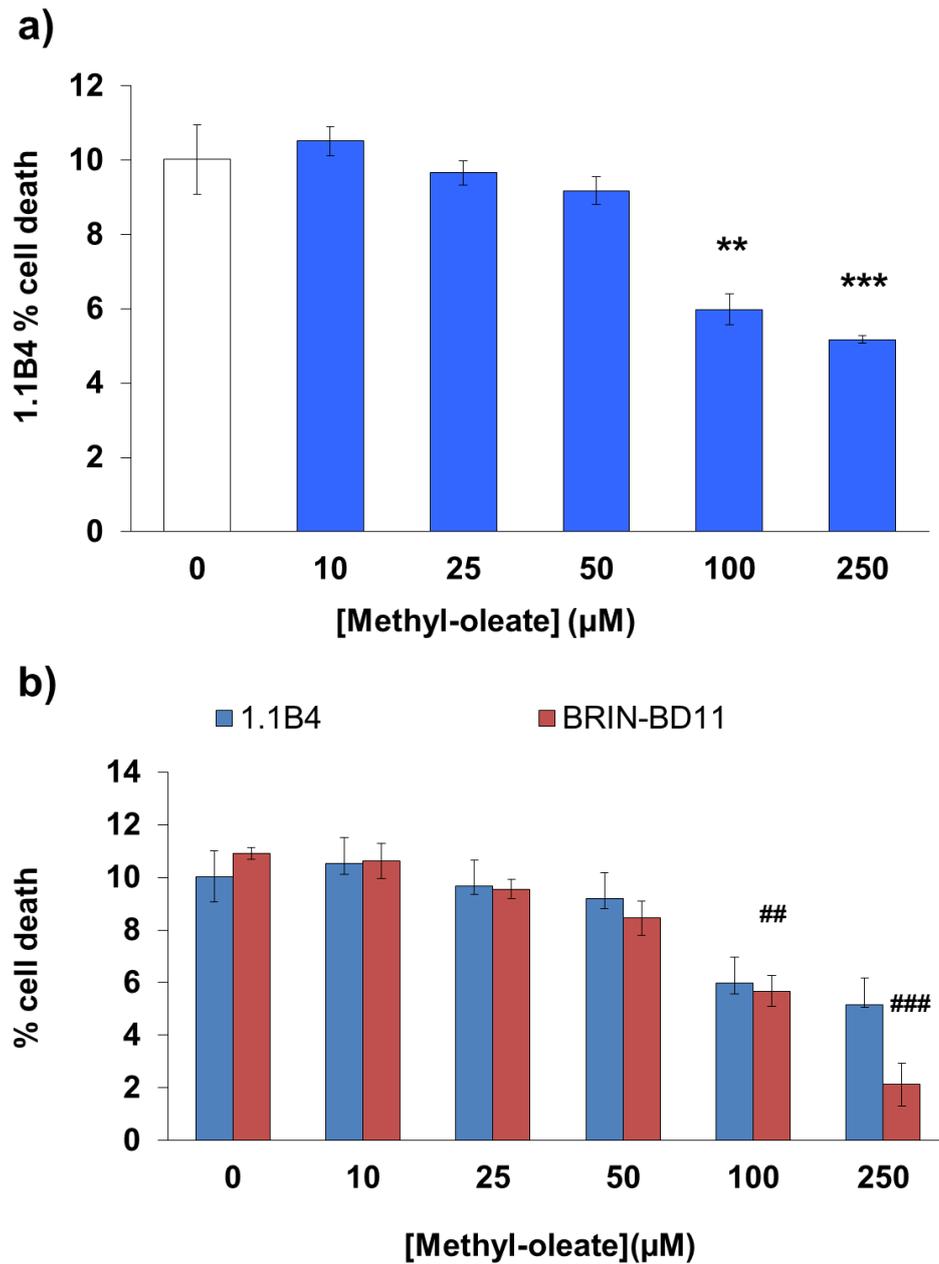


Fig. 4.15 The effect of methyl-oleate on cultured pancreatic β -cells

1.1B4 human pancreatic β -cells and BRIN-BD11 rat pancreatic β -cells were treated with 250 μ M palmitate and increasing concentrations of methyl-oleate: a) 1.1B4 human β -cells were incubated for 24 h, b) BRIN-BD11 cells were incubated for 18 h and effect on cell viability was compared. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$, ** $p < 0.01$ vs. control, ### $p < 0.001$, ## $p < 0.01$ vs. BRIN-BD11 control.

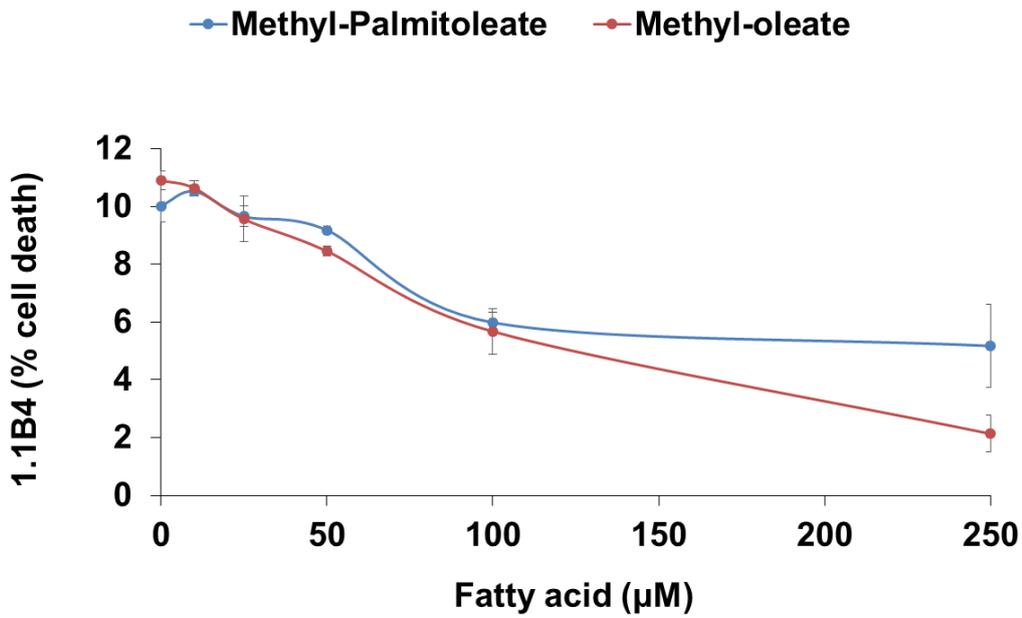


Fig. 4.16 Comparison of the effect of incubating methyl-MUFA species on 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with different length methyl-fatty acids to assess their effect on cell viability over a 24 h incubation period. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3).

4.4 Summary of novel results

- Medium-chain SFAs (C12:0 and C14:0) are well tolerated in 1.1B4 human pancreatic β -cells under chronic incubation conditions (Fig. 4.1 – 4.2).
- Incubation of 1.1B4 cells with palmitate (C16:0) leads to a dose-dependent increase in percentage cell death under chronic incubation conditions, significant at 100 and 250 μ M ($p < 0.01$ and $p < 0.001$ respectively) (Fig. 4.3).
- Incubation of 1.1B4 cells with stearate (C18:0) (Fig. 4.4) leads to a dose-dependent increase in percentage cell death under chronic incubation conditions, significant at 100 and 250 μ M ($p < 0.001$). Stearate does not exert a greater cytotoxic effect than palmitate (Fig. 4.5).
- Co-incubation of 1.1B4 cells with palmitate and palmitoleate (C16:1), leads to a significant dose-dependent decrease in percentage cell death ($p < 0.001$) under chronic incubation conditions (Fig. 4.7).
- Co-incubation of 1.1B4 cells with palmitate and oleate (C18:1) leads to a dose-dependent decrease in percentage cell death under chronic incubation conditions, significant at 10 μ M ($p < 0.01$) and 25 – 250 μ M ($p < 0.001$) (Fig. 4.8). Oleate is not more cytoprotective effect than palmitoleate (Fig. 4.9).
- Compound-16 is significantly cytotoxic to 1.1B4 cells (Fig. 4.10) at all concentrations tested (1 – 25 μ M, $p < 0.001$).
- The incubation of 1.1B4 or BRIN-BD11 cells with methyl-SFA species (C16:0 and C18:0) does not affect cell viability (Fig. 4.11 – 4.12).
- Methyl-palmitoleate (C16:1) exerts a dose-dependent cytoprotective effect on palmitate-induced cell death in 1.1B4 MUFA species under chronic incubation conditions (Fig. 4.14a), significant at 100 and 250 μ M concentrations ($p < 0.001$).

- Methyl-oleate (C18:1) exerts a dose-dependent cytoprotective effect on palmitate-induced cell death in 1.1B4 MUFA species under chronic incubation conditions (Fig. 4.15a)), significant at 100 and 250 μM concentrations ($p < 0.01$ and $p < 0.001$ respectively). Methyl-oleate was less cytoprotective than C16:1 methyl-palmitoleate.
- In BRIN-BD11 cells (Fig. 4.15b)), methyl-oleate provides dose-dependent cytoprotection against palmitate-induced cell death, significant at 100 and 250 μM concentrations ($p < 0.01$ and $p < 0.001$ respectively).

4.5 Discussion

The presence of raised concentrations of FFAs in physiological conditions are suggested to exert a damaging effect on pancreatic β -cell viability. It has been reported that the potential of FAs to stimulate insulin secretion increases with chain length, and conversely decreases with the degree of unsaturation of the FA species being studied (Opara *et al.*, 1994; Dobbins *et al.*, 1998). In the event of the chronic supraphysiological presence of FFAs as is suggested to occur during obesity, it is believed that the availability of predominantly LC-SFAs released from adipose tissue contributes towards the long-term detriment to pancreatic β -cell viability, by driving constant pancreatic β -cell stimulation due to persistent insulin release. This is thought to culminate in ' β -cell exhaustion', a mechanism defined as depleted insulin stores due to the chronic exposure to secretagogues. As such, this mechanism may explain why LC-SFAs may induce pancreatic β -cell apoptosis (Poitout & Robertson, 2002).

The effects of chronic exposure to different FA species on rodent pancreatic β -cell lines and in human primary islets have been clearly established. These studies have demonstrated that the structural requirements for the cytotoxic action of SFAs increases as the chain length of the species being studied increases. Long-chain SFAs exert a potent and detrimental dose-dependent effect on cell viability, whereas medium- and shorter-chain SFAs are well-tolerated and do not exhibit any detrimental effects on cell viability (Welters *et al.*, 2004; Eitel *et al.*, 2003; Dhayal *et al.*, 2008). Mono-unsaturated FA species are also well-tolerated and do not exert a detrimental effect on β -cell death and this has been shown to be independent of chain length. Furthermore, LC-MUFA species can be cytoprotective during co-incubation with their LC-SFA

counterparts (Dhayal *et al.*, 2008; Newsholme *et al.*, 2007; Cnop *et al.*, 2001; Welters *et al.*, 2004; Maedler *et al.*, 2001, Eitel *et al.*, 2002; Maedler *et al.*, 2003; Karaskov *et al.*, 2006). Additional studies have shown that this appears to be true for both monounsaturated and polyunsaturated species in human islets and rodent β -cells (Maedler *et al.*, 2003; Morgan *et al.*, 2008; Keane & Newsholme, 2008; Morgan, 2008). However, the effects of FA species, either saturated or monounsaturated have not yet been reported in the human pancreatic β -cell line. Therefore, in these studies, we employed the use of 1.1B4 human pancreatic β -cells to investigate for the first time, how their *in vitro* responses to different FA species compare with existing rodent models and human islets.

We report that medium-chain SFAs are well-tolerated in the 1.1B4 human pancreatic β -cells. Chronic incubation with laurate (C12:0) and myristate (C14:0) did not induce cell death at any of the concentrations tested. The LC-SFA species palmitate (C16:0) and stearate (C18:0) were found to exert toxic effects on the human pancreatic β -cells and these were dose-dependent. These results correlate with reported findings from our previous studies, and work by others using rodent pancreatic β -cell models (Newsholme *et al.*, 2007; Welters *et al.*, 2004; Eitel *et al.*, 2003; Dhayal *et al.*, 2008).

The C14:1 MUFA myristoleate did not affect the percentage cell death observed in the 1.1B4 human pancreatic β -cells, and this observation correlates with the effects of myristoleate that have been previously observed in BRIN-BD11 cells (Dhayal *et al.*, 2008). The LC-MUFA palmitoleate (C16:1) and oleate (C18:1) were able to completely ameliorate the cytotoxic action of palmitate in the human β -cells during co-incubation studies. Our results investigating the effects of

MUFA in human pancreatic β -cells show for the first time, a correlation between the cytoprotective effects observed in human 1.1B4 cells and what has been reported in previous studies using rodent cell lines and human islets (Dhayal *et al.*, 2008; Cnop *et al.*, 2001; Welters *et al.*, 2004; Maedler *et al.*, 2001, Eitel *et al.*, 2002; Maedler *et al.*, 2003; Karaskov *et al.*, 2006).

However, it has been suggested that the length of FA species entering the β -oxidation pathway does not impact upon their mode of metabolism and as such, is unlikely to explain the differences observed in cytotoxicity or cytoprotection as a FA chain length increases. The addition of a methyl group on the functional carboxyl group blocks the initial esterification step of FAs in the β -oxidation pathway, and as such FA methyl esters can prevent FA oxidation. It has been shown that using methyl-MUFA species does not impede their cytoprotective action, as methyl-MUFA species are still able to effectively inhibit rodent β -cell death when co-incubated with palmitate (El-Assaad *et al.*, 2003; Hardy *et al.*, 2003; Newsholme *et al.*, 2007). Furthermore, our group (Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2008) demonstrated that in BRIN-BD11 and INS-1 cells, equipotent protection was achieved using methyl-palmitoleate as with palmitoleate, during co-incubation studies with palmitate, and as such supports the hypothesis that FA length does not affect how they are metabolised.

With this knowledge, it would be reasonable to expect that the C18 SFA species stearate might exert a greater cytotoxic effect than C16 palmitate in the 1.1B4 human pancreatic β -cells. We would have expected to see increased percentage cell death in the 1.1B4 cells incubated with stearate, but this was not the case. Equally, it would have been reasonable to expect that the C18 MUFA species

oleate would exert a greater cytoprotective effect than C16 palmitoleate in the 1.1B4 cells, so we would have anticipated a decrease in percentage cell death when cells were incubated with oleate, but again, the opposite was true. These unexpected findings in this cell line are difficult to interpret in light of the fact that the mechanisms that underlie why increasing carbon chain length, or why the degree of saturation or unsaturation of a fatty acid species elicits cytotoxic or cytoprotective effects in pancreatic β -cells, are still not yet fully understood. It may be that alternative signalling pathways are activated in the human pancreatic β -cell line due to its tumoural origin, or there may be greater redundancy within the 1.1B4 ER stress response pathways, going some way to explain why this cell line responds differently to C18 FA species, compared to rodent β -cells and human islets.

There is currently a paucity of data on the effects of methyl-FA species on pancreatic β -cells. However, research has shown that in human and rat islets, methyl-palmitate does not affect cell viability or lead to the upregulation of ER stress markers such as ATF3, CHOP, BiP and XBP1s (Briaud *et al.*, 2001; Cunha *et al.*, 2008). It has been suggested that elevated TG formation may be a mechanism for the differential effects of FA species (Moffitt *et al.*, 2005). However, the use of methyl-palmitoleate has demonstrated that the cytoprotective action of MUFA species is not through alterations to intracellular TG formation in BRIN-BD11 cells (Diakogiannaki *et al.*, 2007; Dhayal *et al.*, 2008). The use of methyl FA species in viability studies using 1.1B4 human cells, revealed for the first time that both methyl-palmitoleate and methyl-oleate were cytoprotective when cells co-incubated with 250 μ M palmitate. In the BRIN-BD11 β -cells, methyl-palmitoleate co-incubated with 250 μ M palmitate was

cytoprotective at 50, 100 and 250 μ M, and this correlates with previous findings by our group (Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2008). Again, it is interesting that in the 1.1B4 human pancreatic β -cells, while methyl-oleate was protective, it was less potent than C16:1 methyl-palmitoleate. This has not yet been reported elsewhere. A further finding is that in the 1.1B4 cells, no significant detrimental effect on percentage cell death was seen using methyl-SFA species. This is a novel finding in the 1.1B4 cells, although the response in this human cell line correlates with previous work showing that methyl-palmitate is not toxic to rodent β -cell lines (Welters *et al.*, 2004, Cunha *et al.*, 2008).

Our results from the Compound-16 studies demonstrated that this small molecule inhibitor was toxic to the 1.1B4 human pancreatic β -cells. This is in contrast to what we reported in the previous chapter in the BRIN-BD11 cell line, but given that this potent toxicity was observed in both INS-1 and MIN6 rodent pancreatic β -cells, it was not unduly surprising. It is possible that there is redundancy in the BRIN-BD11 nutrient sensing pathway which compensates for the transient knockdown of PKR, which is not present in the INS-1, MIN6 or 1.1B4 β -cells.

In summary, results from our studies presented here demonstrate that the 1.1B4 human pancreatic β -cells may represent a suitable model for the study of lipotoxic mechanisms. Our results show that these cells respond to glucose in a normal physiological range, and importantly, we have shown novel evidence to demonstrate that these cells respond to FA species in a manner demonstrated in primary human islets.

The only published work to date pertaining to the effects of lipotoxicity in the 1.1B4 β -cells has been limited to the group who developed the cell line. They report that 500 μ M palmitate over an 18 h incubation period significantly increased cell death, and this was established using cell death and cell proliferation assays (Vasu *et al.*, 2013). However, no other investigations to characterise 1.1B4 responses to other FA species have been conducted to our knowledge. In conclusion, our results support the use of 1.1B4 human pancreatic β -cells as a useful model for *in vitro* studies for functional responses to FAs, and as such these cells may provide valuable information about the underlying role of FAs in the pathophysiology of T2D.

Chapter 5 The role of TXNIP and inflammatory mediators in pancreatic β -cell apoptosis

5.1 Introduction

Thioredoxin proteins are highly-conserved antioxidant enzymes present abundantly within intracellular compartments (Arner & Holmgren, 2000; Hirota *et al.*, 1997; Hirota *et al.*, 1999; Martin & Dean, 1991). The predominant role of thioredoxin, along with other antioxidant proteins and peptides such as glutathione within the cell, is to maintain an intracellular reducing environment. This occurs by the reduction of the thiol group of the cysteine side chains in proteins (Gilbert, 1990; Berndt *et al.*, 2007). Thioredoxins are integral to one of the primary reducing pathways within a cell (Collet & Messens, 2010), although they also have numerous other roles. These include reductase activity (Holmgren, 1985), protecting cells against stress particularly from ROS (Landino *et al.*, 2004), regulation of apoptosis (Ravi *et al.*, 2005), modulation of the inflammatory response (Nakamura *et al.*, 2005) and the promotion of protein folding within the ER (Kern *et al.*, 2003).

The activity of thioredoxin is regulated by an endogenous inhibitor protein called thioredoxin-interacting protein (TXNIP). TXNIP has been identified as one of the most highly inducible genes expressed in cultured human islets during elevated glucose conditions (Shalev *et al.*, 2002). TXNIP also modulates the cellular redox state but by an opposite mechanism to thioredoxin, making this protein a known initiator of oxidative stress within the cell (Nishiyama *et al.*, 1999; Nishiyama *et al.*, 2001). TXNIP has been shown to be a proapoptotic factor in pancreatic β -cells during hyperglycaemia although the exact mechanism by which this occurs is still unclear (Shalev, 2008). Overexpression of TXNIP can be induced by

hyperglycaemia, a condition which has been demonstrated to induce apoptosis in pancreatic β -cell models (Minn *et al.*, 2005, Junn *et al.*, 2000). This suggests that TXNIP may have a role in glucotoxicity-associated pancreatic β -cell apoptosis, as seen in T2D (Chen *et al.*, 2008).

There are several other mechanisms suggested to contribute to pancreatic β -cell toxicity in response to hyperglycaemic conditions. One is the production of ROS during the course of normal oxidative phosphorylation (Robertson, 2006). This may result in heightened cell death due to reduced redox potential, thereby rendering the pancreatic β -cell more susceptible to ROS and oxidative stress (Junn *et al.*, 2000; Wang *et al.*, 2002). It is already known that the pancreatic β -cell is very susceptible to oxidative stress, having some of the lowest levels of antioxidant enzyme expression compared to most other tissues (Robertson & Harmon, 2006; Lenzen, 2008). The production of pro-inflammatory cytokines such as IL-1 β is now a commonly accepted mechanism that is involved in apoptotic pathways seen in chronic inflammatory conditions such as obesity and T2D (Maedler & Donath, 2004). It has further been suggested that chronic hyperglycaemia induces ER stress which may drive β -cell apoptosis (Marchetti *et al.*, 2007; Maedler & Donath; 2004; Eizirik *et al.*, 2008). These mechanisms may individually, or more likely collectively, contribute to the ultimate decline of pancreatic β -cell mass and the induction of an insulin-resistant state.

The use of antioxidants and inhibitors of ROS production as a means to reduce mitochondrial oxidative damage, insulin resistance and inflammatory signalling have been areas of great interest in attempts to minimise the risk of diabetic complications (Green *et al.*, 2004; Kim *et al.*, 2007). As such, the TCA cycle

intermediate succinate had been proposed as a potential cytoprotective agent via its ability to inhibit the production of ROS and ameliorate dysfunctional mitochondrial oxidative phosphorylation. Succinate has a potent action to stimulate insulin release, and is suggested to limit oxidative stress mechanisms that may drive mitochondrial dysfunction under glucolipotoxic conditions (Fahien and MacDonald, 2002; Vengerovskii *et al.*, 2007; Zavodnik *et al.*, 2011). Protection has also been suggested to occur through the prevention of peroxidative damage, protein cross-linking and by stabilising mitochondrial membrane permeability (Tretter *et al.*, 1987). It has been suggested that glucolipotoxicity results in impaired metabolism due to the depletion of TCA cycle intermediates, and it was proposed that supplementation with intermediary metabolites may enhance anaplerosis, a mechanism that may protect cells against the detrimental effects of high glucose and high SFA concentrations (Choi *et al.*, 2011).

The links between a chronic inflammatory response and the progression of insulin resistant states such as obesity and T2D are well established. Under metabolically dysfunctional conditions, ER stress and activation of the ERS are suggested to be critical players in driving pancreatic β -cell apoptosis (Hummasti and Hotamisligil, 2012). Deposition of excess FFA in adipose tissue is suggested as a further key process that triggers an ER stress response driven by increased peripheral insulin resistance. This mechanism, in combination with hyperglycaemia, is suggested to drive a glucolipotoxic environment believed to ultimately culminate in the pancreatic β -cell demise (Gregor and Hotamisligil, 2007; Hotamisligil, 2010; Cnop *et al.*, 2011). Evidence now suggests that metabolism, the immune inflammatory response and the ERS are tightly

regulated adaptive mechanisms that collectively respond to changes in energy flux, making these mechanisms intricately related to, and essential for cell survival (Hotamisligil, 2010; Cao *et al.*, 2008). However, this could also lead to eventual dysfunctional responses due to chronic toxic stimuli such as excess glucose or SFA levels (Hummasti and Hotamisligil, 2012; Wellen and Hotamisligil, 2005; Kaufman *et al.*, 2002; Hotamisligil and Erbay, 2008).

Activation of NLRs, in particular the NLRP3 inflammasome is proposed to occur via a two signal activation step, triggered by both exogenous and endogenous danger signals. The activation of NLR links the activation of proinflammatory caspase-1 and increased secretion of IL-1 β levels, suggested to have an integral role in pancreatic β -cell apoptosis (Franchi *et al.*, 2012; Mankan *et al.*, 2012; Tannahill and O'Neill, 2011; Harder *et al.*, 2009; Muñoz-Planillo *et al.*, 2009). Recent studies have demonstrated that TXNIP interacts directly with NLRP3 in a ROS-sensitive manner (Zhou *et al.*, 2010), suggesting a role for TXNIP as a NLRP3 inflammasome ligand. This suggests a pathway where glucolipotoxic conditions may activate the inflammasome, leading to increased IL-1 β levels that are of particular interest in obesity and T2D as a cytotoxic pro-inflammatory trigger.

The aims of the present studies were to investigate the role of TXNIP in pancreatic β -cell apoptosis during experimental conditions of glucotoxicity and glucolipotoxicity. Experiments were conducted using an INS-1 cell line with a TET-inducible stable-INS-1 TXNIP overexpression vector created by our group. An INS-1-empty vector (EV) cell line with endogenous TXNIP expression was

also created as a control cell line. The involvement of IL-1 β in pancreatic β -cells subjected to glucotoxicity and glucolipotoxicity was also investigated.

5.2 Materials and methods

5.2.1 Chemicals and reagents

Chemicals, reagents and antibiotics were purchased from Sigma Aldrich. Hygromycin B was obtained from PAA laboratories.

5.2.2 Cell culture conditions

The culture of rodent pancreatic β -cell lines were performed as described in Section 2.2. The stably transfected INS-1 cells with the TXNIP transgene under the control of a TET-inducible promoter (denoted as INS-TXNIP) and the empty vector control cells (denoted as INS-EV) were generated by our group (Kansikas, 2012). Cells were cultured as described in Section 2.2 in culture medium maintained with hygromycin B. For experiments, the cells were cultured overnight in low glucose conditions (5 mM), then 1 μ g/ml TET or doxycycline (DOX) was added to culture medium with various glucose concentrations and incubated for 48 h.

Fatty acid solutions were prepared and experiments conducted using serum free culture medium as previously detailed in Section 2.3. Cell viability was assessed using PI staining via flow cytometry as previously described in Section 2.4.2. Western blotting was performed as described in Section 2.5. PCR to detect the presence of IL-1 β in rat and 1.1B4 human pancreatic β -cells was conducted as detailed in Section 2.7. Immunoassay using competitive ELISA was carried out

according to the protocol described in Section 2.8. Immunocytochemistry to probe for IL-1 β and NLRP3 was conducted as detailed in Section 2.9.

5.3 Results

5.3.1 Tetracycline induces TXNIP protein overexpression in INS-TXNIP cells but not in INS-EV cells

To investigate the proposed proapoptotic role of TXNIP, a TET-inducible overexpression of TXNIP in the rat cell model INS-1 was used. Initial experiments were conducted to verify that TET induced expression of TXNIP in the INS-TXNIP cells but not the INS-EV cells, as demonstrated previously by our group.

Western blotting of whole cell lysates probed with TXNIP antibody showed that increasing concentrations of TET induced a clear dose-dependent upregulation of TXNIP. There was no detectable expression of TXNIP when the INS-EV control cells were incubated with any concentrations of TET (Fig. 5.1). These results were found to be significant at all TET concentrations compared to the TET-free control group ($p < 0.001$). The fold increases compared to the control were: 25 ng/ml TET, 2.1-fold increase; 50 ng/ml TET, 2.5-fold increase, 100 ng/ml TET, 2.8-fold increase; 500 ng/ml TET, 3.2-fold increase and 1000 ng/ml TET, 3.9-fold increase. There was no detectable level of TXNIP expression in the INS-EV cells \pm TET compared to the TET-free INS-TXNIP control group.

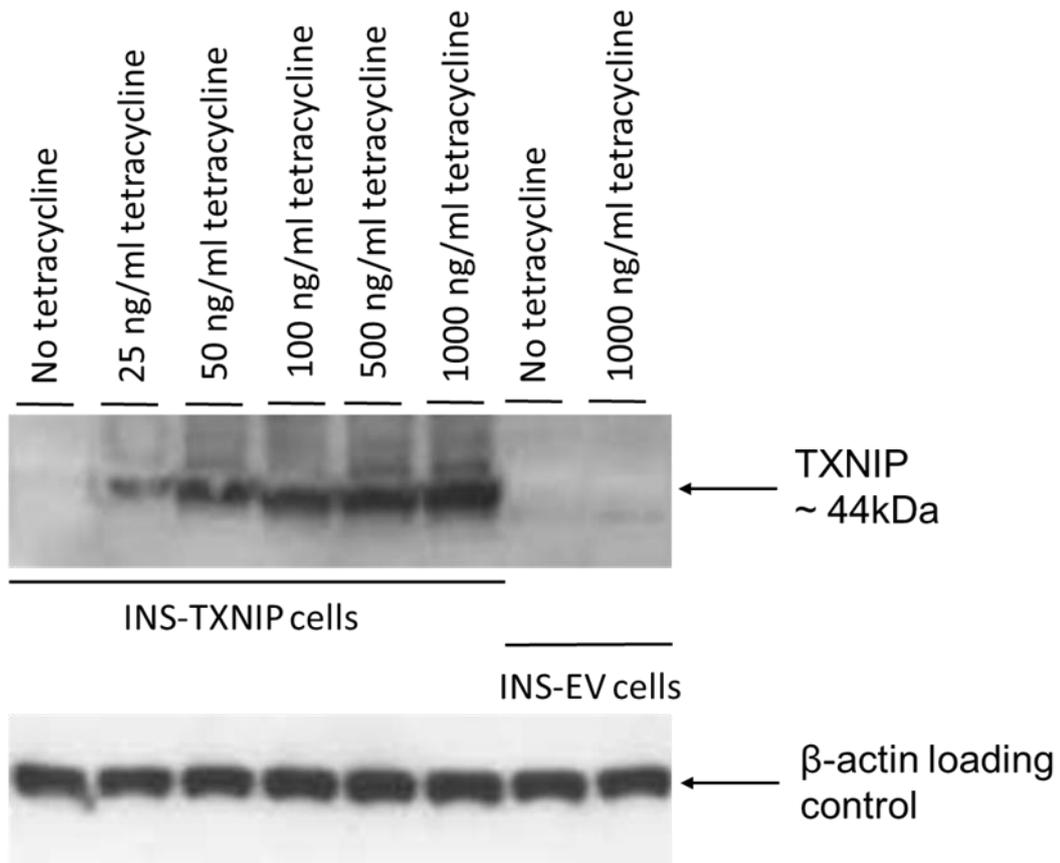


Fig. 5.1 Tetracycline-inducible overexpression of TXNIP in INS-TXNIP and INS-EV pancreatic β -cells

INS-TXNIP pancreatic β -cells and their empty vector control cells INS-EV were incubated overnight in 5 mM glucose culture conditions. Cells were treated with increasing concentrations of tetracycline and incubated for 48 h. Whole cell lysates were probed using TXNIP antibody. Results represent blots from experiments performed in triplicate (N=3).

5.3.2 Hyperglycaemia induces TXNIP overexpression in INS-TXNIP cells but not in INS-EV cells

Our group (Kansikas, 2012) and others (Minn *et al.*, 2005) demonstrated that TXNIP overexpression could also be induced by hyperglycaemia. Therefore, Western blotting was conducted to ascertain whether this was reproducible. Cells were pre-incubated overnight in 5 mM glucose concentrations, to ensure that basal TXNIP expression was established before the cell lines were subjected to 11 and 31 mM glucose concentrations. Western blotting of whole cell lysates from these treatments were probed using a TXNIP antibody. There was a 2.4-fold increase between the 11 mM glucose group and the 5 mM glucose control ($p<0.001$), and significant 7.6-fold increase in TXNIP expression was observed in the INS-TXNIP cells at 31 mM glucose compared to the 5 mM glucose control ($p<0.001$) (Fig. 5.2). By comparison, a 1.7-fold expression of TXNIP was detected in the INS-EV cell line under 31 mM glucose culture conditions when compared to the 5 mM INS-TXNIP glucose control ($p<0.01$).

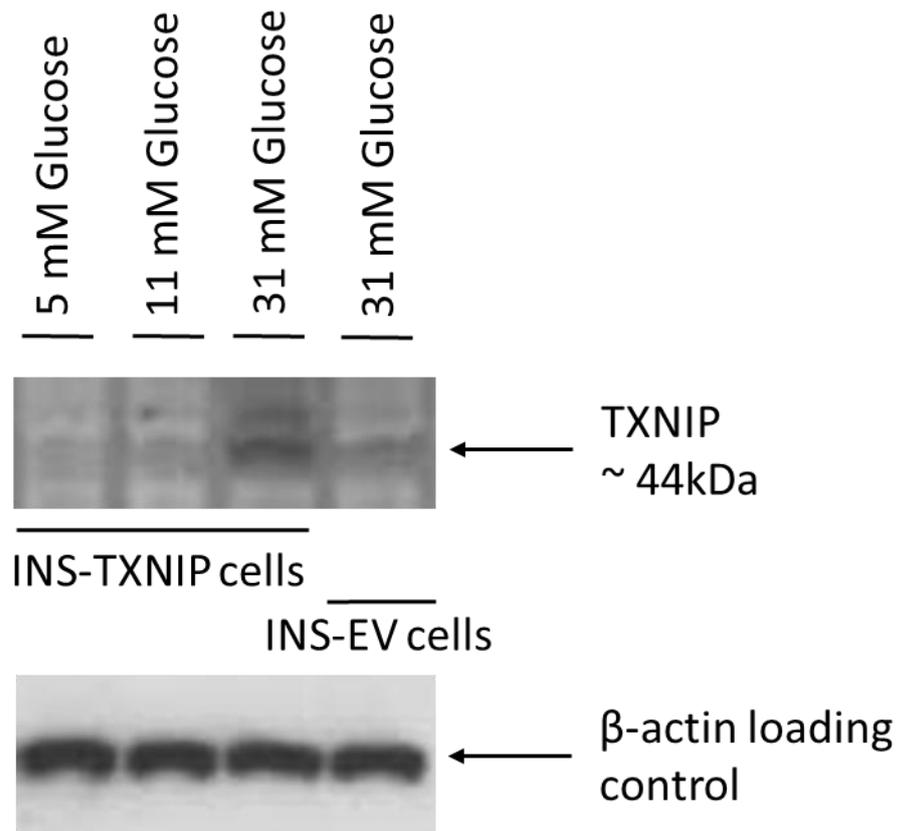


Fig. 5.2 Glucose-inducible overexpression of TXNIP in INS-TXNIP and INS-EV pancreatic β -cells

INS-TXNIP pancreatic β -cells and control cells INS-EV were incubated overnight in 5 mM glucose culture conditions. They were incubated with various concentrations of glucose for 48 h. Whole cell lysates were probed using TXNIP antibody. Results represent blots from experiments performed in triplicate (N=3).

5.3.3 The effects of tetracycline-induced TXNIP overexpression on cell death in INS-TXNIP and INS-EV pancreatic β -cells

Having established in Sections 5.3.1 and 5.3.2 that both TET and high glucose incubation conditions can induce TXNIP expression, cell viability assays were conducted to investigate whether over-expression of TXNIP affected cell death in our model.

INS-TXNIP rodent pancreatic β -cells and INS-EV control cells were pre-incubated overnight with 5 mM glucose, then treated with 11 or 31 mM concentrations of glucose \pm 1000 ng/ml TET. The percentage cell death observed between the various glucose concentrations alone, or the various glucose concentrations co-incubated with TET were compared. In the INS-TXNIP cells incubated with only glucose, percentage cell death was as follows: 5 mM glucose control, 6.4%; 11 mM glucose, 7% and 31 mM glucose, 7.2% (Fig. 5.3 a)). There was no significant difference between the 11 or 31 mM glucose treatments when compared with the 5 mM glucose control.

In the INS-TXNIP cells cultured with TET, percentage cell death was found to be: 5 mM glucose, 6.4%; 11 mM glucose, 11.7% and 31 mM glucose, 21.1%. There was a 3.3-fold increase in percentage cell death seen when comparing the 31 mM glucose group to the 5 mM glucose control ($p < 0.001$). Furthermore, our results showed that there was a 2.9-fold increase in percentage cell death when comparing the 31 mM glucose group in the absence of TET with the 31 mM group in the presence of TET ($p < 0.001$).

In the INS-EV cells incubated with only glucose, percentage cell death was found to be: 5 mM glucose control, 4.5%; 11 mM glucose, 4% and 31 mM glucose, 4.7%. There was no significant difference observed between the 11 or 31 mM glucose treatments compared to the 5 mM control.

In the INS-EV cell cultured in the presence of TET (Fig. 5.3b)), percentage cell death was: 5 mM glucose, 6.4%; 11 mM glucose, 5.5% and 31 mM glucose, 19.6%. There was a 3.1-fold increase in percentage cell death seen when comparing the 31 mM glucose to the 5 mM glucose control ($p < 0.001$). In addition to this, our results showed a 4.1-fold increase in percentage cell death when comparing the 31 mM glucose group in the absence of TET with the 31 mM group treated with TET ($p < 0.001$).

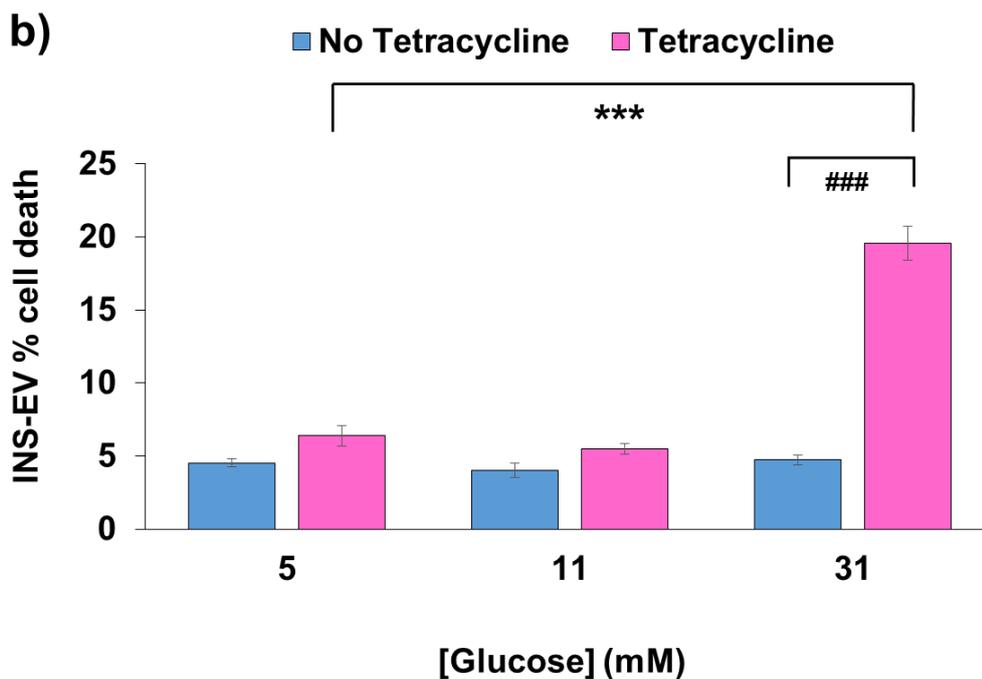
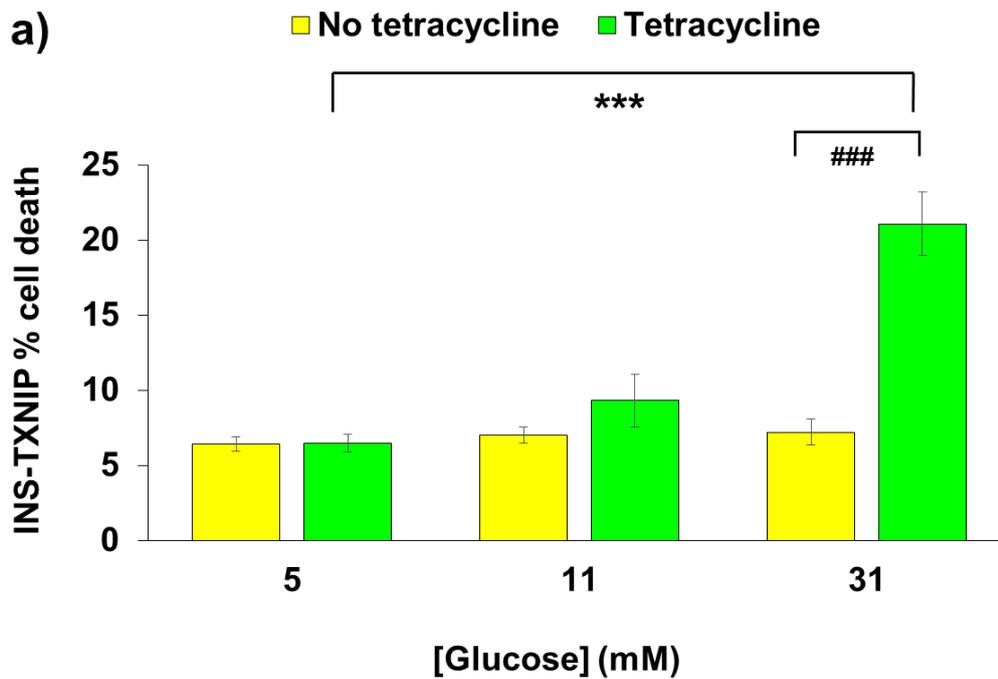


Fig. 5.3 Tetracycline- and glucose-induced TXNIP overexpression on cell death in INS-TXNIP and INS-EV pancreatic β -cells

INS-TXNIP (a) and INS-EV (b) cells were incubated overnight in 5 mM glucose culture conditions, then treated with 11 or 31 mM glucose concentrations. Cells were incubated for 48 h with or without tetracycline (1 μ g/ml). Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. 5 mM glucose, ### $p < 0.001$ vs. no tetracycline

5.3.4 The effects of high glucose and palmitate concentrations on cell death in INS-TXNIP and INS-EV pancreatic β -cells

The mechanism of glucolipotoxicity has been shown to exert a detrimental effect on cell viability, as demonstrated by others (El-Assaad *et al.*, 2003; Poitout and Robertson, 2008), and by our results reported in rodent cell models earlier in these studies. Therefore, we sought to investigate the effect of glucolipotoxic conditions in this cell model, to compare variability between the INS-TXNIP and the INS-EV cells. This would also allow comparisons to be made between results seen in this cell model with results from other rodent cell models under glucolipotoxic conditions that we have reported in earlier studies. Therefore, these next assays were designed so each cell line was co-incubated with 11 or 31 mM glucose and 250 μ M palmitate.

Our results for the INS-EV cells showed a 3.7-fold increase in percentage cell death, when comparing the co-incubation of 11 mM glucose and 250 μ M palmitate with the 11 mM glucose control ($17.3 \pm 1.3\%$ vs. $4.7 \pm 0.3\%$ respectively, $p < 0.001$). A 9.4-fold increase in percentage cell death was seen when comparing the co-incubation of 31 mM glucose and 250 μ M palmitate to the control ($44.1 \pm 1.9\%$ vs. $4.7 \pm 0.3\%$ respectively, $p < 0.001$).

Results for the INS-TXNIP cells demonstrated a 3.7-fold increase in percentage cell death when comparing the co-incubation of 11 mM glucose and 250 μ M palmitate with the control ($14.3 \pm 0.4\%$ vs. $3.9 \pm 1.2\%$, $p < 0.001$). This was followed by a 9.7-fold increase in percentage cell death which was evident when comparing the co-incubation of 31 mM glucose and 250 μ M palmitate to the control ($37.7 \pm 2\%$ vs. $3.9 \pm 1.2\%$, $p < 0.001$). There was however, no significant

difference in the percentage cell death observed in any treatment group when comparing the INS-TXNIP cells with the INS-EV cells (Fig. 5.4). The typical percentage cell death we report here in this cell model correlates with results that we have shown in earlier sections in other rodent pancreatic β -cell models.

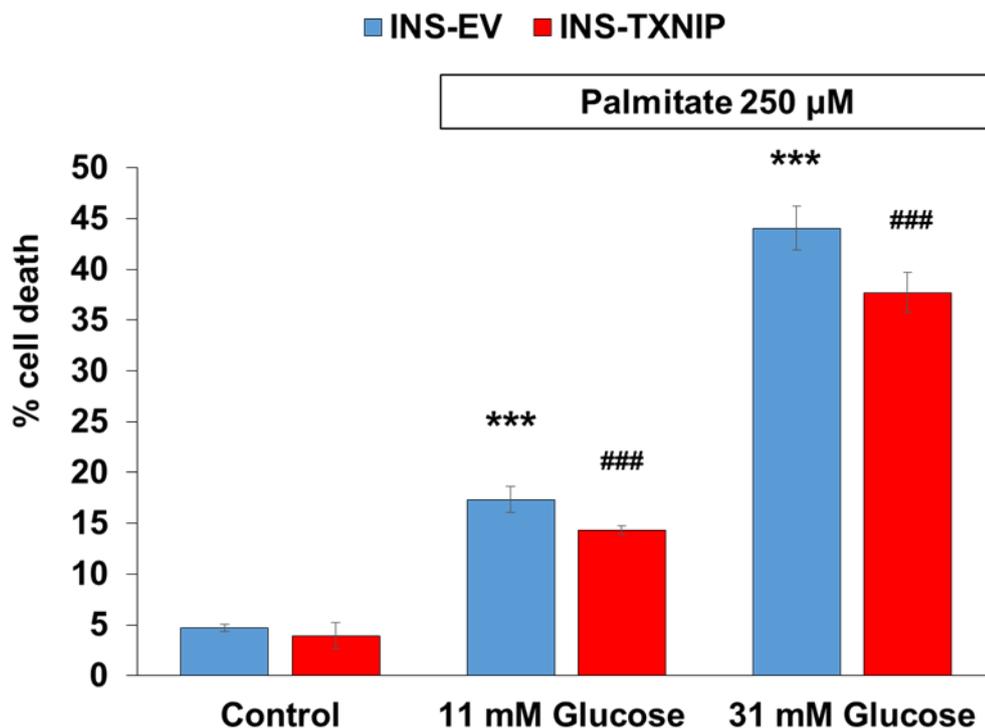


Fig. 5.4 The effect of high glucose and palmitate on cell death in INS-TXNIP and INS-EV pancreatic β -cells

INS-TXNIP pancreatic β -cells and INS-EV control cells were incubated overnight in 5 mM glucose culture conditions. Cells were treated with 11 mM glucose (control), or co-incubated with 250 μ M palmitate and 11 or 31 mM glucose concentrations for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. control, ### $p < 0.001$ vs. control

5.3.5 The effect of tetracycline vs. doxycycline on cell death in INS-TXNIP pancreatic β -cells

Tetracycline is a commonly used antibiotic in TET-regulatory systems. Doxycycline is a TET analogue, and is suggested to be more stable than TET in cell culture conditions, and can also be used in TET-on inducible cell model systems as used here. Both antibiotics were used here at the same concentration for comparison purposes, to consider whether the antibiotic itself was responsible for alterations to percentage cell death seen. The aim of these studies was to further define experimental conditions whereby culture with these antibiotics may induce changes in cell death in this β -cell model. Results of percentage cell death in the INS-TXNIP control cells, i.e. cells cultured without TET or DOX, were found to be: 5 mM glucose, 7.9%, 11 mM glucose, 8.6% and 31 mM, 9.4%. There was no significant difference between the 11 or 31 mM glucose groups compared to the 5 mM glucose control.

The percentage cell death observed when INS-TXNIP cells were incubated with TET were as follows: 5 mM glucose, 7.5%; 11 mM glucose, 10.3% and 31 mM, 17.3%. There was a 1.7-fold increase in percentage cell death seen between 31 mM glucose and the 5 mM glucose ($p < 0.001$) and this is in line with our previous findings in Section 5.3.3. Furthermore, a 1.6-fold increase in percentage cell death was seen when comparing the 31 mM glucose group incubated with TET and the 31 mM glucose control group ($p < 0.001$).

Finally, we report the percentage cell death seen when INS-TXNIP cells were incubated with DOX: 5 mM glucose, 7.1%; 11 mM glucose, 9.86% and 31 mM, 16.8%. There was a 1.5-fold increase in percentage cell death observed between

31 mM glucose when compared to the 5 mM glucose group ($p < 0.001$). This was coupled with a 1.6-fold increase in percentage cell death seen when comparing the 31 mM glucose group incubated with DOX and the 31 mM glucose control group ($p < 0.001$) (Fig. 5.5). These fold increases in percentage cell death were comparable to findings in the TET-treated groups. Indeed, there was no observable difference between the TET and DOX groups at any glucose concentration. It was only at the highest 31 mM glucose concentration that an increase in percentage cell death was observed when cells were co-incubated with each antibiotic.

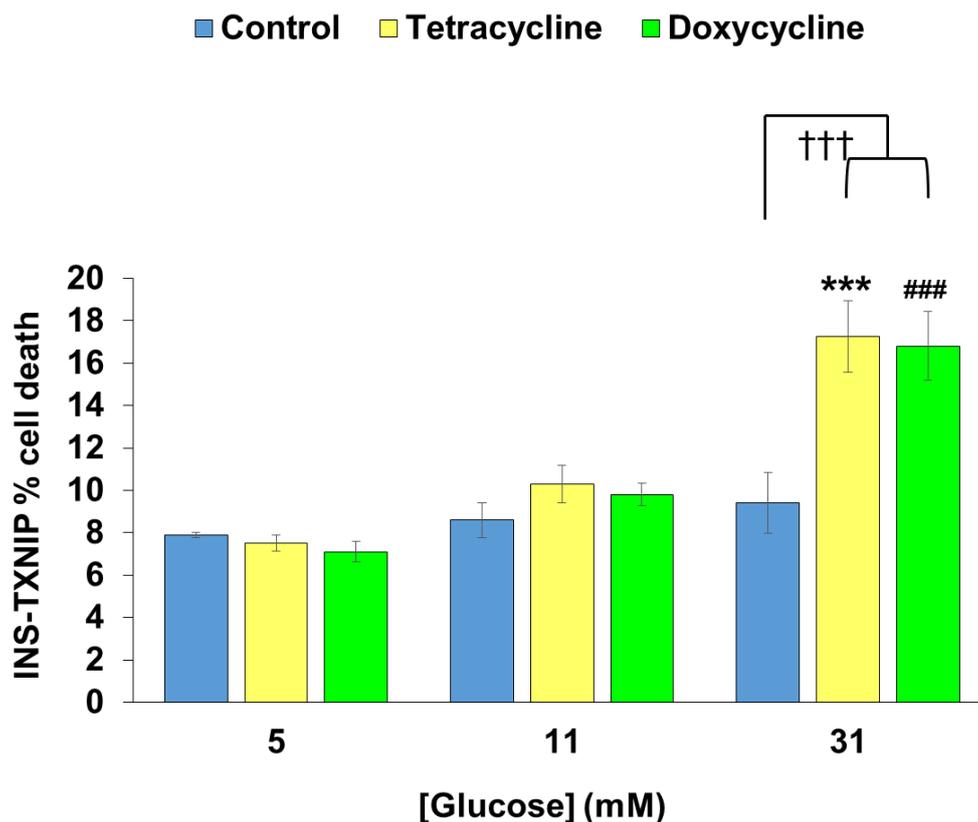


Fig. 5.5 The effect of tetracycline vs. doxycycline on cell death in INS-TXNIP pancreatic β -cells

INS-TXNIP cells were incubated overnight in 5 mM glucose culture conditions, then treated with 11 or 31 mM glucose, or with tetracycline (1 μ g/ml) or doxycycline (1 μ g/ml), and incubated for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. 5 mM glucose, ### $p < 0.001$ vs. 5 mM glucose, ††† $p < 0.001$ vs. 31 mM glucose

5.3.6. The effect of L-glucose and D-mannitol osmotic controls on cell death in INS-TXNIP pancreatic β -cells

L-glucose and mannitol are commonly used as osmotic control 'sugars' for high-glucose experiments, to determine whether increased percentage cell death observed under chronic hyperglycaemic conditions is due to osmotic stress. L-glucose is a glucose stereoisomer and mannitol is a sugar alcohol. Neither sugar can enter cells, thus remain within the extracellular compartment (Boland and Garland, 1993) although both sugars exert the same osmotic effect as D-glucose. Our results here demonstrated for the first time in this cell model that there was a 5.9-fold increase ($p < 0.001$) in INS-TXNIP percentage cell death seen when the 11 mM L-glucose group ($41 \pm 1.2\%$) was compared with 11 mM D-glucose ($7 \pm 0.6\%$) (Fig. 5.6a). Additionally, our results demonstrated a 2.5-fold increase in percentage cell death ($p < 0.01$) when comparing INS-TXNIP cells incubated with D-mannitol ($17.8 \pm 1.3\%$) with D-glucose (Fig. 5.6b)).

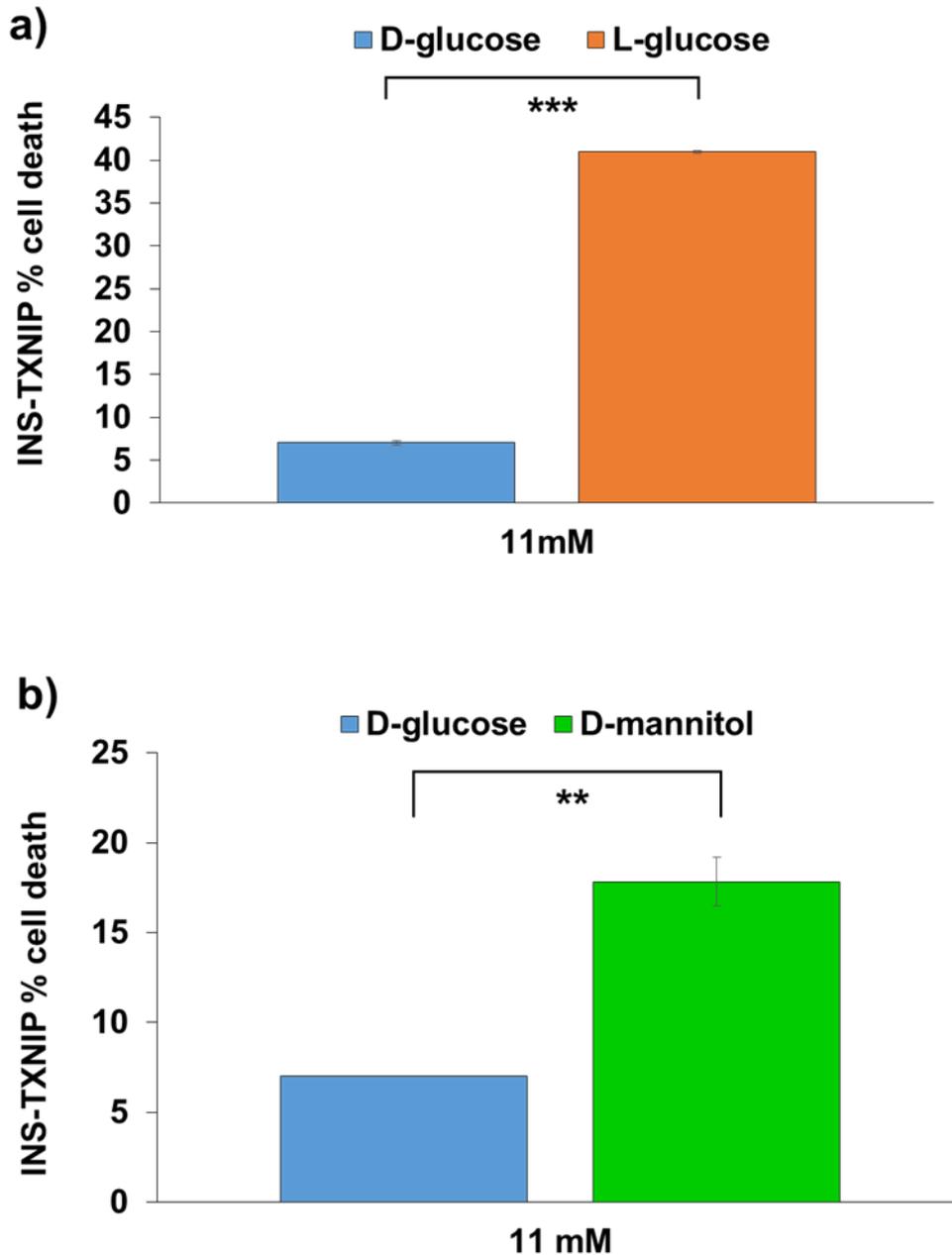


Fig. 5.6 The effect of L-glucose and D-mannitol osmotic controls on cell death in INS-TXNIP pancreatic β -cells

INS-TXNIP cells were incubated overnight in 11 mM glucose culture conditions, then replaced with 11 mM L-glucose (a) or D-mannitol (b) as osmotic controls and incubated for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. D-glucose, ** $p < 0.01$ vs. D-glucose

5.3.7 The effect of succinate on cell death in INS-TXNIP rat pancreatic β -cells

We earlier proposed that succinate may act as a cytoprotective agent against high glucose and high FA conditions, through its ability to potently stimulate insulin release, thereby potentially inhibiting ROS production and mitochondrial dysfunction. To date, there is very limited data regarding the use of succinate as a protective compound in relation to T2D, and certainly we are not aware of any reports regarding its action in this cell model. Therefore, in the next series of experiments, we sought to define experimental conditions whereby succinate may be cytoprotective in the INS-TXNIP β -cell model.

INS-TXNIP cells were initially incubated in normal culture conditions with 11 mM glucose, and increasing concentrations of succinate were tested. Our results show that succinate was well-tolerated up to 10 mM; there was no difference in the percentage cell death seen at 1, 5 or 10 mM succinate compared to the control group. However, at the higher succinate concentrations tested (15 and 20 mM), there was a significant increase in percentage cell death compared to the control ($p < 0.001$) (Fig. 5.7a)). Overall, our results showed percentage cell death to be: control, 1.6%; 1 mM succinate, 2.2%; 5 mM succinate, 1.6%; 10 mM succinate, 2.6%; 15 mM succinate, 7.2% and 20 mM succinate, 85.8%.

In a parallel treatment group, cells were co-incubated with 250 μ M palmitate and 11 mM glucose, and increasing concentrations of succinate. The addition of succinate did not confer any protection at all when palmitate was present (Fig. 5.7b)). Indeed, the addition of succinate greatly increased the percentage cell death seen across all concentrations tested in this culture condition. The results

in percentage cell death for this study were: control (no palmitate or succinate), 6.2%; 250 μ M palmitate, 18.9%, 1 mM succinate, 29.8%; 5 mM succinate, 47.5%; 10 mM succinate, 53.5%; 15 mM succinate, 69.4% and 20 mM succinate, 96.4%.

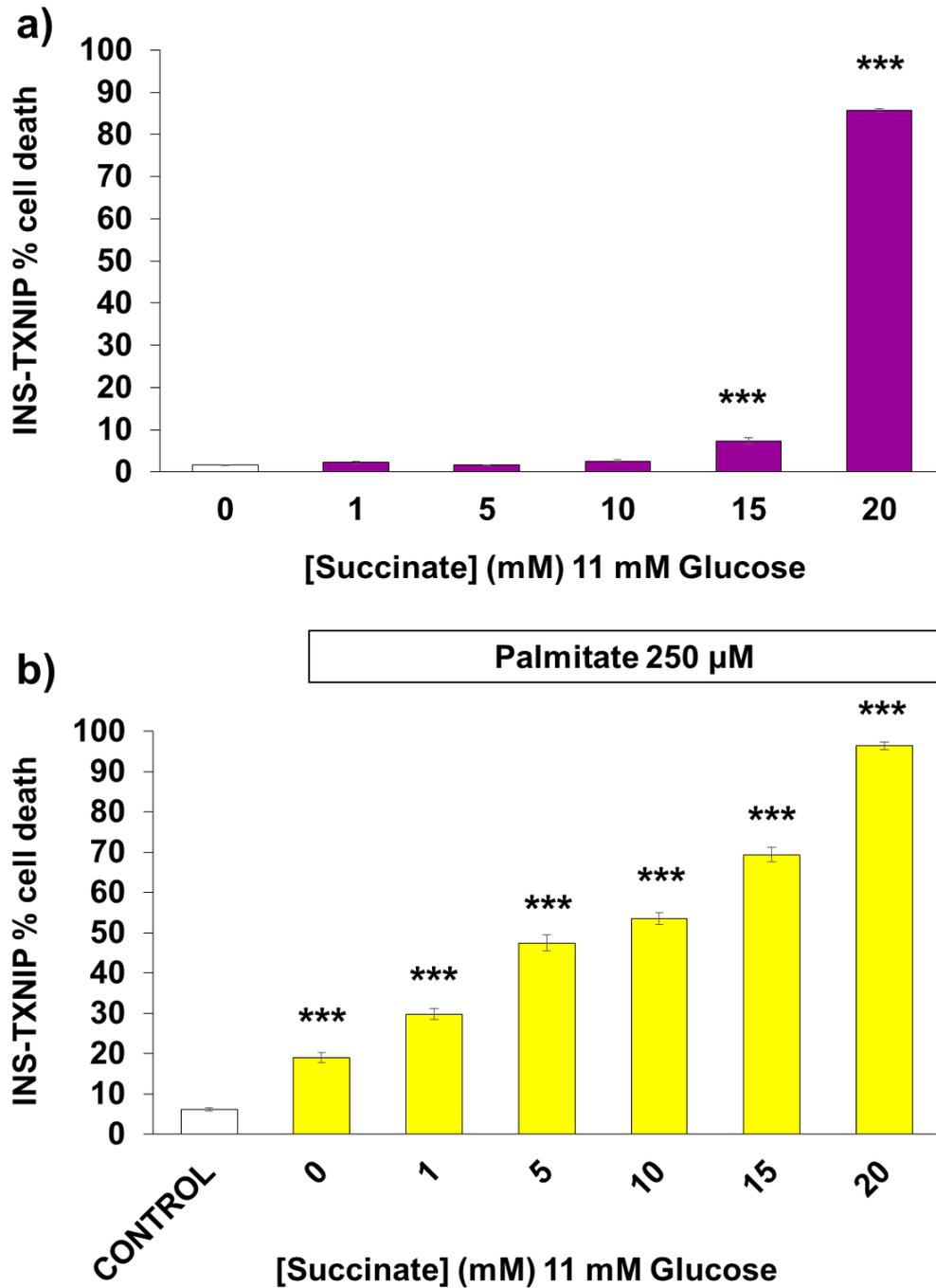


Fig. 5.7 The effect of succinate on cell death in INS-TXNIP pancreatic β -cells: 11 mM glucose \pm palmitate

INS-TXNIP pancreatic β -cells were incubated overnight in 5 mM glucose, then treated with succinate in 11 mM glucose (a) or co-incubated with 250 μ M palmitate and 11 mM glucose, and succinate (b). Cells were incubated for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** p < 0.001 vs. control

These experiments were repeated using 31 mM glucose culture conditions (Fig. 5.8a)). In this instance, succinate was well-tolerated up to 15 mM, but showed a 6.7-fold increase in percentage cell death of INS-TXNIP cells at 20 mM ($p<0.001$). The results for percentage cell death were: control, 4.2%; 1 mM succinate, 3.6%; 5 mM succinate, 2.3%; 10 mM succinate, 2.2%; 15 mM succinate, 2.9% and 20 mM succinate, 28%.

In a parallel study, INS-TXNIP cells were co-incubated with 250 μ M palmitate and 31 mM glucose, and increasing concentrations of succinate. Again, in line with our findings reported above, the addition of succinate significantly exacerbated the percentage cell death seen in the INS-TXNIP cells at all concentrations tested (Fig. 5.8b)). Results for percentage cell death from this experiment were: control (no palmitate or succinate), 4.2%; 250 μ M palmitate, 52.6%, 1 mM succinate, 52%; 5 mM succinate, 58.6%; 10 mM succinate, 59.3%; 15 mM succinate, 80.3% and 20 mM succinate, 82.8%.

Interestingly, the percentage cell death seen in the INS-TXNIP cells at 11 mM glucose was significantly higher than compared to 31 mM glucose conditions when 20 mM succinate was added ($85.8\pm0.4\%$ vs. $28\pm0.3\%$ respectively, $p<0.001$) (Fig. 5.9a)). The addition of succinate did not ameliorate the cytotoxicity caused by co-incubation with palmitate: significantly higher cell death was observed with all succinate concentrations except 20 mM ($p<0.001$), when comparing the 31 mM glucose group to the 11 mM glucose group (Fig. 5.9b)).

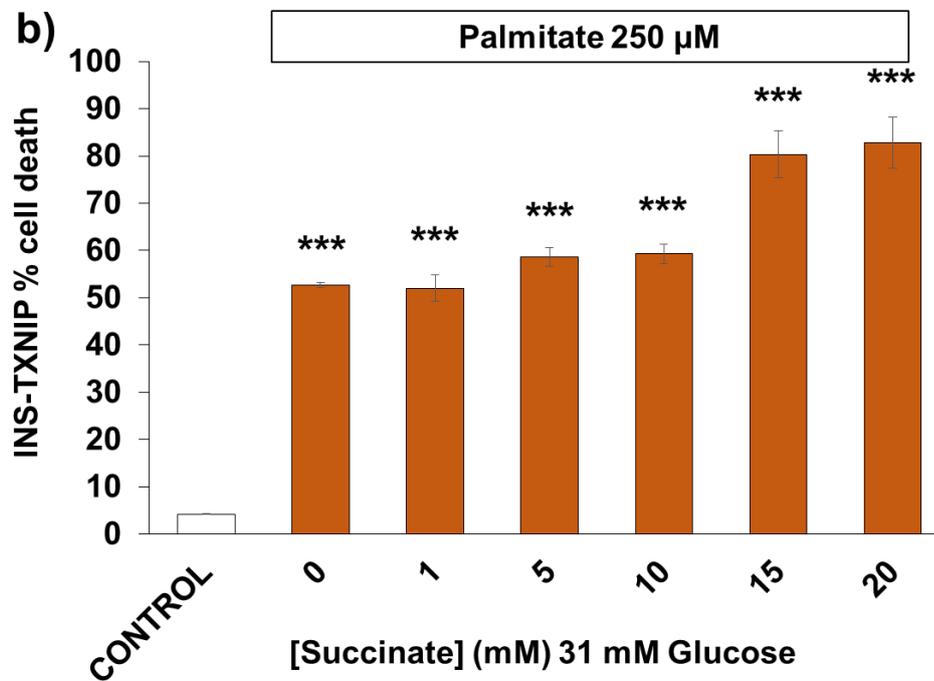
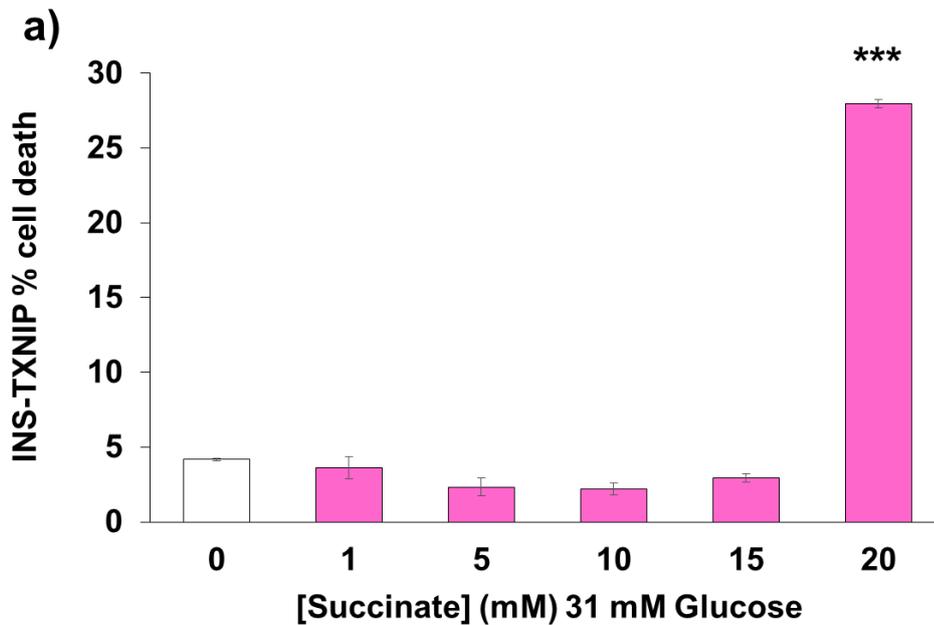


Fig. 5.8 The effect of succinate on cell death in INS-TXNIP pancreatic β -cells: 31 mM glucose \pm palmitate

INS-TXNIP pancreatic β -cells were incubated overnight in 5 mM glucose, then treated with succinate in 31 mM glucose (a), or co-incubated with 250 μ M palmitate and 31 mM glucose, and succinate (b). Cells were incubated for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. control

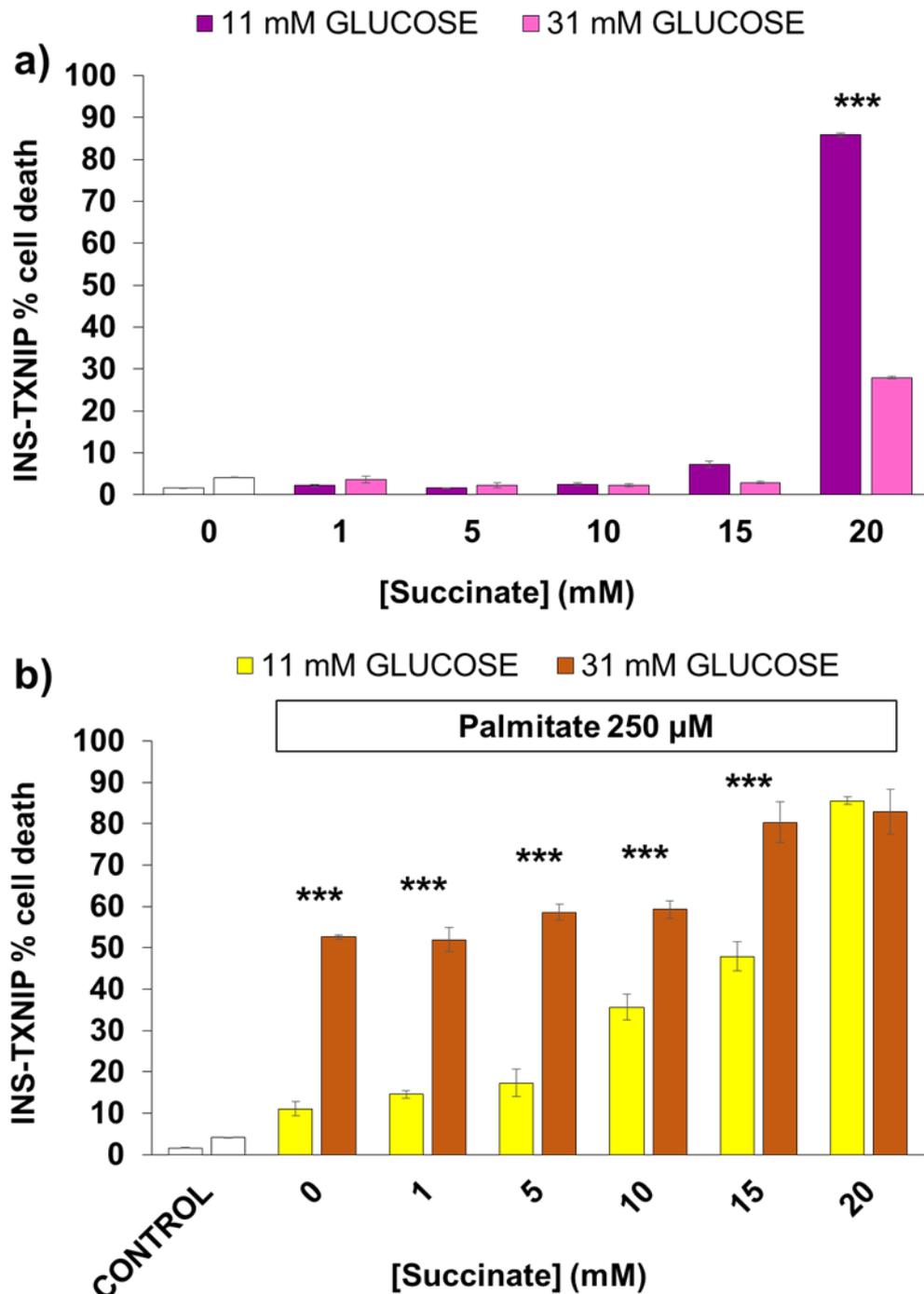


Fig. 5.9 Comparison of the effect of succinate on cell death in INS-TXNIP pancreatic β -cells: 11 vs. 31 mM glucose \pm palmitate

INS-TXNIP pancreatic β -cells were incubated overnight in 5 mM glucose, then treated with succinate in 11 or 31 mM glucose conditions (a), or co-incubated with 250 μ M palmitate and 11 or 31 mM glucose and succinate (b). Cells were incubated for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. control

Following on from this, these studies were again replicated, but with the addition of TET (1000 ng/ml) to investigate whether TXNIP overexpression in this cell line would affect the percentage cell death observed under these experimental conditions (Fig. 5.10a)). Cells were co-incubated with 250 μ M palmitate and 11 mM glucose, and increasing concentrations of succinate. In line with our previous findings, succinate was not shown to be cytoprotective. The percentage cell death seen at each succinate concentration was significantly greater than when compared to the control. Results for percentage cell death were: control (no palmitate, TET or succinate), 6.3%; 250 μ M palmitate, 23.6%; 250 μ M palmitate and 1 mM succinate, 30.3%; 250 μ M palmitate and 5 mM succinate, 40.3%; 250 μ M palmitate and 10 mM succinate, 56.7%; 250 μ M palmitate and 15 mM succinate, 79.9% and finally, 250 μ M palmitate and 20 mM succinate, 97.7%.

For comparison purposes, results for percentage cell death between cells incubated in the presence of TET were compared to those incubated in the absence of TET. There was no significant difference between any treatment combinations (Fig. 5.10b)).

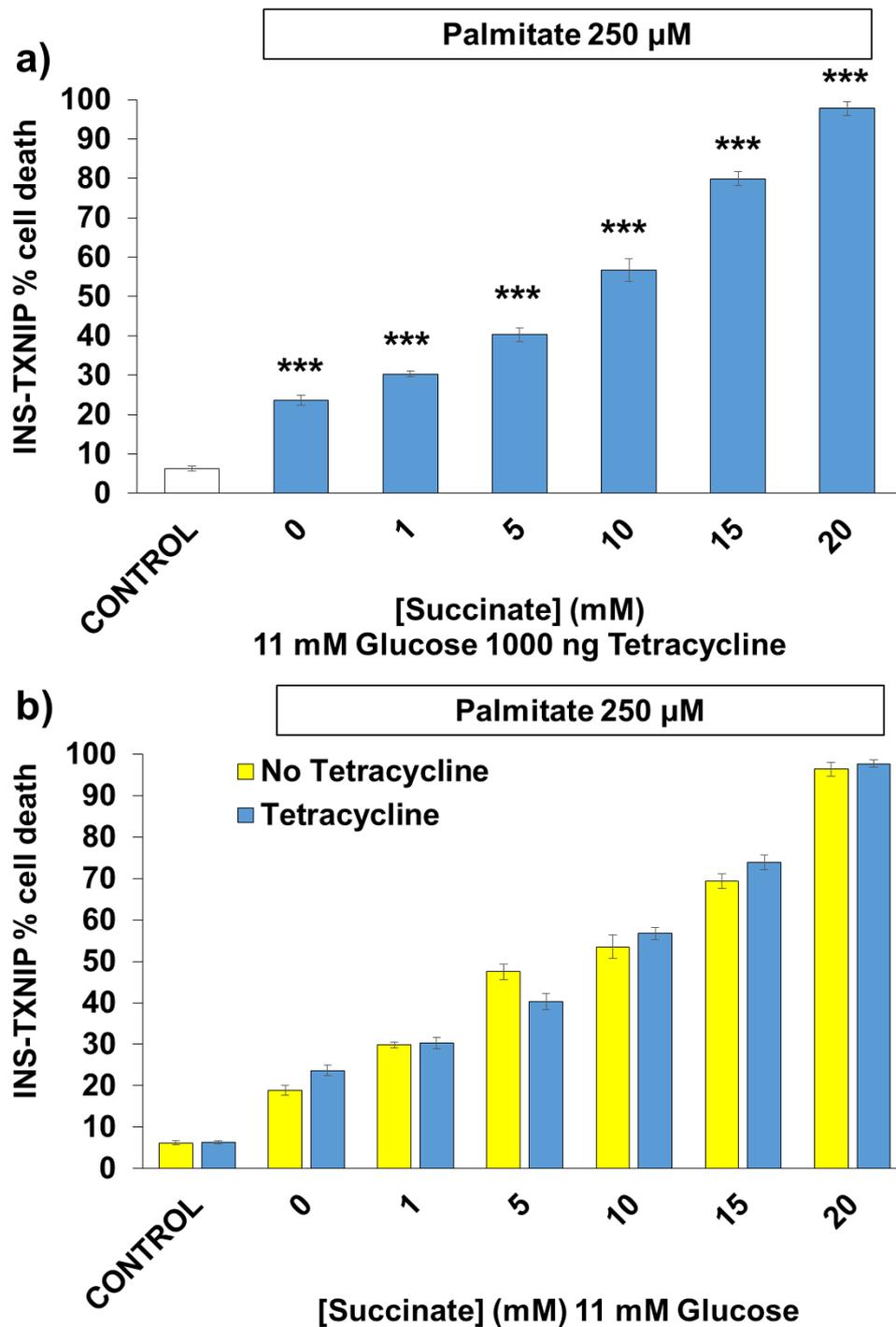


Fig. 5.10 The effect of succinate on cell death in INS-TXNIP pancreatic β -cells: 11 mM glucose, 250 μ M palmitate \pm tetracycline

INS-TXNIP pancreatic β -cells were incubated overnight in 5 mM glucose, then treated with succinate in 11 mM glucose, 250 μ M palmitate and tetracycline at 1000 ng/ml (a). Cells were incubated for 48 h. Comparison of cells treated with or without tetracycline (b). Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. control

In the final set of experiments in this series, INS-TXNIP cells were co-incubated with a higher 31 mM glucose concentration and 250 μ M palmitate, and increasing concentrations of succinate (Fig.5.11a)). Again, similar to our reported findings earlier, succinate was not shown to be cytoprotective when palmitate was present. The percentage of cell death observed at each succinate concentration was significantly greater than when compared to the control. Results for percentage cell death here were: control (no palmitate, TET or succinate), 8.6%; 250 μ M palmitate, 46.8%; 250 μ M palmitate and 1 mM succinate, 51.5%; 250 μ M palmitate and 5 mM succinate, 57.5%; 250 μ M palmitate and 10 mM succinate, 72.2%; 250 μ M palmitate and 15 mM succinate, 90.5% and finally, 250 μ M palmitate and 20 mM succinate, 100%.

The percentage cell death seen in cells incubated in the presence of TET were compared to those incubated in the absence of TET at the 31 mM glucose concentration (Fig. 5.11b)). There was no significant difference between any treatment combinations.

A final comparison of the percentage cell death was made between cells that were co-incubated with palmitate, TET and either 11 or 31 mM two glucose concentrations. These results showed that the percentage of cell death was significantly higher in the cells incubated with 31 mM glucose (Fig. 5.12). This was the case for 250 μ M palmitate treatment, which is consistent with all our previous evidence ($p<0.001$), however with the addition of succinate, the percentage cell death was significantly higher in 1, 5 and 10 mM ($p<0.001$) concentrations, and in the 15 mM succinate group ($p<0.01$).

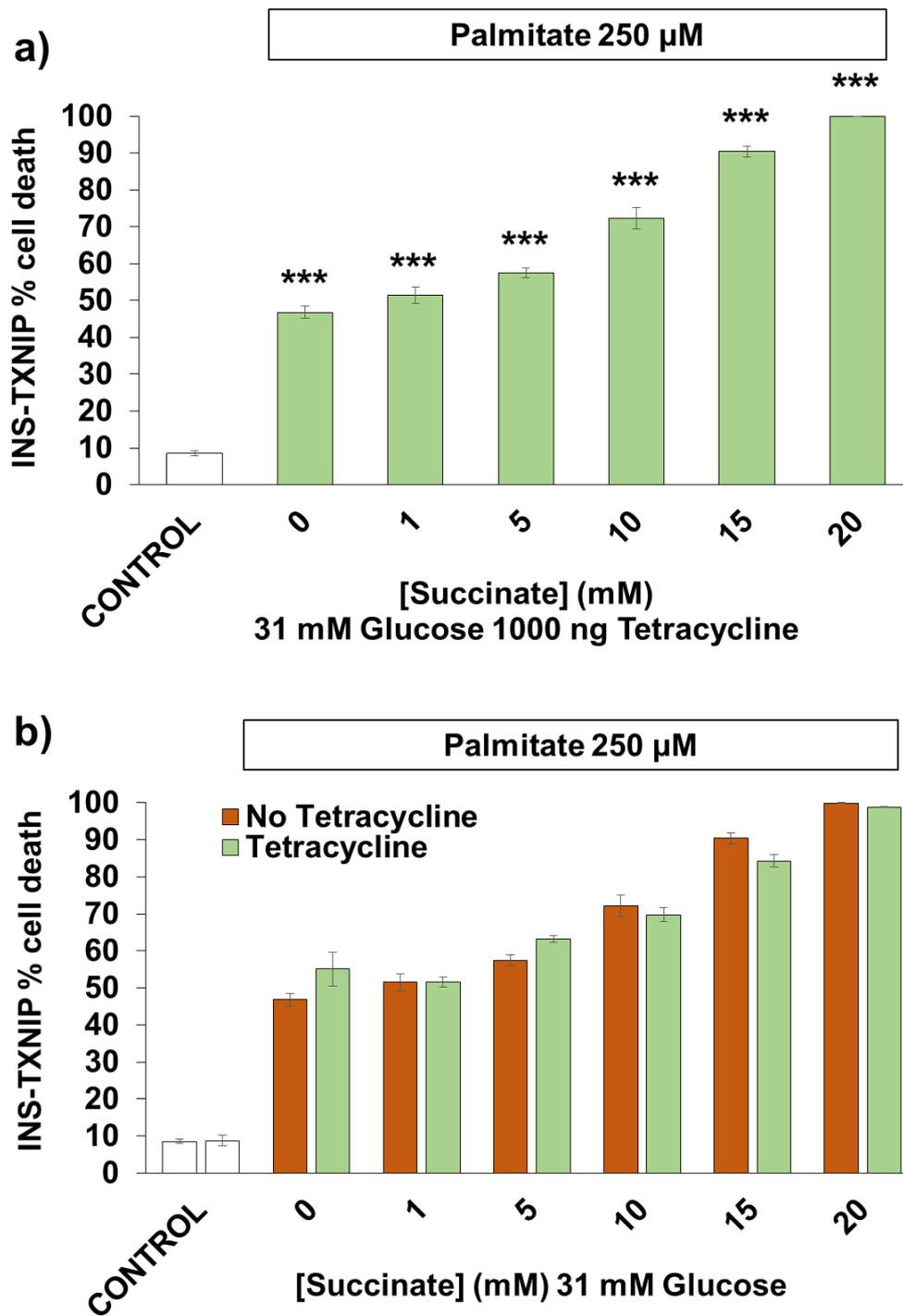


Fig. 5.11 The effect of succinate on cell death in INS-TXNIP pancreatic β -cells: 31 mM glucose, 250 μ M palmitate \pm tetracycline

INS-TXNIP pancreatic β -cells were incubated overnight in 5 mM glucose, then treated with succinate in 31 mM glucose, 250 μ M palmitate and tetracycline (1000 ng/ml) (a). Cells were incubated for 48 h. Comparison of cells treated with or without tetracycline (b). Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. control

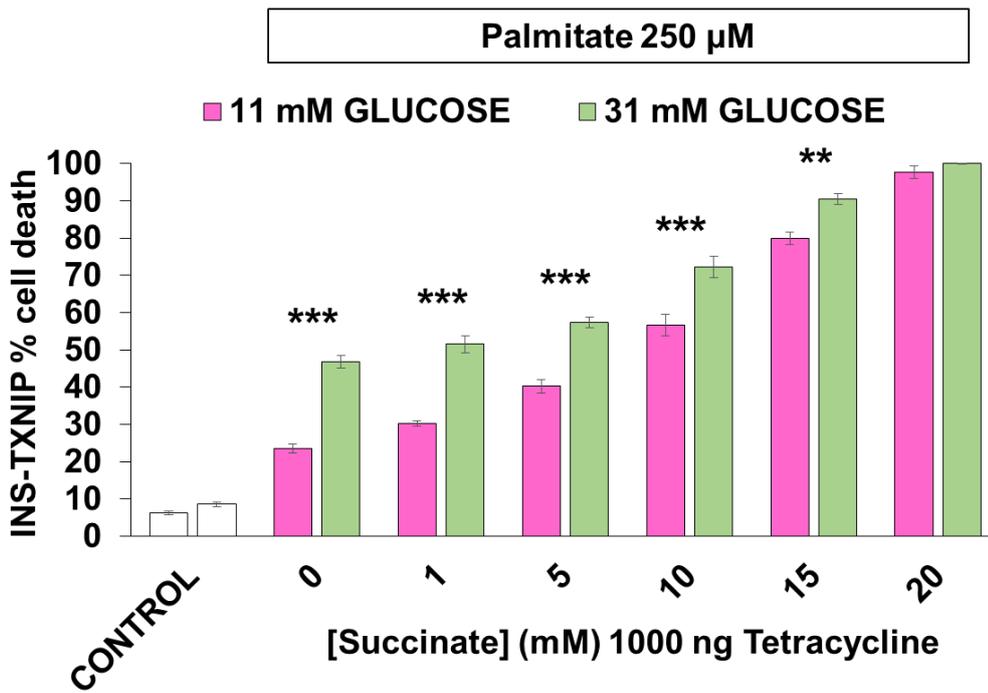


Fig. 5.12 Comparison of the effect of succinate on cell death in INS-TXNIP pancreatic β -cells: 11 vs. 31 mM glucose, 250 μ M palmitate \pm tetracycline

INS-TXNIP pancreatic β -cells were incubated overnight in 5 mM glucose culture conditions. They were co-incubated with succinate in 11 mM or 31 mM glucose conditions, 250 μ M palmitate and tetracycline (1000 ng/ml). Cells were incubated for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$, ** $p < 0.01$ vs. 11 mM treatment groups

5.3.8 The effect of succinate on cell death in 1.1B4 human pancreatic β -cells

We have previously mentioned the paucity of data with regard to the functional responses of 1.1B4 human pancreatic β -cells to FA *in vitro*. Furthermore, to the best of our knowledge, the use of succinate as a cytoprotective agent in this cell model has not been reported. Therefore, for the first time we provide results from experiments that investigated the response of 1.1B4 cells to succinate, in experimental conditions of glucolipotoxicity. Studies were conducted as described in Section 5.3.7. Our results here show that when the 1.1B4 human pancreatic β -cells were incubated with 11 mM glucose (Fig. 5.13a)), succinate was well tolerated up to 15 mM as no significant change in percentage cell death was seen. However, at 20 mM, succinate caused a significant increase in percentage cell death compared to the control ($94.6\pm 3.5\%$ vs. $21\pm 0.6\%$ respectively, $p < 0.001$). This was in line with our findings in the INX-TXNIP rodent pancreatic β -cells. To quantify our results, the following percentage cell deaths were observed when succinate concentrations were compared to the control: 1 mM succinate, 14.6%; 5 mM succinate, 12.8%; 10 mM succinate, 13.2%; 15 mM succinate, 18.2% and finally, 20 mM succinate, 94.6%.

Co-incubation of the 1.1B4 cells with 11 mM glucose and 250 μ M palmitate (Fig. 5.13b) showed a significant increase in percentage cell death vs. the control ($89.6\pm 4.5\%$ vs. $20.5\pm 0.6\%$ respectively, $p < 0.001$). However, the addition of succinate led to a dose-dependent decrease in percentage cell death, found to be significant at both 10 and 15 mM concentrations ($p < 0.001$) when compared to the palmitate and glucose control group. This was in contrast to our results reported in the INS-TXNIP cells. At 20 mM, succinate did not afford any

protection against palmitate-induced apoptosis, and was 1.4-fold higher than compared to the palmitate and glucose control. To fully quantify our results, changes in percentage cell death were as follows: control (no palmitate or succinate), 20.5% cell death; 250 μ M palmitate, 71.5% cell death; 1 mM succinate, 67.7% cell death; 5 mM succinate, 63% cell death; 10 mM succinate, 46% cell death; 15 mM succinate, 26.8% cell death and finally, 20 mM succinate, 89.6% cell death.

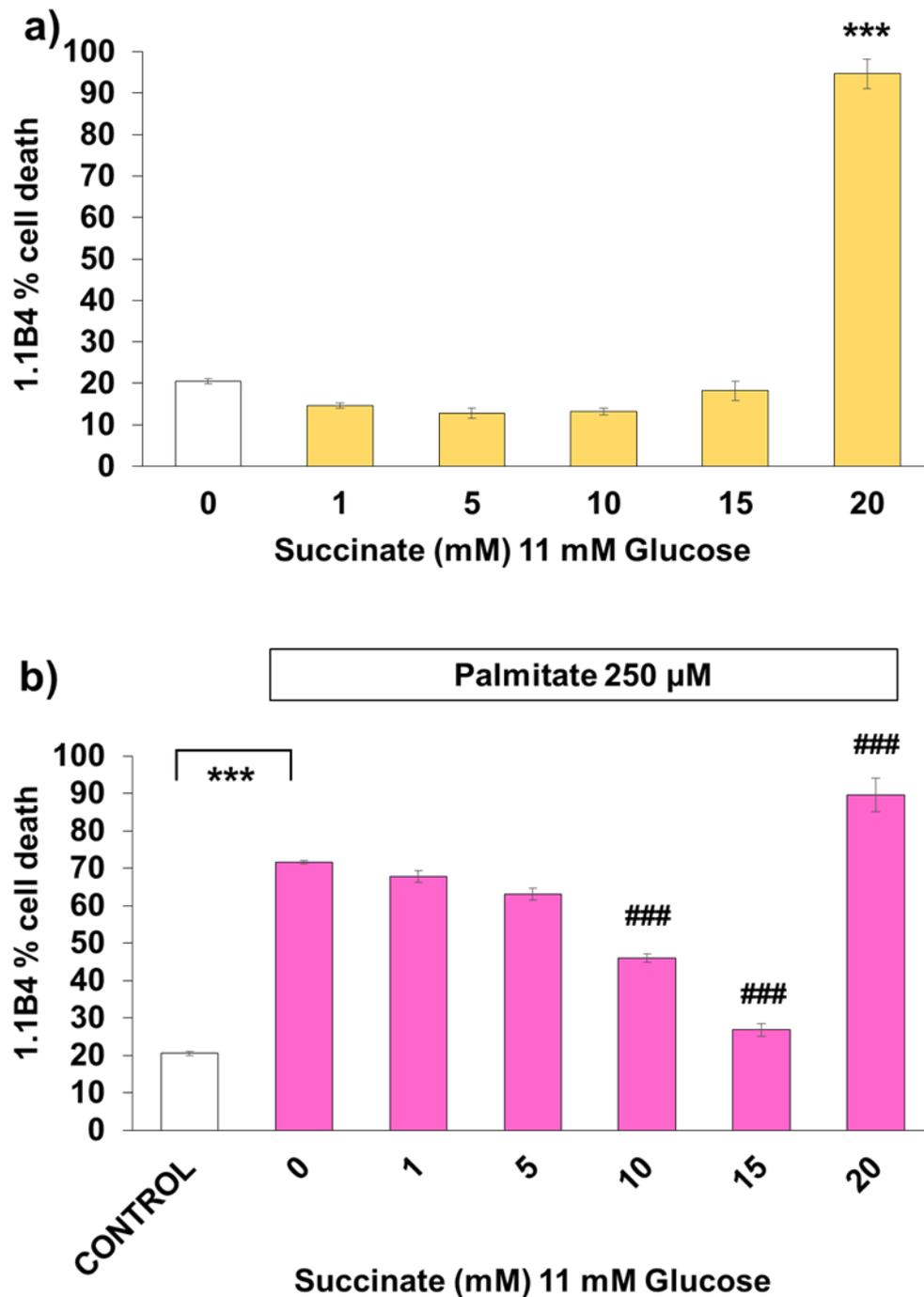


Fig. 5.13 The effect of succinate on cell death in 1.1B4 human pancreatic β -cells: 11 mM glucose \pm palmitate

1.1B4 human pancreatic β -cells were co-incubated with succinate in 11 mM glucose (a) or co-incubated with 250 μ M palmitate (b) for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. control, ### $p < 0.001$ vs. no succinate

In the next set of experiments, 1.1B4 cells were co-incubated with a higher 31 mM glucose concentration and increasing concentrations of succinate (Fig. 5.14a)). The results from this experiment showed that at 31 mM glucose, succinate was again found to be well-tolerated up to 15 mM, and percentage cell death was significantly less than the control group ($p < 0.001$ for 1, 5, 10 and 15 mM succinate concentrations). Similar to our findings in the 11 mM glucose concentrations, no protection was seen using 20 mM succinate, where the percentage cell death was found to be 1.2-fold higher than the control group ($p < 0.001$). Detailed results from this experiment were as follows: control, 23.7% cell death; 1 mM succinate, 11.5% cell death, 5 mM succinate, 14.9% cell death; 10 mM succinate, 13.6% cell death; 15 mM succinate, 13% cell death and 20 mM succinate, 29.6% cell death.

The co-incubation of cells with 31 mM glucose and 250 μ M palmitate led to a 3.8-fold increase in percentage cell death vs. the control group ($89.5 \pm 4.3\%$ vs. $23.7 \pm 0.4\%$ cell death respectively, $p < 0.001$). However, the addition of succinate led to a dose-dependent decrease in percentage cell death when compared to the succinate-free group (significant at 5 mM succinate, $p < 0.01$, and both 10 and 15 mM succinate, $p < 0.001$). Again, 20 mM succinate did not confer any protection against high concentrations of glucose and palmitate (Fig. 5.14b)), and was 1.2-fold higher than the palmitate and glucose control ($p < 0.001$). To further quantify these results, percentage cell deaths were as follows: control (no palmitate or succinate), 23.7% cell death; 250 μ M palmitate, 76.5% cell death; 1 mM succinate, 73.5% cell death; 5 mM succinate, 63.8% cell death; 10 mM succinate, 49.2% cell death; 15 mM succinate, 28.3% cell death and finally, 20 mM succinate, 89.5% cell death.

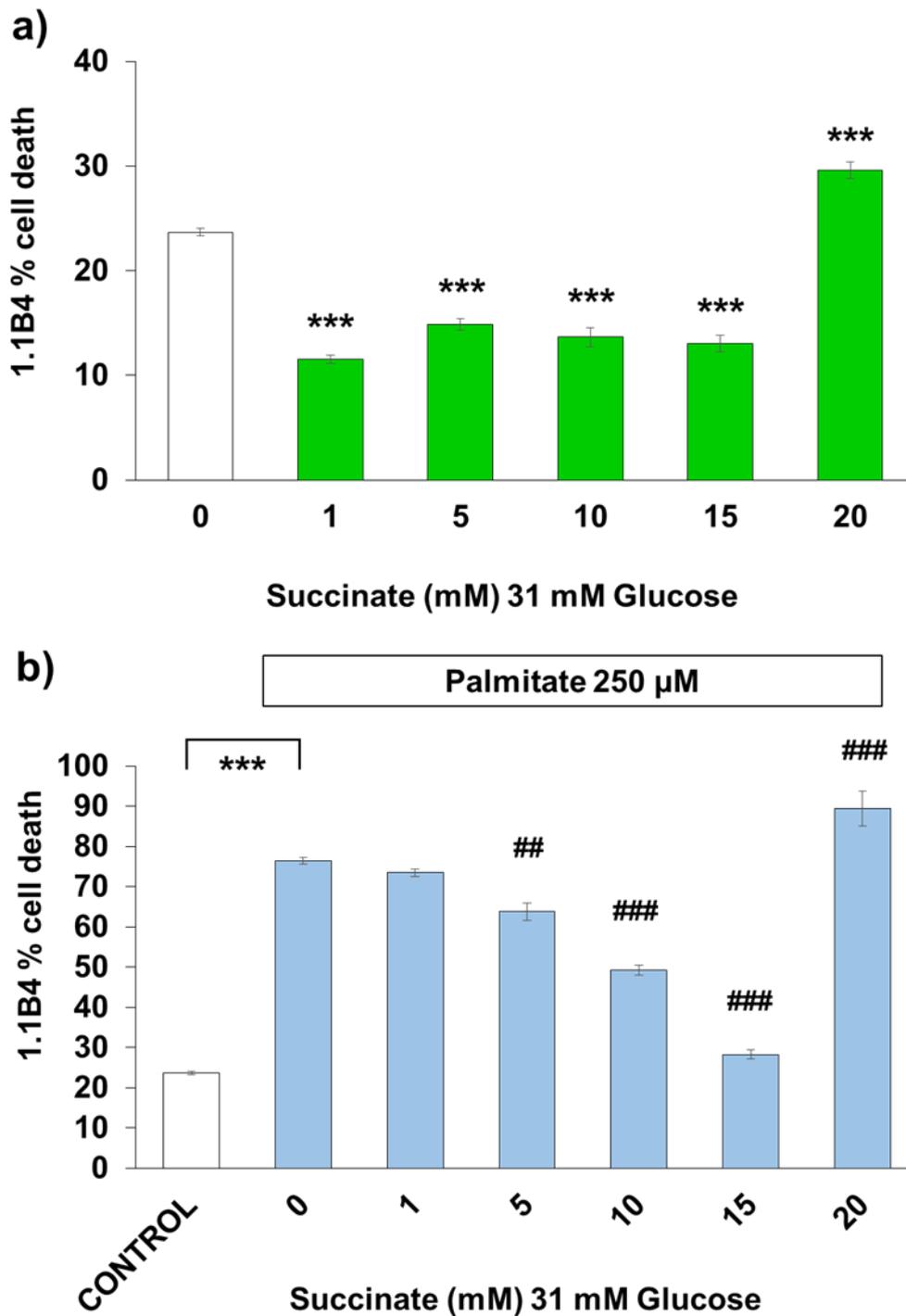


Fig. 5.14 The effect of succinate on cell death in 1.1B4 human pancreatic β -cells: 31 mM glucose \pm palmitate

1.1B4 human pancreatic β -cells were co-incubated with succinate in 31 mM glucose (a) or co-incubated with 250 μ M palmitate (b) for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. control, ### $p < 0.001$, ## $p < 0.01$ vs. succinate

When comparing the glucose concentrations, there was a 3.2-fold reduction in percentage cell death ($p < 0.001$) seen in the 20 mM succinate group in the 31 mM vs. the 11 mM glucose group, and this is in line with our findings previously noted in the INS-TXNIP cells (Fig. 5.15a)). There was no statistically significant difference in the percentage of cell death seen between groups co-incubated with palmitate at either glucose concentration (Fig. 5.15b)).

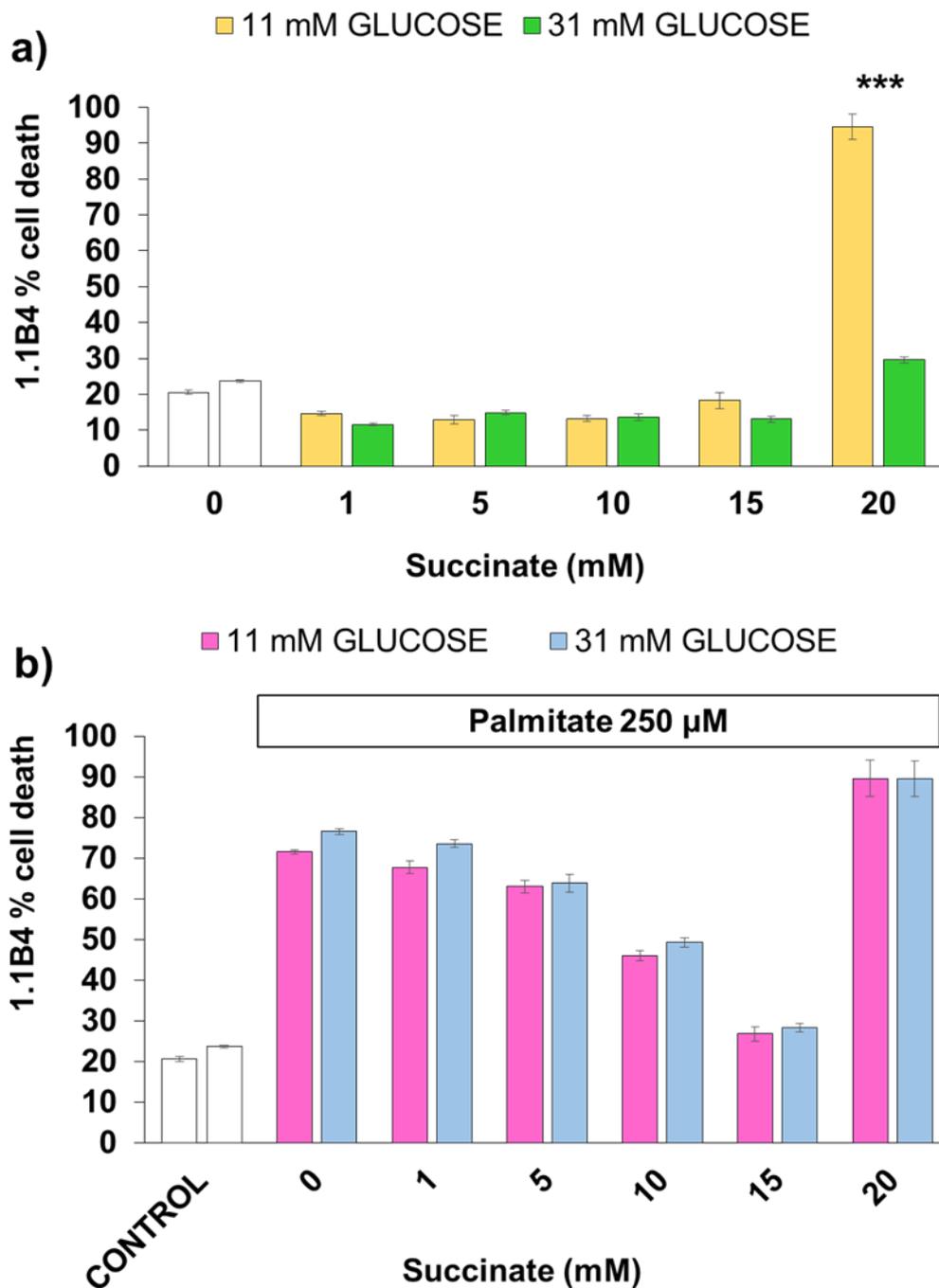


Fig. 5.15 The effect of succinate on cell death in 1.1B human pancreatic β -cells: 11 mM vs. 31 mM glucose \pm palmitate

1.1B4 human pancreatic β -cells treated with succinate in 11 mM or 31 mM glucose conditions (a). A second treatment group was co-incubated with 250 μ M palmitate (b). Cells were incubated for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments performed in triplicate (N=3). *** $p < 0.001$ vs. 11 mM glucose

5.3.9 Detecting the expression of IL-1 β mRNA in rodent INS-TXNIP pancreatic β -cells by RT-PCR

We have previously described the link between chronic inflammation, upregulation of the NLRP3 inflammasome, activation of pro-inflammatory caspase-1 and the subsequent secretion of IL-1 β as an underlying mechanism observed in T2D, which may drive pancreatic β -cell apoptosis. However, the expression of IL-1 β mRNA has not yet been reported in the INS-TXNIP cells to our knowledge, therefore the expression of IL-1 β mRNA in INS-TXNIP cells under experimental conditions of lipotoxicity and glucolipotoxicity was investigated using RT-PCR.

Amplicons equivalent to the size expected for IL-1 β were obtained when RNA from the INS-TXNIP cells was amplified by RT-PCR. A qualitative assessment and densitometric analysis revealed that the INS-TXNIP cells exhibited a 3.2-fold higher level of IL-1 β mRNA expression induced by incubation with the 31 mM glucose concentration compared to the 11 mM glucose concentration when co-incubated with palmitate (Fig. 5.16).

DNA sequencing of the amplicon products returned only a partial read (Fig. 5.17). Nonetheless, this suggested that IL-1 β expression was induced by the INS-TXNIP pancreatic β -cells under incubation conditions of glucolipotoxicity.

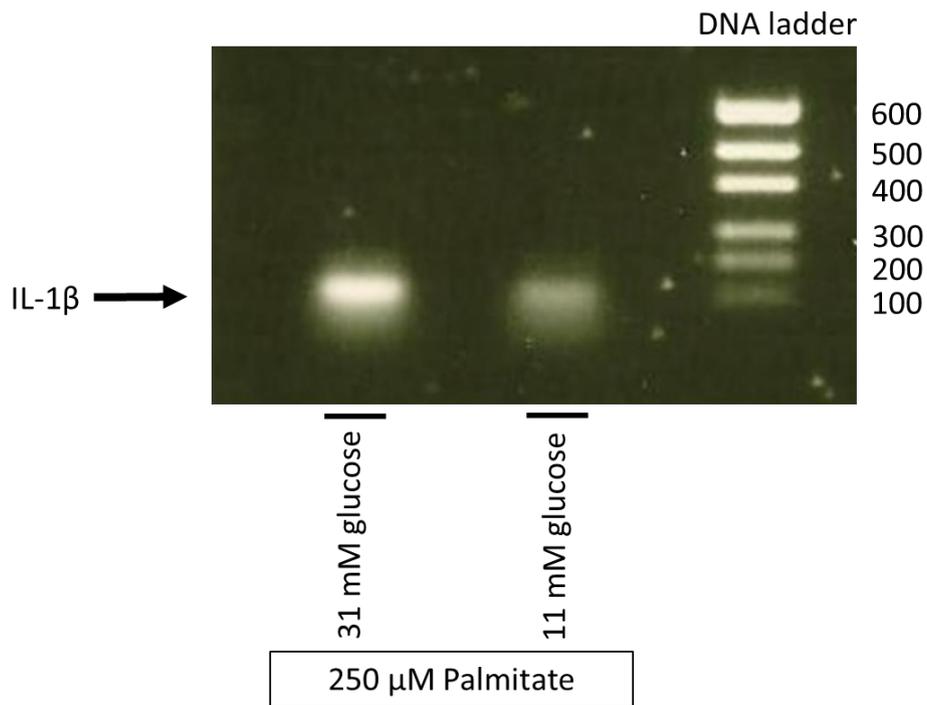


Fig. 5.16 IL-1 β mRNA expression in rodent INS-TXNIP pancreatic β -cells determined by RT-PCR

RT-PCR was carried out where cDNA was generated from mRNA extracted from INS-TXNIP cells. Bands were viewed under UV light after staining of the agarose gel with Gel-Red.

Rat IL-1 β nucleotide sequence

ggctcatctgggatcctctccagtcaggctccttgcaagtgctgaagcagctatggcaactgtccctga
actcaactgtgaaatagcagcttccgacagtgaggagaatgacctgttcttgaggctgacagaccccaa
aagattaaggattgctccaagccctgacttgggctgtccagatgagagcatccagctcaaatctaca
gcagcatctcgacaagagctcaggaaggcagtgctactcattgtggctgtggagaagctgtggcagcta
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ccgtcctctgtgactcgtgggatgatgacgacctgctagtgtgtgatgtccattagacagctgcaactgcag
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atgagtatgaaataaatgtcactaaaacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Partial sequence: atggctcgggacatagttgacttcacatggaacct

Fig. 5.17 IL-1 β amplicon identity in INS-TXNIP pancreatic β -cells

Amplicon identity was confirmed by direct sequencing of the excised and purified DNA product by Eurofins MWG Operon (Germany). Primers are indicated in green.

5.3.10 Detecting the expression of IL-1 β mRNA in human 1.1B4 pancreatic β -cells by RT-PCR

The expression of IL-1 β in 1.1B4 human pancreatic β -cells under experimental conditions of glucolipotoxicity was also investigated using RT-PCR. Again, a qualitative assessment and densitometry of the gel suggested that the 1.1B4 human pancreatic β -cells exhibited a 1.6-fold higher level of IL-1 β mRNA expression upon co-incubation with 11 mM glucose and 250 μ M palmitate, and a 1.8-fold higher level of IL-1 β expression upon co-incubation with 11 mM glucose and 250 μ M palmitate than when incubated with 11 mM glucose alone (Fig. 5.18). DNA sequencing of the amplicons again returned only a partial read, but nonetheless suggested that IL-1 β was expressed by the 1.1B4 pancreatic β -cells (Fig. 5.19) under incubation conditions of glucolipotoxicity.

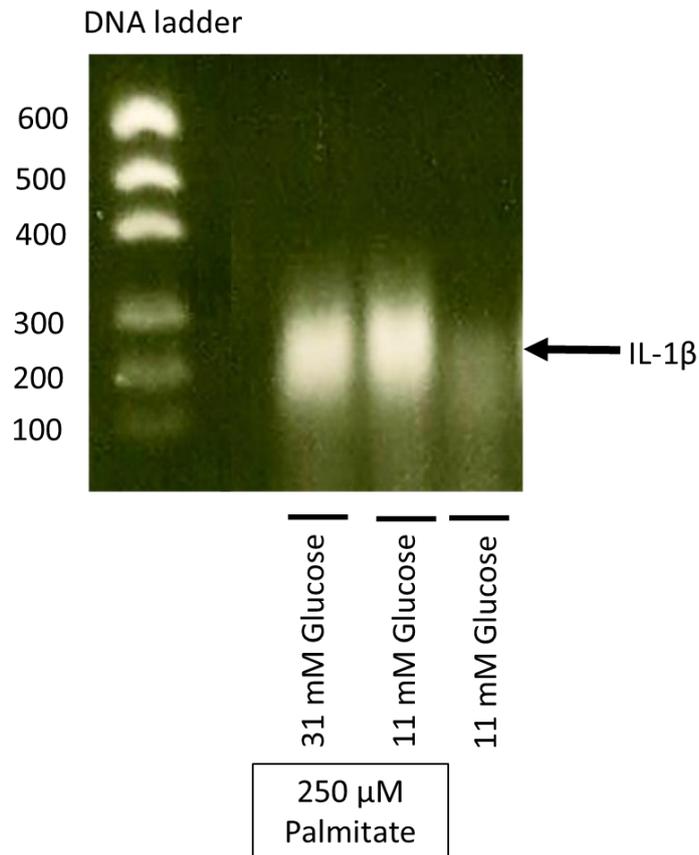


Fig. 5.18 IL-1 β mRNA expression in human 1.1B4 pancreatic β -cells determined by RT-PCR

RT-PCR was carried out where cDNA was generated from mRNA extracted from 1.1B4 human pancreatic β -cells. Bands were viewed under UV light after staining of the agarose gel with Gel-Red.

Human IL-1 β mRNA nucleotide sequence

accaaacctcttgaggcacaaggcacaacaggctgctctgggattctctcagccaatcttcattgctcaagtgtctga
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gctacgaatctccgaccaccactacagcaagggtcaggcaggccgctcagttgttgccatggacaagctga
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Partial sequence: **gctgatggccctaaacagatgaagtgctcctccaggacctggacctgcccctg**

Fig. 5.19 IL-1 β amplicon identity in human 1.1B4 pancreatic β -cells

Amplicon identity from primer set 1 was confirmed by direct sequencing of the excised and purified DNA product by Eurofins MWG Operon (Germany). Primers are indicated in green.

5.3.11 Detecting the secretion of IL-1 β by INS-TXNIP and 1.1B4 pancreatic β -cells by ELISA

As an additional method to detect the expression of the pro-inflammatory cytokine IL-1 β in both INS-TXNIP and 1.1B4 human pancreatic β -cells, a commercially available ELISA kit was used to assess IL-1 β secretion. The cells were treated with concentrations of glucose and palmitate previously demonstrated to consistently induce cytotoxicity. However, our results did not detect secretion of IL-1 β from either rodent or human pancreatic β -cell lines at any of the combinations of hyperglycaemia and hyperlipidaemia tested.

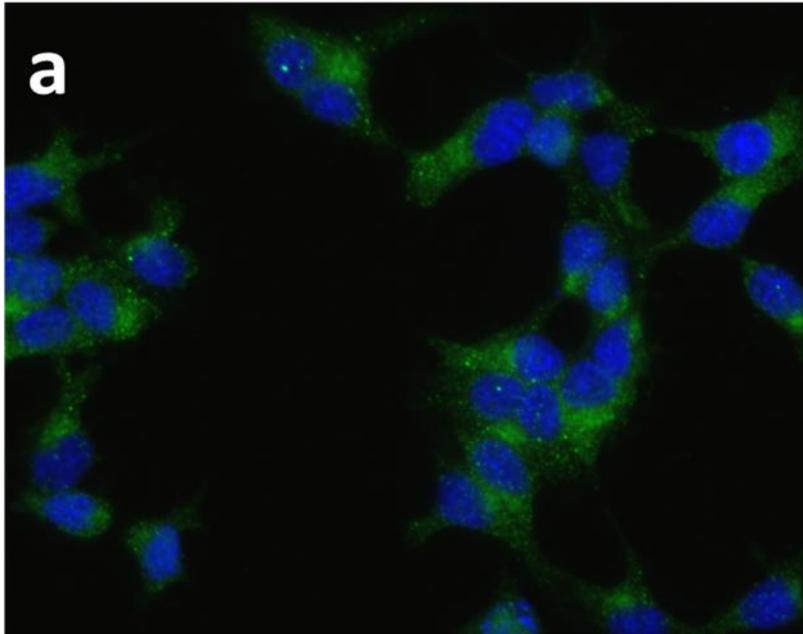
5.3.12 Detecting the secretion of IL-1 β and NLRP3 proteins in INS-TXNIP and 1.1B4 pancreatic β -cells by ICC

The mechanism between the expression of the pro-inflammatory cytokine IL-1 β , and TXNIP expression driving upregulation of the NLRP3 inflammasome is currently still unclear, although IL-1 β has been demonstrated to be secreted by both rodent cell models and human subjects with T2D. However, the secretion of IL-1 β and NLRP3 has not yet been reported in INS-TXNIP or 1.1B4 cells, so this was investigated for the first time in these next set of experiments. Immunocytochemistry was carried out using anti-IL-1 β and anti-NLRP3 antibodies on cultured INS-TXNIP rodent pancreatic β -cells and 1.1B4 human pancreatic β -cells. Both cell models were subjected to treatment conditions known to consistently induce cytotoxicity.

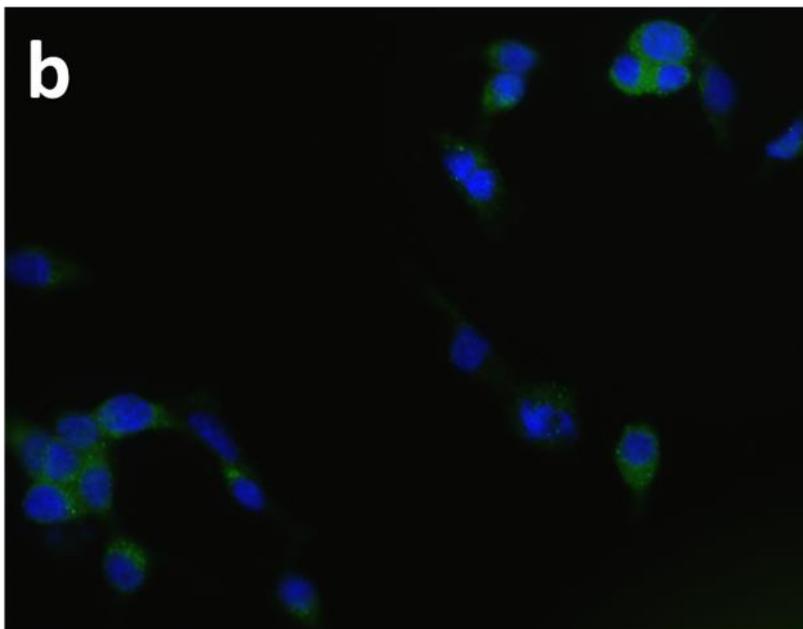
In the INS-TXNIP cells, when comparing the control cells subjected to normal culture conditions (Fig. 5.20a)) with the glucolipotoxic conditions (Fig. 5.20b)), the IL-1 β expression appeared to have undergone a change in distribution, from

ubiquitous cytoplasmic expression to punctate expression localised to areas of the cell. This also appeared to be the case for the 1.1B4 human pancreatic β -cells (Fig. 5.21a) control vs. Fig. 5.21b) the treatment group).

Probing with the NLRP3 antibody did not show any apparent difference in distribution or expression between the control group (Fig. 5.22a)) or the glucolipotoxic conditions in INS-TXNIP cells (Fig. 5.22b)), or in 1.1B4 human pancreatic β -cells (Fig. 5.23a) and b)).



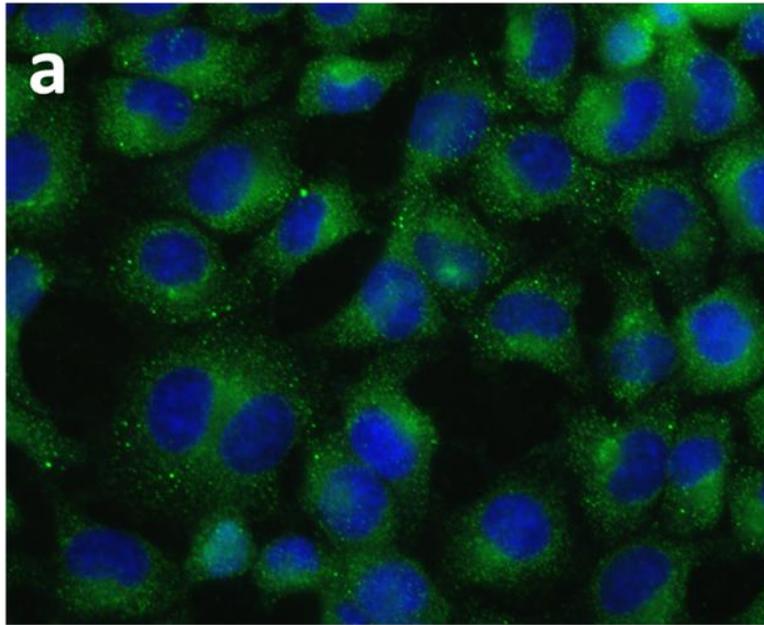
INS-TXNIP pancreatic β -cells
IL-1 β 11 mM glucose control



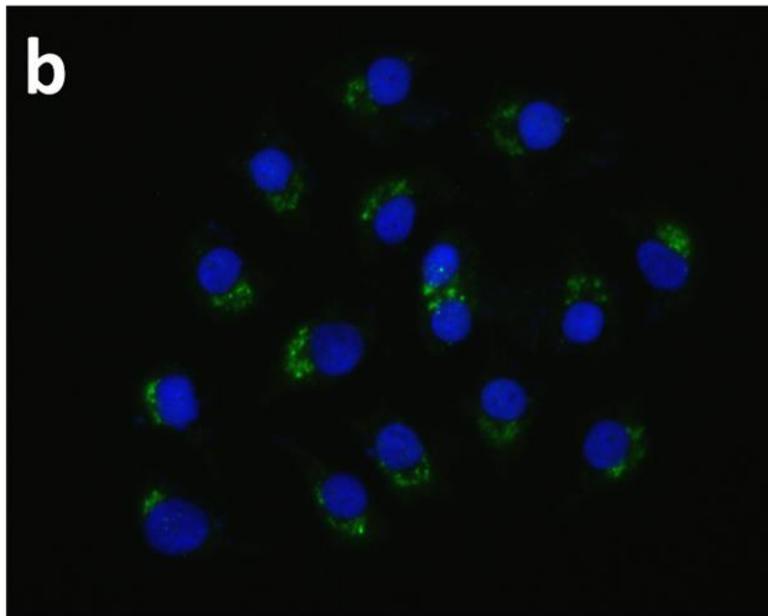
INS-TXNIP pancreatic β -cells
IL-1 β 31 mM glucose & 250 μ M palmitate

Fig. 5.20 IL-1 β secretion by INS-TXNIP rodent pancreatic β -cells

Secretion of IL-1 β (green) using immunocytochemistry in cultured INS-TXNIP pancreatic β -cells in a) control treatment group and b) 31 mM glucose and 250 μ M palmitate conditions for 48 h. Cell nuclei were stained using DAPI (blue).



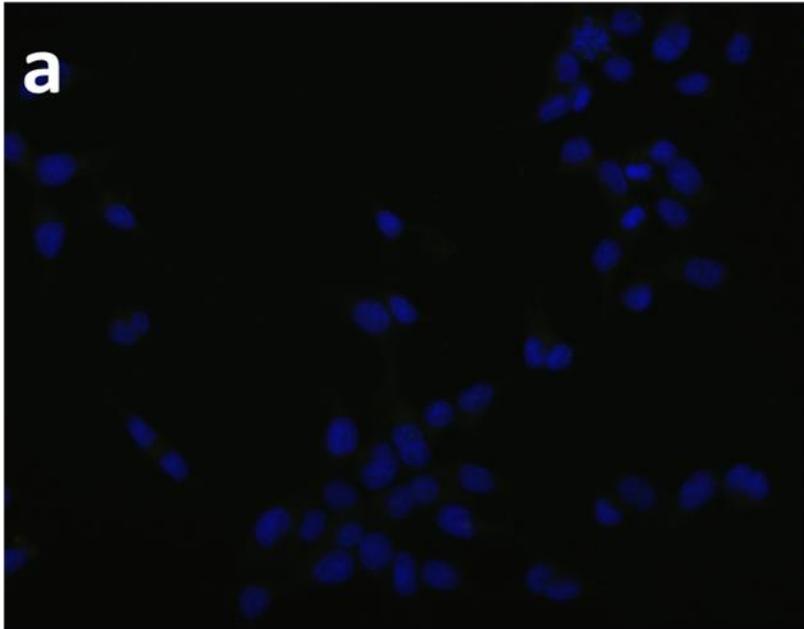
1.1B4 pancreatic β -cells
IL-1 β 11 mM glucose control



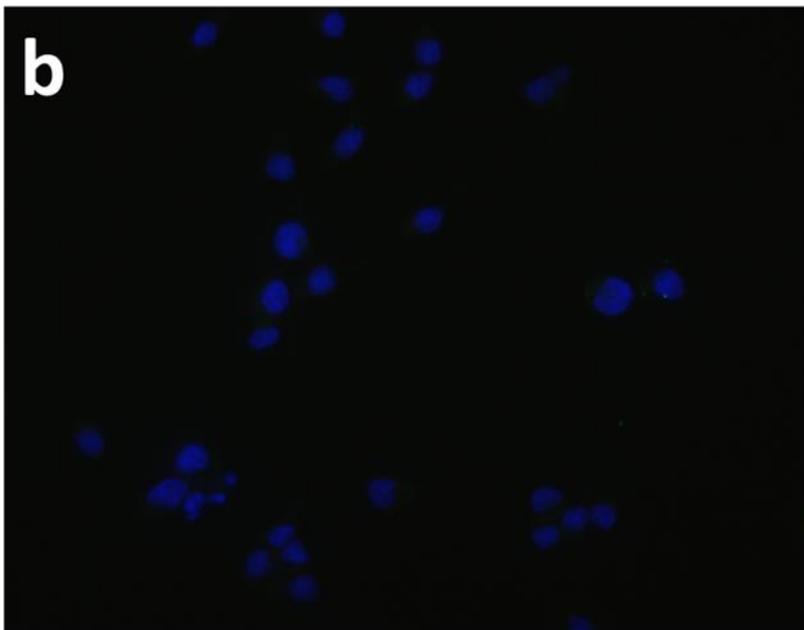
1.1B4 pancreatic β -cells
IL-1 β 31 mM glucose & 250 μ M palmitate

Fig. 5.21 IL-1 β secretion by 1.1B4 human pancreatic β -cells

Secretion of IL-1 β (green) using immunocytochemistry in cultured 1.1B4 human pancreatic β -cells in a) control treatment group and b) 31 mM glucose and 250 μ M palmitate conditions for 24 h. Cell nuclei were stained using DAPI (blue).



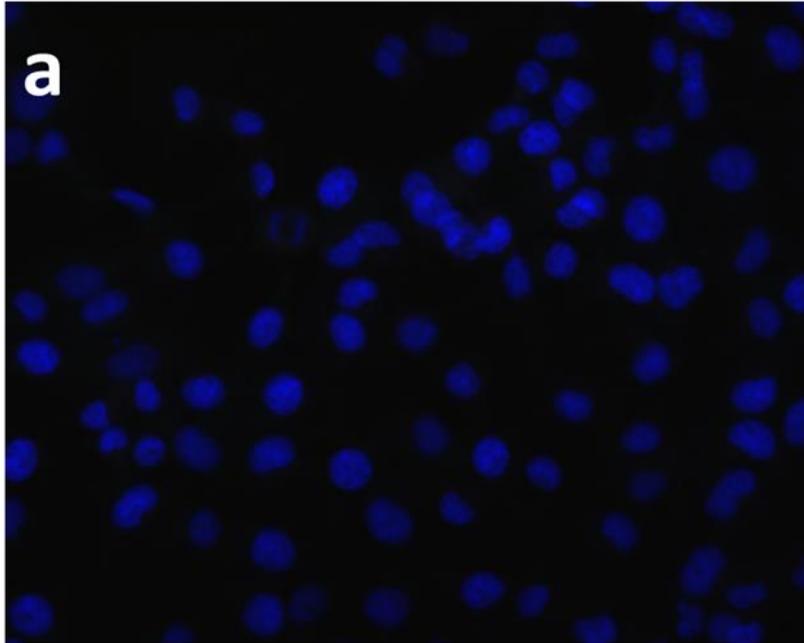
INS-TXNIP pancreatic β -cells
NLRP3 11 mM glucose control



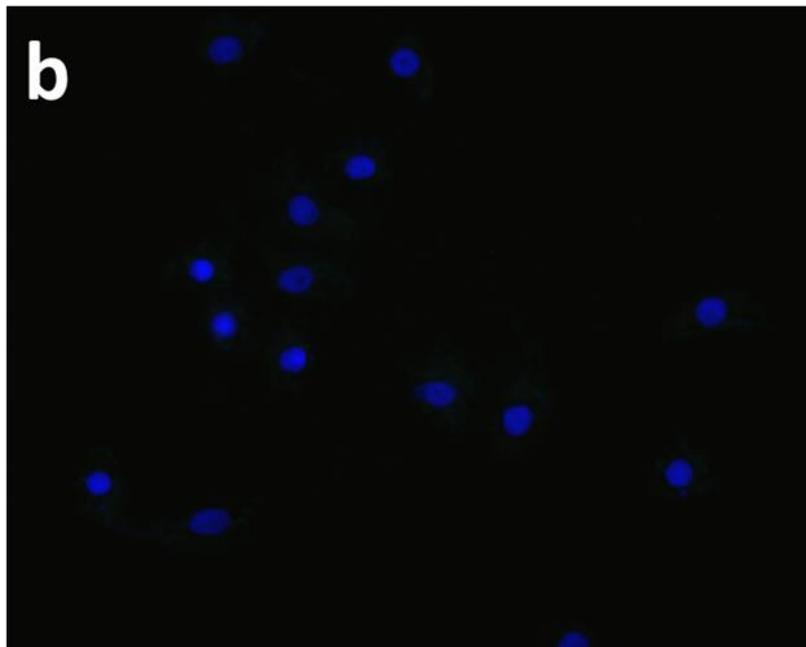
INS-TXNIP pancreatic β -cells
NLRP3 31 mM glucose control & 250 μ M palmitate

Fig. 5.22 NLRP3 secretion by in INS-TXNIP rodent pancreatic β -cells

Secretion of NLRP3 (green) using immunocytochemistry in cultured INS-TXNIP pancreatic β -cells in a) control treatment group and b) 31 mM glucose and 250 μ M palmitate conditions for 48 h. Cell nuclei were stained using DAPI (blue).



1.1B4 human pancreatic β -cells
NLRP3 5 mM glucose control



1.1B4 human pancreatic β -cells
NLRP3 31 mM glucose & 250 μ M palmitate

Fig. 5.23 NLRP3 secretion by 1.1B4 human pancreatic β -cells

Secretion of NLRP3 (green) using immunocytochemistry in cultured 1.1B4 human pancreatic β -cells in a) control treatment group and b) 31 mM glucose and 250 μ M palmitate conditions for 24 h. Cell nuclei were stained using DAPI (blue).

5.3.13 The effect of pro-inflammatory cytokines on cell death in 1.1B4 human pancreatic β -cells

It has been shown in rodent models and human islets that chronic exposure to pro-inflammatory cytokines such as IL-1 β can have a detrimental effect on pancreatic β -cell viability. However, the effect of pro-inflammatory cytokines on cell death has not yet been reported in the 1.1B4 human pancreatic β -cells. Therefore, from our final set of experiments, we report novel data from 1.1B4 β -cells subjected to exogenous pro-inflammatory cytokines.

Our results demonstrated that the incubation of 1.1B4 cells with IL-1 β alone (Fig. 5.24), although slightly increased compared to the control, did not significantly increase percentage cell death. There was a significant 2.3-fold increase in percentage cell death when the 1.1B4 cells were subjected to TNF- α compared to the control ($p < 0.01$), but again, there was no statistically significant effect on percentage cell death when comparing cells incubated with IFN- γ to the control. The co-incubation of cells with IL-1 β and TNF- α , or TNF- α and IFN- γ , increased percentage cell death by 2.1-fold and 2-fold respectively ($p < 0.05$) compared to the control. Interestingly, the co-incubation of IL-1 β and IFN- γ did not affect cell percentage death, as there was no statistically significant difference between the treatment group and control, although perhaps not unduly surprising given the results of the 1.1B4 cells being incubated with these two cytokines alone. Finally, the incubation of 1.1B4 cells with all three pro-inflammatory cytokines significantly increased the percentage cell death by 2.4-fold. To fully quantify our results, percentage cell death were as follows: control, 9.6%; IL-1 β , 13.7%; TNF- α , 21.9%; IFN- γ , 9.5%; IL-1 β and TNF- α , 20.2%; IL-1 β and IFN- γ , 11.9%, TNF- α and IFN- γ , 19% and all pro-inflammatory cytokines, 23%.

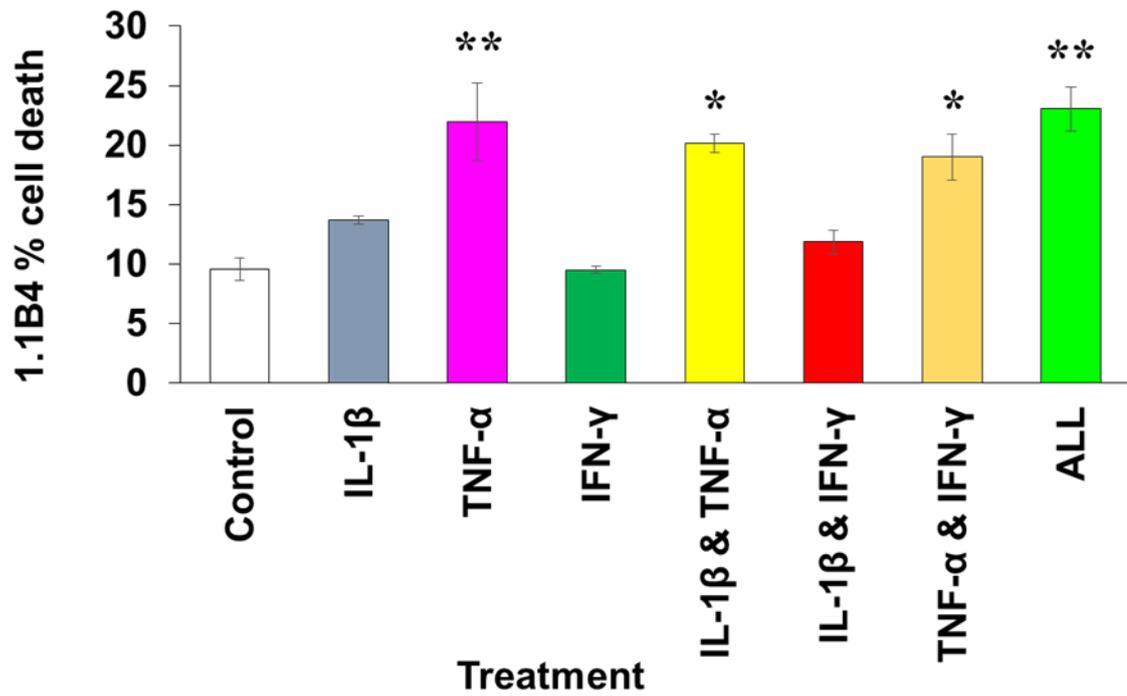


Fig. 5.24 The effect of pro-inflammatory cytokines on cell death in 1.1B4 human pancreatic β -cells

1.1B4 human pancreatic β -cells were treated with various combinations of pro-inflammatory cytokines at 20ng/ml, and incubated for 48 h. Cell death was assessed by flow cytometry using propidium iodide staining. Results represent mean \pm SEM from experiments (N=5). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control

5.4 Summary of novel results

- The overexpression of TXNIP in the TET-induced INS-TXNIP cell model under experimental conditions of glucotoxicity, leads to a significant increase in percentage cell death ($p < 0.001$) (Fig. 5.3), but not in conditions of glucolipotoxicity (Fig. 5.10 and 5.11).
- Succinate up to 15 mM is well-tolerated during glucotoxicity, but not glucolipotoxicity in INS-TXNIP cells (Fig. 5.8).
- Succinate up to 15 mM is significantly cytoprotective ($p < 0.001$) against glucotoxicity and glucolipotoxicity in 1.1B4 human pancreatic β -cells (Fig. 5.13 – 5.15).
- The pro-inflammatory cytokine IL-1 β is expressed by INS-TXNIP and 1.1B4 cells under experimental conditions of glucolipotoxicity (Fig. 5.16 – 5.19).
- The pro-inflammatory cytokine TNF- α significantly increases percentage cell death ($p < 0.05$) in 1.1B4 human β -cells under chronic incubation conditions (Fig. 5.24). Co-incubation of 1.1B4 β -cells with IL-1 β and TNF- α , and TNF- α and IFN- γ significantly increases percentage cell death ($p < 0.05$). The combined treatment of 1.1B4 cells with IL-1 β , TNF- α and IFN- γ significantly increases percentage cell death ($p < 0.01$).
- The sole incubation of 1.1B4 cells with the endogenous pro-inflammatory cytokine IL-1 β does not significantly affect percentage cell death.

5.5 Discussion

The detrimental effect of glucolipotoxicity on the pancreatic β -cell has been well established (El-Assaad *et al.*, 2003; Poitout and Robertson, 2008; Gregor and Hotamisligil, 2007; Hotamisligil, 2010; Cnop *et al.*, 2011). It has also been well recognised that chronic exposure to pro-inflammatory cytokines such as IL-1 β has a damaging effect on pancreatic β -cell viability (Böni-Schnetzler *et al.*, 2009; Dinarello *et al.*, 2010; Donath *et al.*, 2009; Maedler *et al.*, 2002; Masters *et al.*, 2010; Masters *et al.*, 2011). Glucolipotoxicity has been shown to stimulate the secretion of both IL-1 β -dependent and IL-1 β -independent proinflammatory cytokines in cultured human islets and rodent models (Wen *et al.*, 2012; Vandanmagsar *et al.*, 2011; Wen *et al.*, 2011; Masters *et al.*, 2012; Zhou *et al.*, 2010; Westermark *et al.*, 2011). This suggests a likely pathway by which an inflammatory response may drive recruitment and activation of the inflammasome (Donath *et al.*, 2010). More recently, a link has been made between the activation of metabolic stress due to chronic nutrient overload, and the recruitment of pro-inflammatory mediators suggested to underlie progressive metabolic dysfunction such as seen in obesity and T2D (Donath, 2011). However, the exact underlying mechanisms are still not well understood, and we sought to investigate the role of potential mediators involved in inflammatory activation and progression due to glucolipotoxicity.

In the first instance, we sought to establish that the INS-TXNIP/INS-EV cell model used in our investigations responded appropriately to TET, as it has been suggested that TET-on systems may be prone to leakage (Pham *et al.*, 2008). Increasing TET concentrations resulted in a clear dose-dependent increase in expression of TXNIP in the INS-TXNIP cells, and there was no TXNIP expression

when cells were incubated in the absence of TET. The INS-EV cells did not exhibit any expression of TXNIP, even when incubated with the highest TET concentration, thus our Western blots confirmed that there is no leakage in this model and were in line with results reported by our group previously.

High glucose concentrations have been reported to induce TXNIP overexpression in the EV cell model by our group (Kansikas, 2012). This phenomenon has also been shown by Minn and colleagues (2005) in INS-1 cells and rodent islets. Results from Western blots from our present studies confirmed that indeed, TXNIP overexpression was induced in INS-TXNIP cells incubated in high glucose concentrations. Furthermore, significant endogenous expression of TXNIP in the INS-EV cells was apparent under high glucose conditions, thus confirming that our results are in line with previous findings from our group.

The TXNIP protein inhibits thioredoxin activity, and inhibiting TXNIP action has been shown to protect against glucotoxicity in the isolated islets of a diabetic mouse model (Shalev, 2008). This implies that TXNIP overexpression may exacerbate cell death during hyperglycaemic conditions, and indeed, TXNIP has been demonstrated to be a proapoptotic mediator under hyperglycaemic conditions in mouse and rat cell models (Junn *et al.*, 2000; Wang *et al.*, 2002; Chen *et al.*, 2008; Shalev, 2008; Chen *et al.*, 2010). The addition of TET when INS-TXNIP cells were incubated with 31 mM glucose led to a significant increase in the percentage cell death seen compared to the 5 and 11 mM groups treated with TET, and this correlated with significantly increased levels of TXNIP expression as seen from our Western blot results. However, in our study, glucose-induced TXNIP overexpression in the INS-TXNIP cells without TET did

not result in increased percentage cell death over and above the control despite an increase in TXNIP expression. These results support evidence previously reported by our group (Kansikas, 2012), and suggest that the extent of TXNIP induction achieved by TET is far higher than the TXNIP level achieved by high glucose conditions alone. Interestingly, results from our group previously showed that TET-overexpression of TXNIP does not lead to increased percentage cell death when cells are incubated with palmitate (Kansikas, 2012), and our results also corroborate this.

Results gleaned from the INS-EV cells showed that again, high glucose conditions do not result in increased percentage cell death over and above the control, even though our Western blot results demonstrated an increase in endogenous TXNIP expression in this cell-line at 31 mM glucose concentrations. It was unexpected however, that the addition of TET in the 31 mM glucose experimental conditions resulted in a significant increase in percentage cell death in the INS-EV cells. The Western blot results clearly showed that incubation with TET does not induce TXNIP expression in the empty vector cells, so increased TXNIP expression cannot be an explanation for the increased cell death observed. Nevertheless, the endogenous expression of TXNIP in the empty vector under conditions of high glucose does question whether it is appropriate to utilise this cell line as a control in high glucose treatment conditions. Therefore, given the unusual findings reported here, it was deemed practical to exclude the INS-EV cell line from future studies. The INS-TXNIP cells incubated at 5 mM glucose conditions, where basal expression of TXNIP was not detectable by Western blot, and no detrimental effect on cell death was observed upon incubation with TET, served as a more appropriate control for future experiments in this cell model.

This unusual finding in the INS-EV cells does raise the question whether TET may be responsible for the increased percentage cell death seen in this cell model. It is well-reported in the literature that TET is well-tolerated under normal cell culture conditions. Indeed, our group demonstrated previously that TET was not toxic to this cell model at the concentrations used in our experimental conditions (Kansikas, 2012). Our results reported in Section 5.3.5 show that at 5 and 11 mM glucose conditions TET was well tolerated, and hence, also support the principle that TET is not toxic to our cell model. However, clearly under 31 mM glucose conditions, the addition of TET does appear to significantly increase percentage cell death compared to cells incubated at the same glucose concentration without TET, so is this cytotoxicity due to TXNIP overexpression, or from TET itself? The use of DOX as an alternative to TET in TET-on systems is becoming more commonplace due to the stability of DOX, and because DOX can be used at lower concentrations than TET. Under our experimental conditions, results demonstrated that DOX was also well-tolerated at 5 and 11 mM glucose concentrations, but a significant increase in percentage cell death was seen at 31 mM glucose concentrations just as seen with TET. It could be inferred from these results that it is the antibiotics *per se* that are responsible for the increased cell death seen, but clearly at lower glucose concentrations they do not affect cell viability, irrespective of increased TXNIP expression as demonstrated by our Western blot results. Furthermore, an extensive search of the literature does not suggest that the use of either TET or its analogue DOX, induces cytotoxicity in TET-regulated systems. Therefore, this supports the likelihood that the over-expression of TXNIP induced by the presence of either antibiotic in combination with a high glucose concentration, is the cause of increased cell death rather than antibiotic-toxicity alone.

Taken together, our results support a role for TXNIP as a proapoptotic factor in the TET-induced INS-TXNIP cell model under experimental conditions of glucotoxicity. It is interesting that under glucolipotoxic conditions, TET-induced TXNIP overexpression does not exacerbate cell death over and above the percentage cell death seen when cells are incubated with high glucose and high palmitate conditions in the absence of TET. This does not rule out TXNIP as a proapoptotic intermediary, but may suggest that ultimately, the role of TXNIP may only be relevant in glucotoxic conditions, rather than glucolipotoxic conditions.

To consider whether osmotic stress due to high glucose concentrations was the cause of elevated cell death in our model, cells were also incubated with L-glucose and D-mannitol osmotic controls, which exert the same osmotic effect as D-glucose. These sugars are not metabolised because of their inability to be phosphorylated by glucokinase, the first enzyme in the glycolysis pathway. It is also thought that these sugars are unable to enter cells during incubation conditions, thus remaining strictly within the extracellular compartments (Boland and Garland, 1993). L-glucose may still be able to participate in extracellular glycation reactions contributing to osmotic effects, but D-mannitol cannot thus acts as a true osmotic control (Yu *et al.*, 2007). The percentage cell death seen when cells were incubated with these sugar analogues was much higher than the percentage of death seen when cells were incubated with D-glucose. This suggests that the ability of cells to metabolise D-glucose during chronic incubation conditions thereby reducing glucose concentrations over time, may afford a compensatory mechanism against glucotoxicity. It further suggests that cell death observed during hyperglycaemic conditions is not entirely due to osmotic effects. Our group also reported that the glucose-induced upregulation

of TXNIP was not an osmotic effect of high glucose incubation, as no increase in TXNIP expression was seen when cells were incubated with L-glucose (Kansikas, 2012).

There has been some limited evidence to suggest a role for succinate as a cytoprotective agent, by way of reduced ROS production due to the ability of succinate to potentially stimulate insulin release (Fahien and MacDonald, 2002; Vengerovskii *et al.*, 2007; Zavodnik *et al.*, 2011). It has been suggested that supplementation of the TCA cycle with intermediate metabolites enhances anaplerotic reactions and protects against glucolipotoxic conditions (Choi *et al.*, 2011). We sought to investigate whether succinate afforded any cytoprotection in pancreatic β -cells incubated in glucotoxic or glucolipotoxic conditions. Our results suggested that succinate is protective against glucotoxic conditions in the INS-TXNIP cell line up to concentrations of 15 mM. However, succinate was not cytoprotective in our experiments at any concentration when the INS-TXNIP cells were subjected to glucolipotoxic conditions. The addition of palmitate clearly antagonised the protective effect of succinate in this cell model. This is in contrast to results reported in glucolipotoxicity studies by Choi and colleagues who reported that succinate afforded a protective effect in INS-1 cells under experimental conditions of glucolipotoxicity (2011). Taken together, our results support a cytoprotective role for succinate under glucotoxic conditions in the INS-TXNIP cells, but not in conditions of glucolipotoxicity.

In the 1.1 B4 human pancreatic β -cells, our results showed that succinate was cytoprotective up to concentrations of 15 mM in experimental conditions of hyperglycaemia. Interestingly, at higher glucose conditions, the cytoprotective

effect of succinate was greatly amplified, as percentage cell death was significantly less than the control in these conditions. Furthermore, and in contrast to results gleaned from the INS-TXNIP cell line, succinate was shown to be dose-dependently cytoprotective up to concentrations of 15 mM in the 1.1B4 cells under high glucose and high palmitate conditions. Quite why there was such a difference between the cell lines is unclear, and would need further investigation into the molecular and functional responses of each cell line, to be able to understand and compare the mechanisms responsible for the variations seen. Nonetheless, our results do support a cytoprotective role for succinate in the 1.1B4 human pancreatic β -cells under both glucotoxic and glucolipotoxic conditions.

Emerging links between activation of the NLRP3 inflammasome and upregulation of the pro-inflammatory cytokine IL-1 β have been established in glucolipotoxic conditions. Furthermore, recent evidence from animal studies, *in vitro* work and clinical trials have shown persuasive evidence that a primary cause of pancreatic β -cell loss in T2D is due to IL-1 β (Dinarello *et al.*, 2010). We sought to investigate IL-1 β expression in our cell models. Results confirmed that both INS-TXNIP cells and the 1.1B4 human pancreatic β -cells expressed IL-1 β mRNA under glucolipotoxic incubation conditions, and furthermore mRNA expression was increased in the higher glucose conditions. Although only partial reads were obtained from amplicon sequencing, they did strongly support the expression of IL-1 β upon alignment of the partial sequence with the predicted IL-1 β nucleotide sequence. These findings are in line with other groups who have found a pro-inflammatory response due to nutrient excess in other β -cell lines (Donath *et al.*, 2010; Böni-Schnetzler *et al.*, 2008; Dinarello, 2010; Maedler *et al.*, 2009).

However, it must be emphasised that these results do not necessarily prove that IL-1 β is expressed at the protein level.

Moreover, the ELISA for IL-1 β did not detect expression of the pro-inflammatory cytokine IL-1 β during glucolipotoxic incubation conditions in either INS-TXNIP or 1.1B4 pancreatic β -cells. However, very high percentage of cell death is observed in cells subjected to high glucose and high palmitate treatments as we have previously shown in our *in vitro* studies. Therefore, it is possible that the level of IL-1 β secretion in the supernatant from the limited number of surviving cells was below the limit of detection of the assay, rather than due to little or no IL-1 β expression at a protein level *per se*.

The ICC data was somewhat unusual, in that the subcellular distribution of IL-1 β appeared to be ubiquitous in the control cells, whereas cells subjected to high levels of glucose and palmitate appeared to demonstrate punctate and localised IL-1 β expression. This result was observed in both the INS-TXNIP and 1.1B4 human pancreatic β -cell models when probing for IL-1 β . This could of course mean that IL-1 β was not expressed under conditions of glucolipotoxicity, however positive ICC for IL-1 β has been previously demonstrated in both animal models of diabetes and in humans with T2D (Maedler *et al.*, 2002). It is more likely however, that this localisation of was due to pro-IL-1 β associating with the inflammasome for cleavage into mature IL-1 β for secretion, explaining the pattern of IL-1 β staining. Attempts to determine IL-1 β expression by Western blot using whole cell lysates from parallel treatments were unsuccessful and did not show any detectable expression (data not shown). This was initially assumed to be due to very low levels of IL-1 β secretion as a consequence of greatly elevated

cell death as outlined above. However, in light of the additional ICC data, it is more likely that the antibody was of poor quality or poor specificity.

Although the mechanism of action is unclear, there is little doubt of the role of the NLRP3 inflammasome in chronic inflammatory conditions. Therefore, it was unexpected that ICC failed to show any NLRP3 expression in either INS-TXNIP or 1.1B4 cells subjected to glucolipotoxic conditions. Our parallel experiments to determine NLRP3 expression by Western blot using whole cell lysates were unsuccessful using the same antibody (data not shown). This was almost certainly for the same reasons as outlined above for IL-1 β , which was likely poor quality or poor specificity antibody. Furthermore, we looked to determine ASC expression by Western blot using whole cell lysates, and through ICC, but again failed to determine any detectable expression.

While the role of pro-inflammatory cytokines such as IL-1 β , TNF- α and IFN- γ have been well established over the last few decades in T1D, but it has only been recently that pancreatic β -cell dysfunction in T2D has been suggested as a result of pro-inflammatory cytokine influence (Wang *et al.*, 2010). We sought to investigate the effect of pro-inflammatory cytokines on the novel 1.1B4 human pancreatic β -cell, and to our knowledge, no other work has yet been reported on these findings in this cell-line.

Incubation with TNF- α , but not any other cytokine, significantly increased percentage cell death compared to the control. Previous evidence of the effects of incubation with pro-inflammatory cytokines either alone or in combination have been greatly varied, and have been dependent on factors such as glucose

concentration, cytokine concentration and cell model used. Although it may have been reasonable to expect to observe increased percentage cell death during chronic incubation with human recombinant IL-1 β , evidence so far suggests that the effects on viability due to incubation with IL-1 β have only been reported in rat islets. Indeed, a combination of pro-inflammatory cytokines has been necessary to induce the same effect in mouse or human islets (Mandrup-Poulsen, 1996; Suk *et al.*, 2001; Donath *et al.*, 2010).

The cellular origin of IL-1 β , at least in human islets, is still not fully understood, and may be from the β -cell itself, or from recruited macrophages or T-cells during an ongoing inflammatory state (Böni-Schnetzler *et al.*, 2008; Donath *et al.*, 2005; Donath *et al.*, 2008). Often, synergism between combinations of pro-inflammatory cytokines has been required to elicit significant cell death (Suk *et al.*, 2001; Wang *et al.*, 2010), and indeed, with the exception of TNF- α , this was seen in the 1.1B4 cells. Interestingly, it has been shown that pro-inflammatory cytokine-induced cell death in pancreatic β -cells does not involve the p-eIF2 α /ATF4/CHOP10 apoptotic pathway, eliminating ER stress in apoptosis under these conditions (Åkerfeldt *et al.*, 2008). Clearly, much additional work is required to more fully elucidate cytokine influence and expression in the human pancreatic β -cell line. However, taken together, our evidence presented offers persuasive argument that IL-1 β expression in the INS-TXNIP and 1.1B4 human pancreatic β -cells is likely.

Chapter 6 Discussion

Obesity is a key risk factor for developing T2D (Vernochet *et al.*, 2009; Ford *et al.*, 1997; Gwiazda *et al.*, 2009). Hyperlipidaemia is often associated with obesity, and can lead to elevated plasma levels of FFAs (Newsholme *et al.*, 2007; Wei *et al.*, 2006). Chronic elevations of FFAs are suggested to have a detrimental effect on β -cell viability and drive the progression of T2D in a process termed lipotoxicity (Butler *et al.*, 2003; Eizirik *et al.*, 2008; Cnop *et al.*, 2005; Maedler, 2008; Eitel *et al.*, 2003; El-Assaad *et al.*, 2003).

Important information regarding the molecular mechanisms of FAs has been gleaned from *in vitro* studies using various pancreatic β -cell models. These studies have demonstrated that FAs exert differential responses in β -cells under chronic incubation conditions. The degree of toxicity or protection exerted by a FA species varies markedly according to the carbon chain length, degree of saturation or configuration of the individual FA species being studied. Long-chain-SFAs (\geq C16:0) exert a detrimental effect on β -cell viability, whereas medium and shorter-chain SFAs (\leq C14:0) or LC-MUFAs (\geq C16:1) are well tolerated by β -cells. A further critical finding is that the *in vitro* co-incubation of LC-MUFAs with LC-SFAs, attenuates the cytotoxic effects observed in β -cells when they are incubated solely with a LC-SFA species (Newsholme *et al.*, 2007; Karaskov *et al.*, 2006; Welters *et al.*, 2004; Eitel *et al.*, 2003; Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2007; Diakogiannaki *et al.*, 2008; Morgan *et al.*, 2008).

The molecular mechanisms that mediate cytotoxic or cytoprotective responses of β -cells to FA species remain unclear. However, increasing evidence implicates a role for ER stress in mediating β -cell death associated with chronic lipotoxicity-

induced stress (Qu *et al.*, 2009; Breckenridge *et al.*, 2003; Laybutt *et al.*, 2007). The primary aim of the ER stress response is to restore cellular homeostasis, but if ER stress is persistent, it may induce β -cell apoptosis (Araki *et al.*, 2003; Laybutt *et al.*, 2007; Karaskov *et al.*, 2006; Breckenridge *et al.*, 2003; Wei *et al.*, 2009; Qu *et al.*, 2009; Fonseca *et al.*, 2009; Scheuner and Kaufman, 2008).

A critical early step in the ER stress pathway is the phosphorylation of eIF2 α (Qu *et al.*, 2009; Fonseca *et al.*, 2009; Cnop *et al.*, 2008; Araki *et al.*, 2003; Herbert, 2007). It is generally accepted that this eIF2 α phosphorylation step is mediated by PERK (Kapoor and Sanyal, 2009; Cunha *et al.*, 2008; Eizirik *et al.*, 2008; Qu *et al.*, 2009; Harding *et al.*, 2000; Herbert, 2007). However, evidence from a more recent study suggests that PERK may adopt a more cytoprotective role, while another eIF2 α kinase, PKR, may assume a role in regulating proapoptotic responses in β -cells (Morgan, 2009; Lee *et al.*, 2007).

The primary aim of this thesis was to investigate whether cell death induced in pancreatic β -cells during lipotoxic incubation conditions, was due to activation of an ER stress response centred on alterations to PKR activation.

Chapter 3: The role of PKR in pancreatic β -cell apoptosis

This chapter focussed on the use of a putative small molecule inhibitor Compound-16, transient PKR KD using adenoviral DN vectors and the creation of stable PKR KD BRIN-BD11 clones using shRNA to manipulate PKR activity. Studies were predominantly viability studies and Western blotting to correlate with ER stress markers.

Key findings

The key findings from this chapter were that Compound-16 (5 – 25 μ M) significantly protected BRIN-BD11 cells against palmitate-induced cell death. Furthermore, Compound-16 significantly protected BRIN-BD11 cells against serum-starvation for up to a 30 h incubation period before significant protection was lost. Western blot analyses of lysates from BRIN-BD11 cells incubated with 5 μ M Compound-16 showed a significant increase in the ER stress marker phospho-eIF2 α despite improving cell viability during chronic palmitate exposure. However, Compound-16 is potently cytotoxic to INS-1 cells and MIN6 cells.

The use of PERK and PKR DN adenoviral vectors demonstrated that transient KD of these kinases individually, or together, significantly reduced the phosphorylation of eIF2 α , thereby confirming a role for both kinases within ER stress pathways in BRIN-BD11 cells. Stable PKR KD clones using BRIN-BD11 parental cells show that the loss of PKR function significantly protects against palmitate-induced cell death in BRIN-BD11 cells and potentiates the cytoprotective action of palmitoleate.

Taken together, our results provide novel evidence that in BRIN-BD11 rodent pancreatic β -cells, PKR has a clear role in mediating cytotoxic and cytoprotective responses during exposure to various FA species. This supports recent evidence that identifies PKR as a central player within nutrient sensing pathways in mouse cell models (Nakamura *et al.*, 2010). Other recent works have shown that PKR is required for inflammasome activation in response to certain PAMPs such as ATP. PKR interacts with the NLRP3 inflammasome to mediate assembly and activation, linking this nutrient sensing protein with chronic inflammation and

inflammasome-dependent IL-1 β secretion (Lu *et al.*, 2012; So *et al.*, 2013). This establishes a central role for PKR within multiple immune and metabolic responses. Taken together, PKR represents a critical target for the design of therapeutic agents aimed to reduce β -cell loss in the progression of T2D.

Limitations of study

The potent toxicity of Compound-16 to INS-1 and MIN6 cells raises questions regarding the suitability of this compound for use with *in vitro* pancreatic β -cell models. The lack of a commercially available rat phospho-PKR antibody, and poor specificity of human and mouse phospho-PKR antibodies prevented critical Western blot corroboration of results obtained from viability studies. Additional challenges with upstream and downstream ER stress marker antibodies made it difficult to make conclusive statements regarding the involvement of potential mediators of the p-eIF2 α /ATF4/CHOP10 apoptotic pathway in lipotoxic conditions.

Areas for future research

A primary aim to further this research would be to commission a custom-made rat phospho-PKR antibody, to allow the opportunity to obtain conclusive evidence of alterations to PKR activity in glucotoxic, lipotoxic and glucolipotoxic culture conditions. Western blot and PCR studies to fully delineate all proteins involved along the ER stress pathway from activating ligands to effector molecules, would provide important information for effective targeted therapeutic intervention of critical pathway mediators. Furthermore, these mechanisms need to be investigated in human pancreatic β -cells and human islets, to complement existing knowledge in rodent models. An additional line of study would be to utilise the islet cells from patients with T2D, recovered post mortem, to look for

evidence of ER stress and apoptosis. Further experiments using Compound-16 are necessary to explore the involvement of related kinases that can phosphorylate eIF2 α to quantify redundant kinase activity, as this was not conducted in the original Jammi work.

Chapter 4: Characterisation of 1.1B4 human pancreatic β -cells

The use of *in vitro* cell models in diabetes research represents a convenient approach to study physiological and pathophysiological mechanisms, and can be advantageous in the development of novel pharmacological agents. Studies using rodent pancreatic β -cell lines have contributed valuable observations regarding the molecular and functional responses of β -cells to experimental conditions that are suggested to drive the progression of T2D *in vivo* (Asfari *et al.*, 1992; Gazdar *et al.*, 1980; Santerre *et al.*, 1981; McClenaghan *et al.*, 1996; Efrat *et al.*, 1988; Miyazaki *et al.*, 1990).

However, despite the usefulness of rodent cell models, there are differences between rodent and human pancreatic β -cell responses to glucotoxicity and lipotoxicity, and it is critical that any findings in rodent cells are verified in human β -cells. Furthermore, aberrant functional responses, such as poor insulin secretion in response to secretagogues or cell dedifferentiation over time, have limited the usefulness of human pancreatic β -cell models in T2D studies (Ulrich *et al.*, 2002; Skelin *et al.*, 2010; Newgard and McGarry *et al.*, 1995; Dufayet de la Tour *et al.*, 2001; Demeterco *et al.*, 2002; Gueli *et al.*, 1987; Levine *et al.*, 1995; Beattie *et al.*, 1999; Russ *et al.*, 2008). Due to the restricted availability of primary human islets, and their poor response to culture conditions, considerable effort has been made into the development of human pancreatic β -cell lines from

human pancreatic sources over the last few decades (Negi *et al.*, 2012; Hohmeier and Newgard, 2004).

The development of a novel human insulin-secreting β -cell line 1.1B4 was described in 2011. These cells were reported to be stable in culture conditions and to display typical characteristics of human pancreatic β -cells. This provided the opportunity to undertake functional studies to characterise the effects of FA species, which have not yet been reported in this β -cell line. Therefore, in these studies, we employed the use of 1.1B4 human pancreatic β -cells to investigate for the first time, their *in vitro* responses to different FA species.

Key findings

The key findings from this chapter showed that medium-chain SFAs are well-tolerated in 1.1B4 human pancreatic β -cells under chronic incubation conditions. In contrast, the LC-SFAs palmitate (C16:0) and stearate (C18:0) induce a dose-dependent increase in percentage cell death under chronic incubation conditions. The longer-chain SFA stearate does not exert a greater cytotoxic effect than palmitate as shown in rodent β -cells. Co-incubation of 1.1B4 cells with palmitate, and the MUFAs palmitoleate (C16:1) or oleate (C18:1), induces a dose-dependent decrease in percentage cell death under chronic incubation conditions. However, the longer-chain MUFA oleate does not exert a greater cytoprotective effect than palmitoleate as demonstrated in rodent β -cells.

In consideration of the primary aim of this thesis, Compound-16 was used in preliminary experiments to investigate the role of PKR KD in mediating cytotoxic or cytoprotective responses in lipotoxic conditions. However, similar to our

findings in both INS-1 and MIN6 cells, Compound-16 is potently cytotoxic to 1.1B4 cells.

The incubation of 1.1B4 cells with methyl-SFA species does not affect cell viability. Moreover, the incubation of 1.1B4 cells with the MUFAs methyl-palmitoleate and methyl-oleate exerts a dose-dependent cytoprotective effect against palmitate-induced cell death. However, the longer-chain methyl-oleate species does not exert a greater cytoprotective effect than methyl-palmitoleate.

These studies establish 1.1B4 responses to various FA species in novel data not shown before in this cell line, suggesting these cells are a suitable model to study β -cell dysfunction during lipotoxic conditions. Our data corroborates previously reported well-tolerated effects of methyl-palmitate and cytoprotective effects of methyl-palmitoleate in BRIN-BD11 cells (Briaud *et al.*, 2001; Cunha *et al.*, 2008). Furthermore, our results using methyl-stearate and methyl-oleate in the 1.1B4 cells adds to the currently limited knowledge regarding the impact of methylated FAs on β -cell viability (Diakogiannaki *et al.*, 2007; Dhayal *et al.*, 2008).

Limitations of study

The main limitation of this study is the method of creation of the 1.1B4 cell line, which were created using an *in vitro* cell line with tumoral origin (McCluskey *et al.*, 2011). The development of *in vitro* cell line typically focusses on cell survival and proliferation, rather than maintaining long-term viability (Alderman *et al.*, 1985). This can impact on secretory capacity, and it has been reported that this cell line does have poor secretory response to secretagogues (McCluskey *et al.*, 2011). The induced pseudo-islet formation of these cells was reported to improve

secretory capacity, suggesting that cell-cell interactions are critical to retain normal physiological responses of β -cells. This highlights the importance of confirmatory work in islets in addition to *in vitro* monolayer cell culture models (Guo-Parke *et al.*, 2012).

Future work

The use of RT-PCR and Western blotting to investigate the activation of ER stress markers would be useful to determine molecular mechanisms of lipotoxicity in this cell line, as these have not yet been studied. Furthermore, the use of next generation sequencing would provide rapid and comprehensive results of gene expression within this cell line. This would afford valuable information to enable comparisons to be made between 1.1B4 cell and human β -cell gene expression. This would also allow the opportunity to investigate whether erroneous gene expression exists due to the tumoral origin of the cell model.

Chapter 5 The role of TXNIP and inflammatory mediators in pancreatic β -cell apoptosis

Thioredoxins have a predominant role as intracellular antioxidant enzymes (Arner & Holmgren, 2000; Hirota *et al.*, 1997; Hirota *et al.*, 1999; Martin & Dean, 1991). Their predominant role is to maintain an intracellular reducing environment, through reduction of thiol groups within protein cysteine side chains (Gilbert, 1990; Berndt *et al.*, 2007). Thioredoxin activity is regulated by TXNIP, a highly inducible gene, expressed in human islets cultured in hyperglycaemic conditions (Shalev *et al.*, 2002). TXNIP initiates oxidative stress within β -cells by a converse mechanism to thioredoxin (Nishiyama *et al.*, 1999; Nishiyama *et al.*, 2001). TXNIP has been shown as a pro-apoptotic mediator in pancreatic β -cells and is

suggested to drive apoptosis in T2D (Shalev, 2008; Minn *et al.*, 2005, Junn *et al.*, 2000).

The use of succinate as a potential cytoprotective compound in T2D has been recently suggested. Succinate stimulates insulin release, which is suggested to inhibit ROS production and mitochondrial oxidative phosphorylation dysfunction in conditions of nutrient excess (Fahien and MacDonald, 2002; Vengerovskii *et al.*, 2007; Zavodnik *et al.*, 2011).

The activation of NLRs by glucolipotoxic conditions links the upregulation of proinflammatory caspase-1 and increased secretion of IL-1 β levels. The production of pro-inflammatory IL-1 β is a commonly accepted mechanism thought to drive apoptotic pathways seen in chronic metabolic conditions such T2D (Maedler & Donath, 2004). TXNIP has been shown to interact with NLRP3, thereby linking activation of the inflammasome with glucolipotoxicity. This mechanism is suggested to drive increased IL-1 β production, which is thought to have an integral role in pancreatic β -cell apoptosis (Franchi *et al.*, 2012; Mankan *et al.*, 2012; Tannahill and O'Neill, 2011; Harder *et al.*, 2009; Muñoz-Planillo *et al.*, 2009).

Key findings

The key findings from this chapter are that TXNIP overexpression in the TET-induced INS-TXNIP cell model leads to a significant increase in percentage cell death in glucotoxic conditions, but not in conditions of glucolipotoxicity. Succinate ($\geq 5 \leq 15$ mM) is cytoprotective against glucotoxicity, but not glucolipotoxicity in

INS-TXNIP cells. However, succinate ($\geq 1 \leq 15$ mM) is cytoprotective against both glucotoxicity and glucolipotoxicity in 1.1B4 human pancreatic β -cells.

The pro-inflammatory cytokine IL-1 β is expressed by INS-TXNIP and 1.1B4 cells under experimental conditions of glucolipotoxicity. Incubation with endogenous TNF- α increases percentage cell death in 1.1B4 human β -cells during chronic incubation conditions, as does co-incubation with IL-1 β and TNF- α , TNF- α and IFN- γ or combined treatment with IL-1 β , TNF- α and IFN- γ . However, the incubation of 1.1B4 cells with endogenous IL-1 β alone does not significantly affect percentage cell death.

Our results from this chapter suggest that the role of TXNIP in β -cells is apoptotic only under conditions of glucotoxicity, and this corroborated findings by Chen and colleagues (2008; 2010), and Shalev (2008) in INS-1 cells and mouse islets. There is little data on the cytoprotective role of succinate in T2D. Choi *et al.*, (2010) used succinate in INS-1 cells, and demonstrated that it can act as a cytoprotective agent in conditions of glucotoxicity and glucolipotoxicity. We have shown here in INS-TXNIP cells, that succinate is only cytoprotective in conditions of glucotoxicity. In contrast however, we established that succinate is protective against cell death induced by glucotoxicity and glucolipotoxicity in 1.1B4 human cells. Clearly, the variable efficacy of succinate within these studies, particularly under different stress-inducing conditions, highlights the importance of further work required to clarify molecular mediators and signalling pathways more fully. However, it does suggest that succinate may have a therapeutic use in protecting β -cell viability.

The cellular origin and mechanism of cell death induced in pancreatic β -cells by IL-1 β is not well understood and studies to date have been limited. The PCR and ICC studies from this chapter suggest that IL-1 β is secreted from INS-TXNIP and 1.1B4 pancreatic β -cells, contributing towards a paucity of information in this area. Interestingly, we have shown that the action of IL-1 β alone does not induce apoptosis in 1.1B4 β -cells under normal glucose concentrations. Previous studies have invariably used conditions of glucolipotoxicity, and/or a combination of proinflammatory cytokines to demonstrate cytotoxic effects on β -cell viability (Maedler et al., 2002). As such, this suggests that although IL-1 β clearly does have a role in the progression of β -cell dysfunction, it requires additional synergistic effect from other mediators to lead to eventual β -cell demise.

Limitations of studies

The results returned from IL-1 β mRNA expression would have been more compelling if full reads had been returned from amplicon sequencing. Furthermore, corroborating Western blot and ELISA detection of IL-1 β protein expression from cell lysates would have provided definitive proof of pancreatic β -cell IL-1 β secretion. By virtue of experimental design, chronic glucotoxicity and glucolipotoxicity usually results in very high percentage β -cell death, highlighting a limitation of using *in vitro* cell culture models to study β -cell dysfunction and apoptosis in experimental conditions of T2D. Poor specificity of NRLP3 and ASC antibodies meant mediators of inflammasome activation could not be corroborated in these studies.

Areas for future research

To strengthen results from these studies, and to potentiate targeting of mediators of inflammasome activation, further work is needed to detect and characterise ligands and integral proteins within the chronic inflammatory pathway, in both *in vitro* human pancreatic β -cells and human islets in T2D experimental conditions. This would mean locating and validating good quality antibodies with which to conduct further Western blotting, ICC and precipitation studies.

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Posters and Publications

The role of Protein Kinase R in the regulation of cytotoxicity by long-chain saturated fatty acids in pancreatic beta-cells



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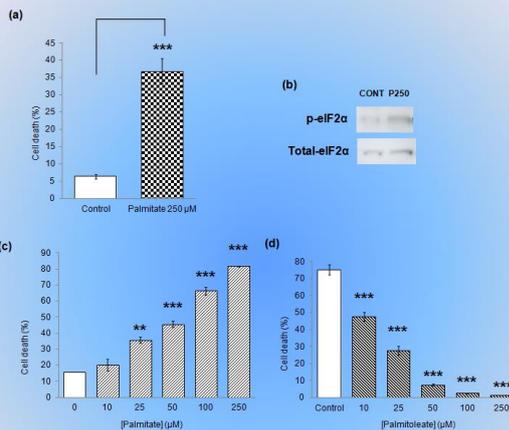
Introduction and Aims

Chronic exposure of pancreatic beta-cells to the long-chain saturated fatty acid palmitate (C16:0), induces an endoplasmic reticulum (ER) stress response which can lead to beta-cell apoptosis. This mechanism could be relevant to the beta-cell loss observed during the progression of Type 2 diabetes (T2D) in humans and, as such, agents attenuating ER stress may have therapeutic potential. Conversely, co-incubation of pancreatic beta-cells with LC-SFAs and long-chain monounsaturated fatty acids (LC-MUFAs) such as palmitoleate (16:1) attenuates ER stress and reduces cytotoxicity. A critical early step in the ER stress response is the phosphorylation of eukaryotic initiation factor-2 alpha (eIF2α) which alleviates cytotoxic insults by inhibiting global protein synthesis, thereby allowing the opportunity for the restoration of normal ER homeostasis. Phosphorylation of eIF2α is catalysed by PERK (double-stranded ribonucleic acid-dependent protein kinase R (PKR)-like ER kinase) and this mechanism may operate in cells exposed to palmitate. However, recent work has suggested that another eIF2α kinase, PKR (protein kinase R), may also play a role in mediating palmitate-induced eIF2α phosphorylation and we have investigated this possibility.

Materials and Methods

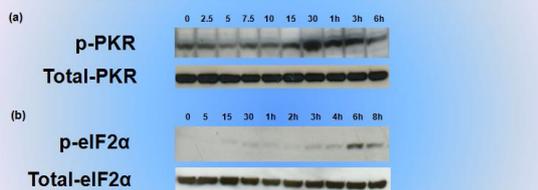
- The rodent beta-cell line BRIN-BD11 was used in this study (though similar results were also obtained in the INS-1E rodent cell line). Cells were cultured and exposed to palmitate (250 μM) and palmitoleate (250 μM) in complex with BSA (1% final concentration).
- Vectors encoding dominant negative kinase isoforms were used to manipulate the expression of both PERK and PKR. The induction of ER stress was assessed by immunoblotting of relevant ER stress marker proteins.
- Formalin-fixed paraffin embedded pancreatic tissue sections from human T2D patients and non-diabetic adult controls were examined for the expression of PKR by immunohistochemistry.

Results 1: Effects of palmitate and palmitoleate on cell viability



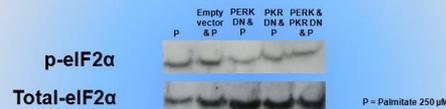
(a) Loss of viability was observed when cells were incubated with 250 μM palmitate for 18 h (*** $p < 0.001$ vs control), and this correlated with increased phosphorylation of eIF2α as observed during Western blotting analysis (b). (c) The loss of viability caused by incubation of cells with palmitate for 18 h was dose-dependent. (d) Co-incubation of cells with palmitate (250 μM) and increasing concentrations of palmitoleate reduced the cytotoxic effects of palmitate. (** $0.001 < P < 0.01$ vs control; *** $P < 0.001$ vs control).

Results 2: PKR and eIF2α become phosphorylated during exposure of pancreatic beta-cells to palmitate



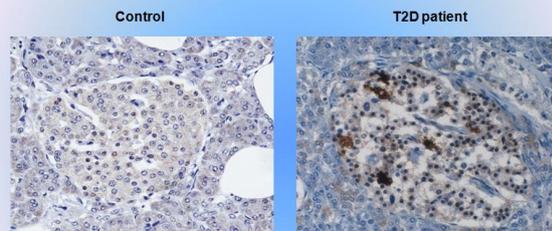
(a) Western blotting analysis revealed an increase in phosphorylation of PKR within 15-30 min of exposure of cells to palmitate, and this response was sustained for ~6 h. Enhanced phosphorylation of eIF2α also occurred but was slightly delayed relative to PKR phosphorylation (b).

Results 3: Effects of dominant negative PERK and PKR isoforms on eIF2α phosphorylation



BRIN-BD11 cells were incubated with adenoviral vectors encoding dominant negative isoforms of PERK or PKR. They were then exposed to 250 μM palmitate for 6 h. Cells infected with luciferase-containing empty vectors served as controls. Expression of dominant negative forms of either PERK or PKR, attenuated eIF2α phosphorylation during incubation with palmitate, when compared to non-palmitate treated control cells.

Results 4: PKR activation in human T2D pancreatic sections



Pancreatic tissue sections from adults with T2D were examined using immunohistochemistry and stained for the presence of PKR. Compared to non-diabetic adult control sections, the majority of T2D sections showed clear positive staining for total PKR in certain endocrine cells.

Conclusions

Immunostaining of human pancreatic tissue sections revealed immunopositivity for PKR in the islets of T2D patients, consistent with the activation of a stress response. However, *in vitro* studies suggest that both PKR and PERK may contribute to the ER stress response seen during exposure of pancreatic beta-cells to palmitate. Further studies will be required, therefore, to establish whether targeting either one of these enzymes alone, is sufficient to attenuate pancreatic beta-cell stress in T2D.