

Regulation of pyruvate dehydrogenase kinase 4 (PDK4) through protein-protein interaction and its role in apoptosis



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A dissertation submitted to the faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

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Declaration

I, Patricia Coliwe Ntlabati, declare that this dissertation is my own work and any added information from other sources has been carefully referenced. The dissertation “The regulation of pyruvate dehydrogenase kinase 4 through protein-protein interaction and its role in apoptosis” is being submitted for the degree of Master of Science at the University of the Witwatersrand, South Africa.

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Patricia Coliwe Ntlabati

Date:.....

Dedication

I would like to dedicate this work to my family at large, my father and mother who always believed, supported and loved me, my two sisters whom I have inspired throughout the years and not forgetting my two brothers (Thapelo and Eddie) who encouraged me when things were difficult. For my uncle who has been there for me ever since I started with my degree until today.

Lastly and foremost I dedicate this thesis to my life partner Makhosini Dlamini who has supported, encouraged and loved me throughout and unconditionally. Your love kept me strong and enabled me to conquer anything including this difficult challenge.

Abstract

Pyruvate dehydrogenase kinase 4 (PDK4), a mammalian mitochondrial serine kinase has emerged as an interesting candidate for diabetes therapy. Due to the high prevalence of this disease especially type 2 diabetes (T2D) and the health complications associated with it, there is extensive effort to find the appropriate treatment. Understanding the regulation of PDK4 would therefore contribute significantly to the development of therapeutic agents.

This research outlines the bioinformatics analysis of PDK4, using tools such as Interweaver, ClustalW and Protein Structure Visualiser. These programs were used to determine potentially interacting partners for PDK4. Interweaver database identified 96 proteins which have possible interaction sites for PDK4. Protein p100/p49, containing a death domain that is known to have a role in suppressing apoptosis, was identified as a potential partner for PDK4. The alignment between p100/p49 primary sequence and that of PDK4 using Gene explorer/ClustalW demonstrated sequence similarity between the two proteins. Swiss PDB Viewer then located the positions of the amino acids that are in the hypothetical protein binding motif of p100/p49 within the 3D structure of hPDK4. These amino acids were found to be located in the region of PDK4 which is known to bind protein substrates of PDK4 and may be accessible to other proteins as well. These findings were very interesting as PDK4 have not previously been associated with apoptosis and this could be the link between apoptosis and insulin resistance.

Cell biology studies were then performed to verify the relationship between PDK4 and apoptosis. In this regard, HeLa and HepG2 cells were treated with apoptosis inducing agents such as TNF α , C2-Ceramide, and Linoleic acid. These cells were then monitored for apoptosis and PDK4 mRNA expression using a DNA laddering assay as well as Real Time PCR. The results showed that these factors to induce apoptosis in a concentration dependent manner. This induction of apoptosis by these three factors indicated to suppress PDK4 mRNA levels. These findings suggested a regulatory role for PDK4 in apoptosis.

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Abbreviations

ADP	Adenosine diphosphate
aPKC	atypical protein kinase C
α	Alpha
β	Beta
bp	base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
Ct	Threshold cycle
°C	Degrees celcius
DD	Death domain
DMEM	Dulbecco's Modification of Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid
FBS	Fetal bovine serum
FF	Full length
FOXO	Forkhead Box O factors
FS	Short length
G	Grams
Glut4	Glucose transporter 4
GR	Glucocorticoids receptor
GRE	Glucocorticoids response elements
GSK3	Glycogen synthase kinase 3

HCl	Hydrochloric acid
HeLa	Human epithelial cell cervical carcinoma
HepG2	Human hepatocellular liver carcinoma
hPDK	Human pyruvate dehydrogenase kinase
IKK	I κ B inhibitor kinase
IR	Insulin receptor
IRSs	Insulin receptor substrates
I κ B	Inhibitory kappa-B
K	Kappa
Kb	Kilo base
LA	Luria agar
LB	Luria broth
Mg	Milligrams
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NCBI	National centre for biotechnology information
NF κ B	Nuclear factor kappa-B
Ng	Nano grams
Ng	Nano grams
NIDDM	Non-insulin dependent diabetes
NLS	Nuclear localization sequence
%	Percentage

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PI3	Phosphatidylinositol 3
PPARs	Peroxisome proliferators-activated receptors
PPs	Peroxisome proliferators
PREs	PPAR response elements
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Real time polymerase chain reaction
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TFB	Standard transformation buffer
TNFR-1	Tumor necrosis factor receptor 1
TNF- α	Tumor necrosis factor – alpha
TPP	Thiamine pyrophosphate
Tris	Tris(hydroxymethyl)-aminomethane
V	Volts
γ	Gamma
δ	Delta
μ M	Micro molar

CHAPTER 1

1 Introduction

Diabetes also known as diabetes mellitus is a disease that has been known to exist for thousands of years and affects approximately 6% of the world population. It is a metabolic disorder characterised by a failure to maintain glucose homeostasis and this results in a variety of severe complications (Malecki, 2005 and Nir and Dor, 2005). These complications which includes blindness, stroke, and renal insufficiency may arise due to the disease being undiagnosed or poorly controlled (Malecki, 2005). Diabetes in most patients is brought about by a combination of genetic and environmental factors (Accili, D, 2000). There are two main types of diabetes namely type 1 (T1D) and type 2 diabetes. (T2D) T1D diabetics frequently have very low insulin levels and depend on insulin intake for survival. The onset of the disease usually occurs at a young age but also may occur at any age (Fatehi-Hassanabad and Chad, 2005). T1D which accounts for 5-10% of diabetic cases results from autoimmune-mediated destruction of pancreatic β cells (Accili, 2000, Behrooz and Bobby, 2007 and Fatehi-Hassanabad and Chad, 2005).

Type 2 diabetes mellitus (T2D), also known as non-insulin dependent diabetes (NIDDM) affects approximately between 2-6% of the adult population mostly in Western countries (Bailey, 2000). It manifests itself in middle or later stages of life and is growing rapidly worldwide (Korc, 2003). Approximately 85% of diabetic patients have T2D (Owen and Hattersley, 2001). Its chronic complications are a leading cause of death in many countries. T2D is caused by insulin resistance which results from the inability of tissues to respond to insulin (Goldstein, 2003; Metzler *et*

al., 2002; Withers *et al.*, 2000 and Florence and Yeager, 1999). Lack of tissue response causes continuous secretion of insulin by pancreatic β cells, which results in β cell failure and a subsequent inability to synthesize and secrete insulin, whose primary biological function is to regulate and maintain glucose homeostasis (Quon, 2001; Rasouli *et al.*, 2005; Kahn, 2003; Goldstein, 2003 and Kawasaki, *et al.*, 2004). Insulin resistance is normally caused by obesity and stress and is frequently associated with a number of diseases including chronic infection and hypertension (Korc, 2003; Bavenholm *et al.*, 2003; Hunter and Garvey, 1998; Baumann and Saltiel, 2001; Banadonna *et al.*, 1990). The resistance results from mutations or post translational modification of the insulin receptor or any of its downstream effector molecules (Le Roith and Zick, 2001 and Storz *et al.*, 1999).

Due to high prevalence of T2D and its health complications, there is intensive effort to find appropriate treatment. Pyruvate dehydrogenase kinase 4 (PDK4) has emerged as an interesting candidate in diabetes therapy (Sugden and Holness, 2002). Understanding its regulation would contribute meaningfully to this effort. We therefore sought to investigate the role of protein-protein interaction in the regulation of PDK4 activity.

1.1 Physiological role of PDK4

PDK4 is a member of a family of mitochondrial serine kinases that are more related to bacterial histidine kinases than eukaryotic serine kinases (Roche *et al.*, 2001 and Gudi *et al.*, 1995). All members of this family contain conserved motifs that form the kinase domain (Rowles *et al.*, 1996). These kinases are involved in glucose clearance

by regulating the activity of pyruvate dehydrogenase complex (PDC) (Randle, 1986 Patel and Roche, 1990 and Sugden and Holness, 2003). PDC is a multi-subunit enzyme composed of multiple copies of three catalytic enzymes pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) (Korotchkina and Patel, 2001). The E1 component is a heterotetramer composing of two α and two β which requires a thiamine pyrophosphate for its functioning (Korotchkina and Patel, 2001). Its α -subunit has three phosphorylation sites, site 1 (Ser-264), site 2 (Ser-271) and site 3 (Ser-203) (Korotchkina and Patel, 1995). The primary role of PDC is to catalyze the oxidative decarboxylation of pyruvate to form acetyl-CoA and to reduce NAD^+ to NADH, a rate limiting step in glucose metabolism (Kolobova *et al.*, 2001). The reaction catalysed by PDC is depicted in the schematic diagram below in figure 1:

Pyruvate dehydrogenase kinases (PDKs) inactivate PDC by phosphorylation of the serine residues on the E1 α -subunit of PDC (Yeaman *et al.*, 1978 and Korotchkina and Patel, 2001). Currently, four PDK isoforms have been identified in humans, PDK1, 2, 3 and 4 (Bowker-Kinley *et al.*, 1998). These isoforms are known to perform similar functions due to strong conservation of their primary structure even though they are expressed differently in different cell types (Bower-Kinley *et al.*, 1998 and Popov, 1993).

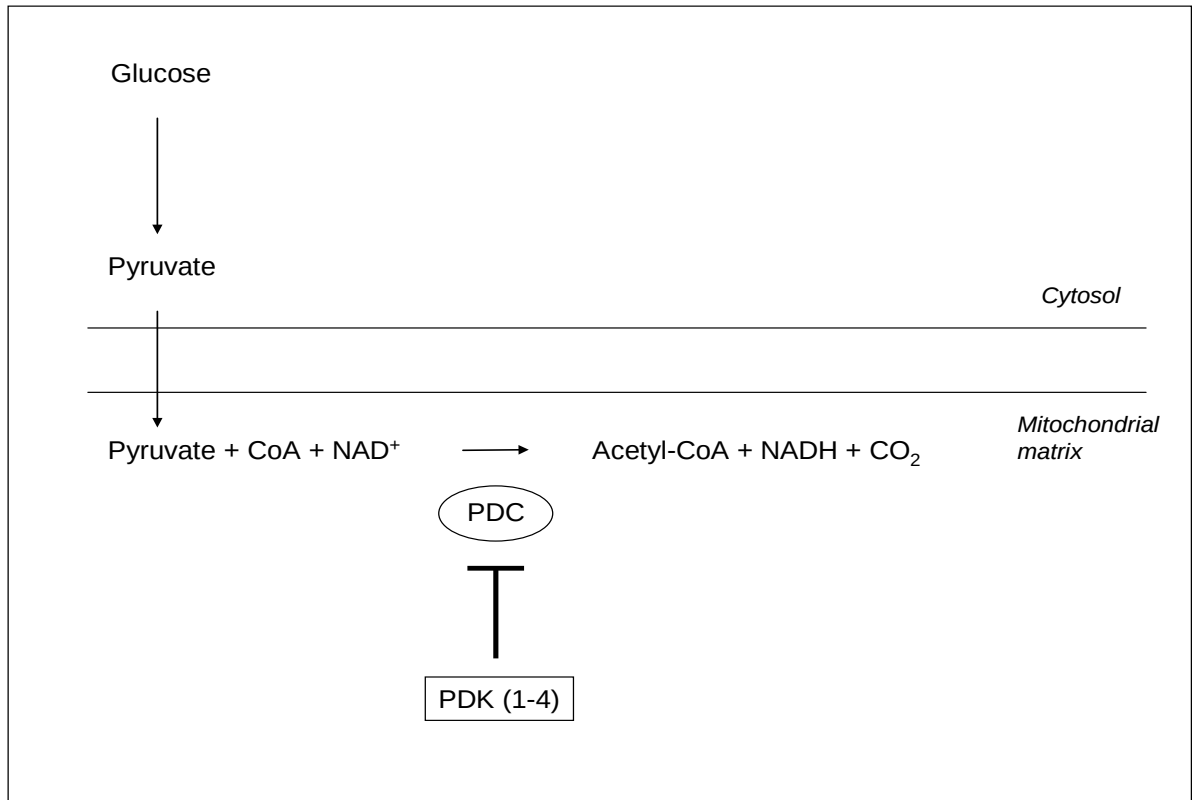


Figure 1. A schematic diagram showing the regulation of PDC by PDKs. PDC catalyses the oxidative decarboxylation of pyruvate to acetyl CoA and reduces NAD^+ to NADH. PDC activity is inhibited by PDKs through phosphorylation (as indicated by a bar).

1.2 Regulation of PDK4

The activity of PDK4 is known to be regulated by short-term (Wieland and Siess, 1971) and long-term (Dennis *et al.*, 1978) mechanisms. In the short-term, the activity of the PDK enzyme is modified by factors that bind to the PDK protein, whereas, the long-term regulation of PDK is achieved by controlling PDK gene expression.

1.2.1 Regulation of PDK enzyme activity

The short-term regulation of PDK has been studied primarily in rat PDK2 which has very high structural similarity to PDK4. These studies revealed that substrates and products of the PDC action regulate PDK activity via allosteric mechanisms (Pratt and Roche, 1979). For example, PDC substrates which include pyruvate, NAD^+ and coenzyme A (CoA) were shown to inhibit PDK activity (Sugden and Holness, 2002 and Kolobova *et al.*, 2001), and the PDC products such as NADH and acetyl-CoA were shown to induce PDK activity as illustrated in figure 2 (Kerbey *et al.*, 1979).

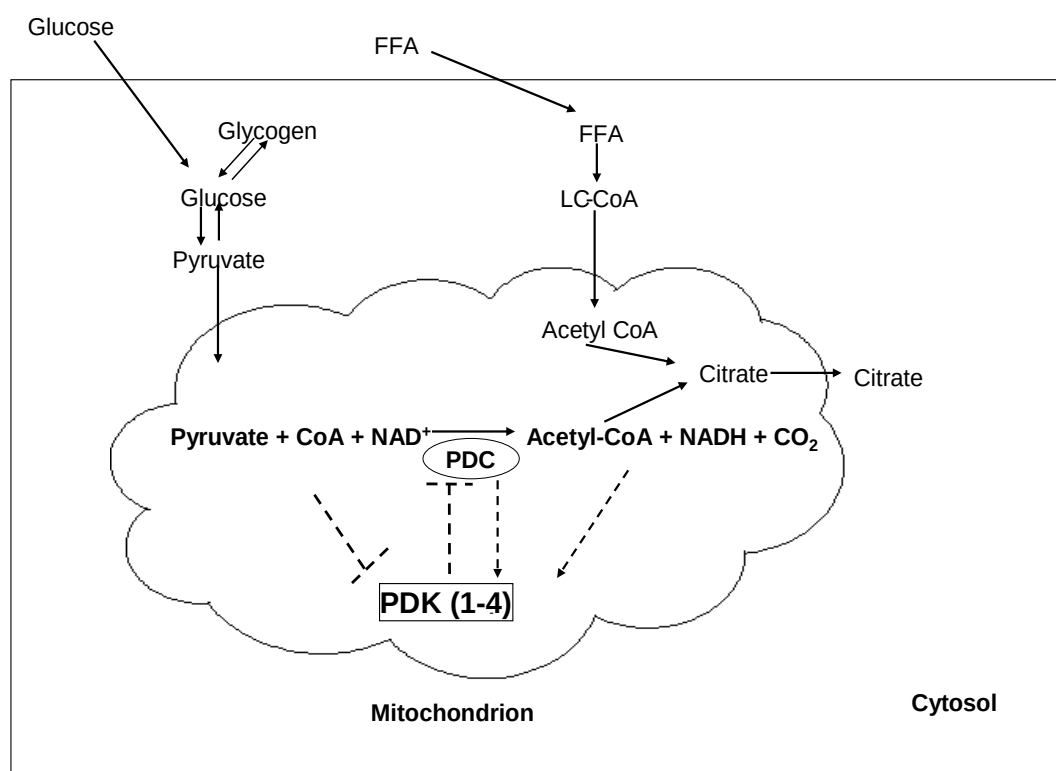


Figure 2. Regulation of PDK enzyme activity. Solid arrows indicate biochemical pathways, dashed arrows indicate positive regulation of enzyme activity, and the dashed bars indicate suppression of enzyme activity.

Fatty acid metabolism is known to increase PDK activity, presumably through production of acetyl-CoA (figure 2, Holness *et al.*, 1989). Further, the interaction between PDC and PDK has also been shown to activate PDK (Korotchkina and Patel, 2001).

1.2.2 Regulation of PDK gene expression

Long-term regulation of PDK is effected by nutritional and hormonal states (Wu *et al.*, 1999; Huang *et al.*, 1998 and Sugden *et al.*, 1998), which regulate PDK activity by controlling PDK gene expression. The primary PDK isoform whose expression is affected by these factors is PDK4. Levels of PDK4 expression are elevated during starvation, high-fat feeding and diabetes (Wu *et al.*, 1999, Wu *et al.*, 1998 and Kwon and Harris, 2004). Hormones such as glucocorticoids have also been shown to induce PDK4 expression (Huang *et al.*, 2002). In addition, it has been shown that insulin regulates the expression of PDK4 mRNA levels by suppressing it (Huang *et al.*, 2002 and Lee *et al.*, 2004). Furthermore PDK4 gene expression has also been shown to be regulated by thyroid hormone, fatty acids, TNF α and ceramide (Kwon and Harris, 2004). Recent studies have shown that estrogen related receptors (ERRs) also induce PDK4 gene expression in hepatic cells (Zhang *et al.*, 2006). These ERRs bind to the PDK4 gene promoter therefore stimulating PDK4 gene expression. Factors that mediate PDK4 gene expression are known to activate several signal transduction pathways which are discussed below.

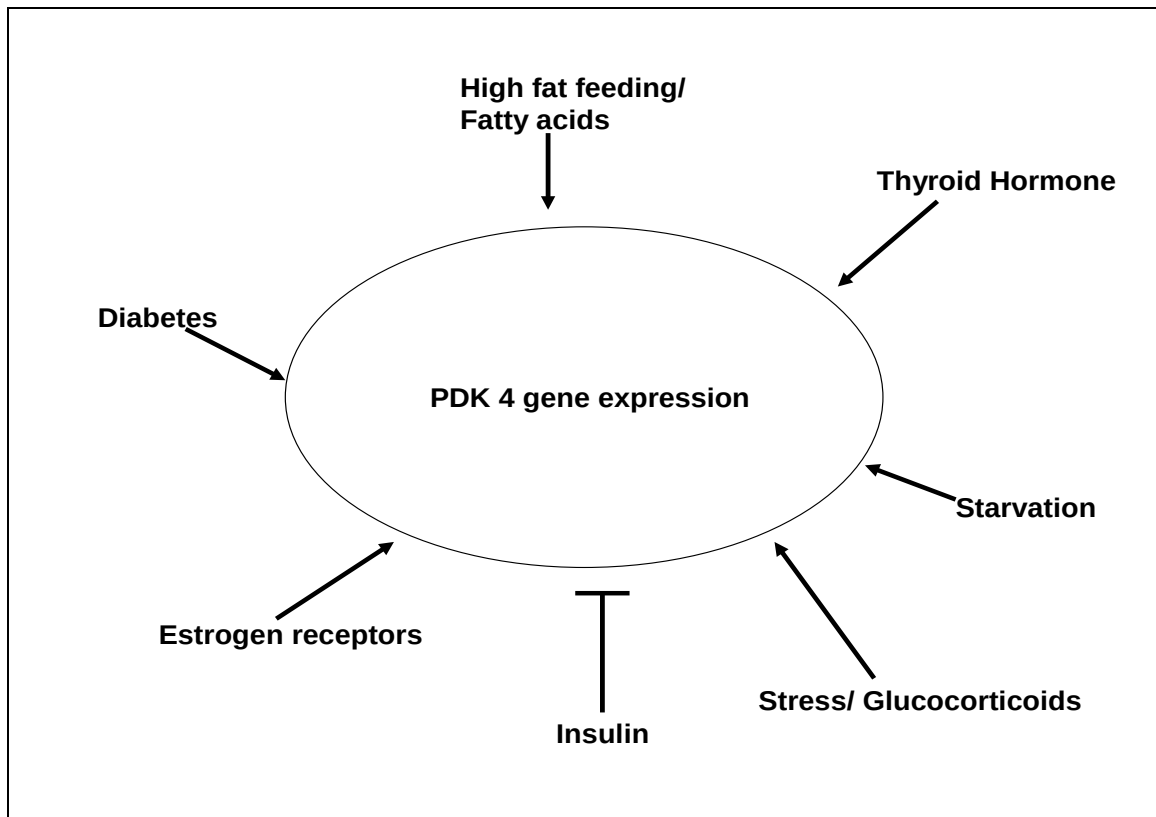


Figure 3. Regulation of PDK4 gene expression. Arrows indicate factors that induce PDK4 gene expression, and the solid bar indicates factors that suppress PDK4 gene expression.

1.2.2.1 Signalling pathways activated by insulin

Insulin plays a prominent role in the regulation of the metabolic clearance of glucose by utilizing the phosphatidylinositol 3 (PI3) kinase signalling pathway (Cheng *et al.*, 2002 and Baumann and Saltiel 2001), Figure 3). Binding of insulin hormone to the insulin receptor (IR) is necessary for initiation of this signalling (Formisano and Benguinot, 2001, White, 1997 and Kaburagi *et al.*, 1993).

The IR is a large transmembrane glycoprotein consisting of two α and β subunits which form a heterotetramer (Le Roith and Zick, 2001, Hunter and Garvey, 1998 and

Kahn and Goldfine, 1993). The α subunits are entirely extracellular and have been shown to interact directly with the ligand, whereas the β subunits are transmembrane proteins and have tyrosine kinase activity on the intracellular domains (Hunter and Garvey, 1998; Kahn and Goldfine, 1993 and Le Roith and Zick, 2001). Ligand binding to the extracellular α subunit induces conformational changes in the β subunits which result in activation of tyrosine kinase activity in the intracellular domain (Cheng *et al.*, 2002 and Whitehead *et al.*, 2000). This results in tyrosine phosphorylation of insulin receptor substrates (IRSs) (Whitehead *et al.*, 2000; White, 1997; Kahn and Goldfine, 1993; and Frattali *et al.*, 1992) and activation of the PI3 kinase pathway (Duan *et al.*, 2004; Egawa *et al.*, 1999 and Kahn and Goldfine, 1993).

The PI3 kinase pathway regulates many of the enzymes that are utilized by insulin to stimulate the metabolic clearance of glucose. For example, activation of atypical protein kinase C (α PKC) by insulin through PI3 kinase stimulates glucose uptake by promoting the translocation of glucose transporter 4 (Glut 4) to the cell membrane (Whitehead, *et al.*, 2000; Formisano and Beguinot, 2001 and Duan, *et al.*, 2004). PI3 kinase also promotes the conversion of glucose to glycogen by inhibiting glycogen synthase kinase3 (GSK3) (Pessin *et al.*, 1999; Clark *et al.*, 1998 and Uchida *et al.*, 2000). GSK3 is a physiologic inhibitor of glycogen synthase (Cross *et al.*, 1995 and Pap and Cooper, 1998). The PI3 kinase dependent inhibition of GSK3 is mediated by Akt (Hunter and Garvey, 1998).

These observations are consistent with those reported by Cross *et al.*, (1995), which demonstrated that Akt activation resulted in phosphorylation and inactivation of GSK-3 which resulted in of glycogen synthase activity.

The PI3 kinase pathway also promotes metabolic utilization of pyruvate by inhibiting the expression of PDK4 (Kwon, *et al.*, 2004; Furuyama, *et al.*, 2003; Egawa, *et al.*, 1999), the inhibitor of pyruvate dehydrogenase complex (Yeaman *et al.*, 1978 and Korotchkina and Patel, 1995). This metabolic effect of PI3 kinase is also mediated by Akt, whose activation has been shown to lead to the phosphorylation of transcriptional factors known as Forkhead Box O factors (FOXO). The FOXO group consists of FOXO1a, FOXO3a and FOXO4 which are transcription factors that act downstream of Akt (Kwon *et al.*, 2004). Two of these factors, FOXO1a and FOXO3a have been shown to bind on hPDK4 promoter through the insulin response elements (Kwon *et al.*, 2004). Binding of these transcriptional factors to the promoter activates hPDK4 gene expression. However, insulin action results in activation and phosphorylation of Akt, which leads to translocation of Akt from the cytoplasm to the nucleus where Akt phosphorylates FOXO transcription factors (Van der Heide and Smidt, 2005). The phosphorylation of FOXO results in reduction of DNA binding ability and FOXO translocation from the nucleus to the cytoplasm. This results in the inactivation of PDK4 gene expression (Kwon and Harris, 2004).

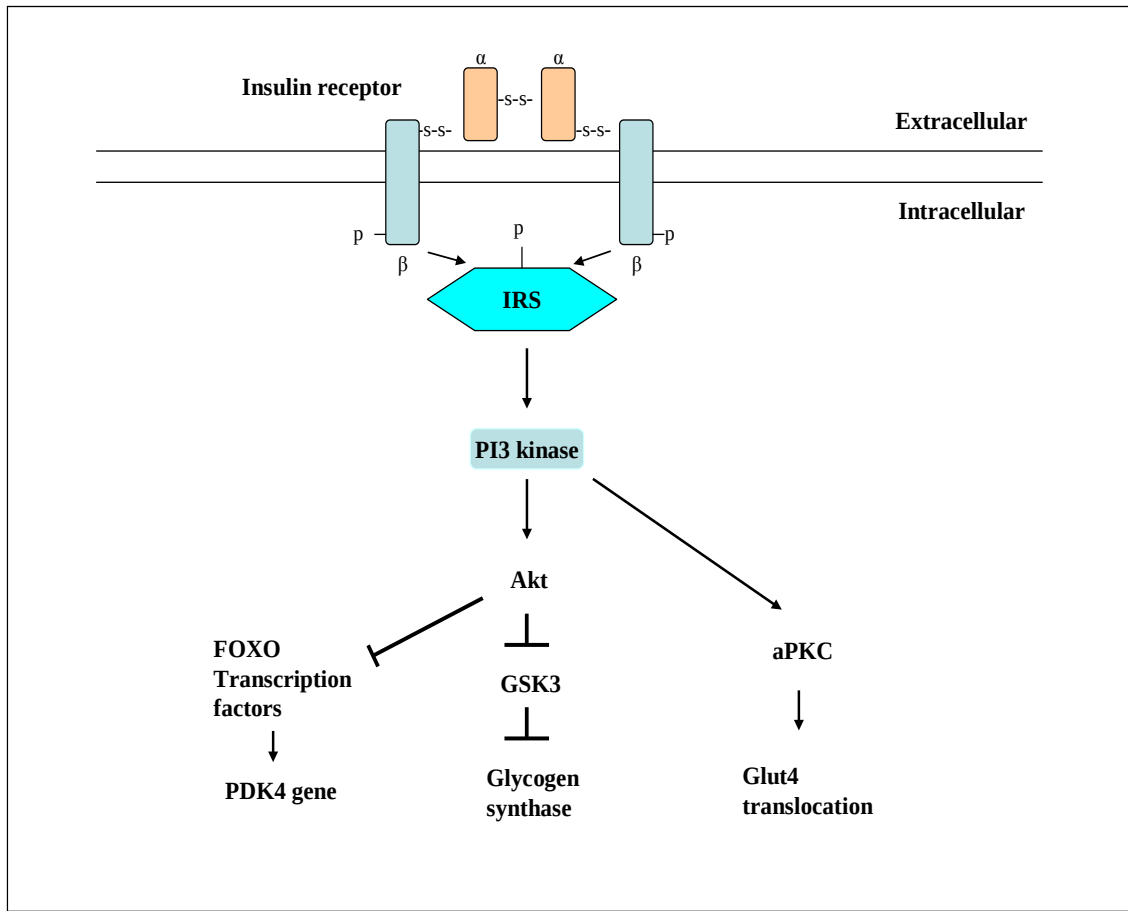


Figure 4. The mechanisms involved in the metabolic signalling pathway initiated by insulin action. IRS-insulin receptor substrate, GLUT4- glucose transport protein 4; GSK3- glycogen synthase kinase 3; PI3 kinase-phosphatidylinositol 3 kinase; aPKC – atypical protein kinase C; FOXO – Forkhead box factors.

1.2.2.2 Signalling pathways activated by Fatty acids

Fatty acid (FA) signalling has been the subject of interest for many years and it has been revolutionized by the discovery of a novel steroid hormone receptor that can be activated by both FAs and fatty acid-like molecules called peroxisome proliferators (PPs) (Issemann and Green, 1990). These receptors are known as peroxisome proliferators-activated receptors (PPARs). PPARs are recently classified into

subfamilies which include PPAR α , PPAR β and PPAR γ (Braissant, 1996), and these are expressed in different cell types (Braissant, 1996). The PPARs are involved in the control of gene expression by interacting with specific DNA response elements (PPAR response elements; PPREs) located upstream of PPAR target genes (Tugwood *et al.*, 1992). The PPARs, which are normally localized in the cytoplasm, are activated by binding of PPs or fatty acids, which causes translocation of PPAR to the nucleus and induces conformational changes that enable the PPARs to bind to the PREs (**Figure 5**). The common examples of fatty acids that bind to the PPARs and induce signalling are lenoleic acid, oleic acid, arachidonic acid and prostaglandin J2 (Heuvel, 1999, Huang *et al.*, 2002, and Holness *et al.*, 2002). The common examples of PPs used regularly are Wy14,643 and clofibric acid (Issemann and Green, 1990 and Dreyer *et al.*, 1992).

Several studies have shown that PPAR α and γ induce PDK4 gene expression (Holness *et al.*, 2002; Huang *et al.*, 2002; Sugden *et al.*, 2001 and Wu *et al.*, 2001). In these studies, the PPAR receptors were activated by Wy14,643, lenoleic acid, and oleic acid (Holness *et al.*, 2002).

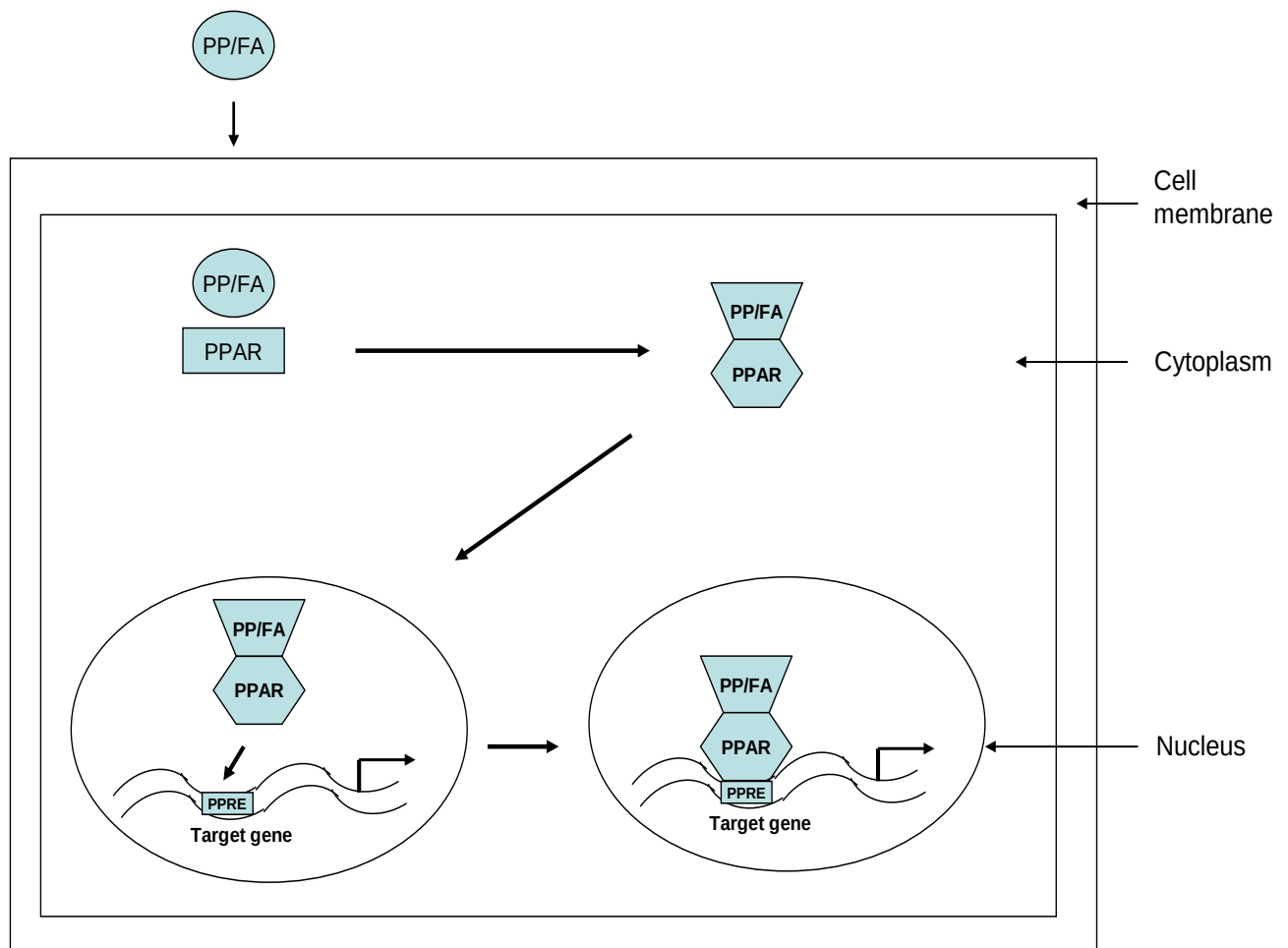


Figure 5. Signalling thorough fatty acids (FAs) and peroxisome proliferators (PPs). Binding of PP or FA to the peroxisome proliferators-activated receptors (PPAR) leads to conformational change that result in the translocation of the complex to the nucleus. The complex then binds to the peroxisome proliferator response elements (PPRE) and activates the target gene.

PPARs are also able to regulate gene expression independently of binding to DNA via receptor-dependent *trans*-repression. This mechanism involves a physical interaction of PPAR α with the nuclear factor kappa B (NF κ B) (Planavila *et al.*, 2005). NF κ B is present in the cytoplasm as an inactive heterodimer consisting of p50 and p65 subunits. However after activation the heterodimer translocates to the nucleus and interact with PPAR β/δ . This association prevents PPAR β/δ from binding to its response element and thereby inhibits its ability to induce gene transcription leading to a reduction in the expression of PDK4 (Planavila *et al.*, 2005).

1.2.2.3 Signalling pathway activated by Glucocorticoids

Glucocorticoids have been shown to be involved in PDK4 gene regulation. This was demonstrated by the discovery of the glucocorticoids response elements (GRE) located within the promoter region of the PDK4 promoter (Kwon *et al.*, 2004). This region has been shown to be utilised by other corticosteroids as well such as dexamethasone. Both glucocorticoids and dexamethasone induce PDK4 gene expression through the GRE (Kwon and Harris, 2004). Activation of the glucocorticoids receptor (GR) through binding by glucocorticoids translocates into the nucleus where it forms a homodimer and binds to the GRE thus induce PDK4 gene expression (Kwon *et al.*, 2004). GR activation of target genes depends on cooperative interaction with p300/CBP to be able to activate its target genes (Van der Heide and Smidt, 2005). P300/CBP is believed to have multiple surfaces that enable cooperative interactions with other corticosteroids as well which includes dexamethasone and FOXO factors (Kwon *et al.*, 2004).

The presence of p300/CBP in any complex may promote the rate of formation of a stable transcriptional initiation complex and thereby enhance the rate of PDK4 transcription.

The presence of the three IRSs near to the GRE may interfere with the interaction between p300/CBP complex and the general transcriptional machinery (Kwon and Harris, 2004). In the presence of insulin FOXO factors are inhibited from binding to the IRSs thereby decreasing the rate of formation of a stable initiation complex. This result in the suppression of the glucocorticoids response thus leads to inhibition of PDK4 gene expression (Kwon *et al.*, 2004).

1.3 Regulation of PDK4 by protein-protein interaction

Protein-protein interactions are essential in cellular events such as protein and vesicle trafficking, cell cycle, apoptosis, gene expression, DNA repair, control of the cytoskeleton and targeted protein degradation (Pawson and Nash 2003). Protein-protein interaction also plays a role in signal transduction. As part of our aim to understand the regulation of PDK4, we explored how PDK4 is regulated by protein-protein interaction. Currently, the understanding of regulation of PDK4 by protein-protein interaction is limited to what is known about its interaction with PDC (Popov *et al.*, 1993). In this instance, the interaction of PDK4 with the lipoyl domain of PDC results in the activation of PDK4 (Bowker-Kinley *et al.*, 1999).

We wondered whether there are other proteins which affect the activity of PDK4 by binding PDK4. We therefore used bioinformatics tools to uncover possible interacting partners for PDK4 in order to further explore the role of protein-protein interactions in PDK4 activity. We identified death domain (DD) containing proteins such as nuclear factor kappa B (NFκB) subunit p100/p49 (referred to as p100), as potential interacting partners for PDK4. These proteins are known to be involved in apoptosis and inhibit NFκB by binding it and therefore retaining it in the cytoplasm (Ghosh and Karin, 2002).

1.4 The role of NFκB and p100 in apoptosis

1.4.1 Signal transduction pathways involved in induction of apoptosis

Apoptosis also known as programmed cell death is an important cellular process that is used by organisms to eliminate unwanted or damaged cells (Desaghnier and Martinou, 2000; Kannan and Jain, 2000; Dlamini *et al.*, 2004). Failure in proper regulation of apoptosis can lead to various genetic diseases such as neurodegenerative disorders, immune deficiency and cancer (Nur-E-Kamal *et al.*, 2004). There are two major pathways that lead to apoptosis and are namely extrinsic (type I) and intrinsic (type II). These two pathways are induced by different factors (Bishopric *et al.*, 2001 and Dlamini *et al.*, 2004).

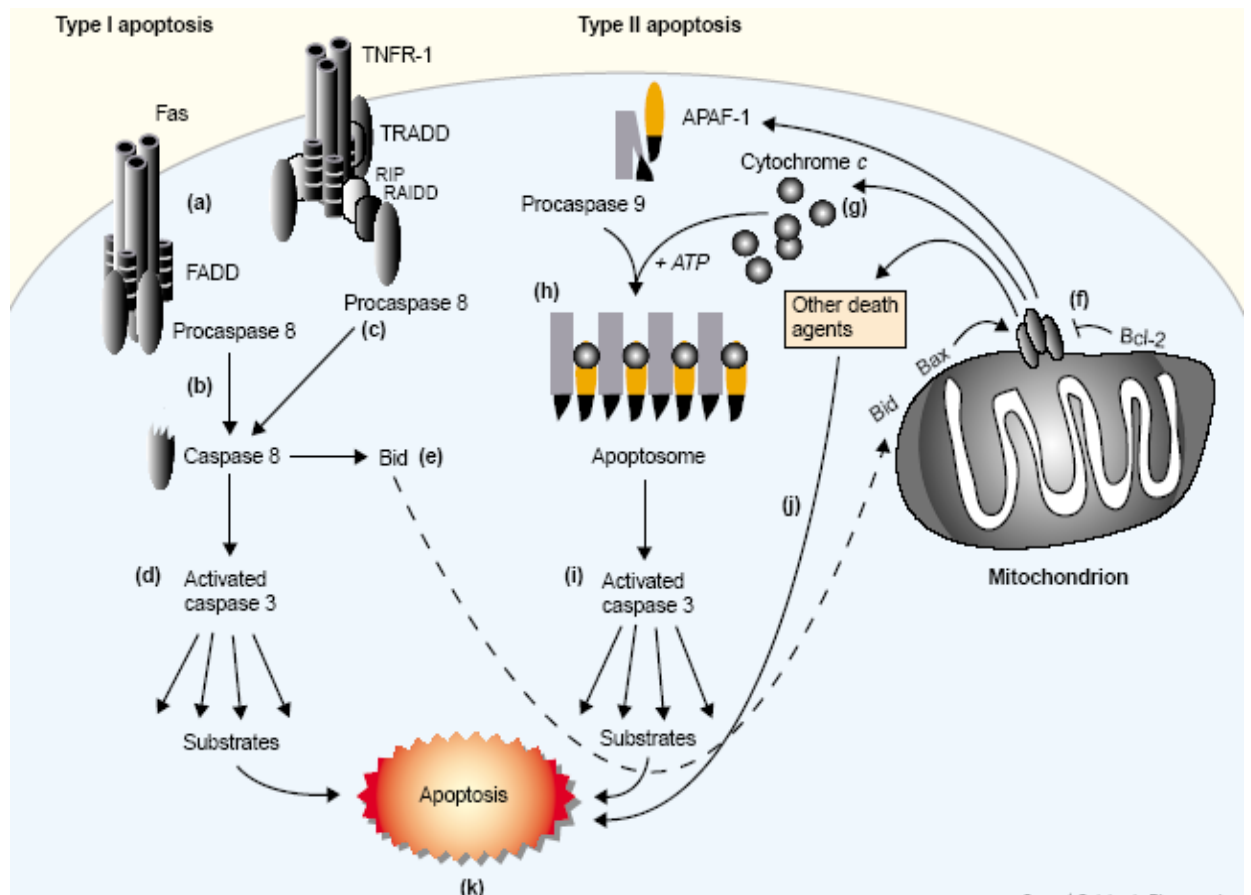


Figure 6. The schematic diagram showing the two main apoptotic pathways. The type I (extrinsic) involves death receptors such as Fas and TNFR1. (a)-(k) Induction of apoptosis by binding of Fas and TNFR1 ligand to the receptors and activation of cascade leading to apoptosis. Type II (intrinsic) pathway involves the release of cytochrome c by the mitochondrion (Adapted from Bishopric *et al.*, 2001).

The type I pathway is mediated by external factors that binds to the transmembrane receptors containing death domain (Bishopric *et al.*, 2001) for example Fas (also known APO-1/CD95) and (tumor necrosis factor- α receptor-1) TNFR1. Binding by Fas ligand and tumor necrosis factor- α (TNF α) induces aggregation of other factors at the receptor which leads to the cleavage and activation of caspase 8 from procaspase which in turn activate caspase 3 (figure 6). Type II pathway is dependent on the release of cytochrome c by the mitochondrion (figure 6). Cytochrome c then forms a

complex with procaspase 9 and its cofactor apoptotic protease activating factor-1 (APAF-1). Sufficient ATP is needed for caspase 9 to undergo conformational change which produces active apoptosome which further activates caspase 3 which results in apoptosis (Bishopric *et al.*, 2001).

1.4.2 NF κ B and the death receptor pathway

One of the two main signalling pathways, the death receptor pathway, is of interest as it involves DD containing proteins (Schlessinger and Ullrich, 1992). This pathway is induced by binding of DD containing ligands such as Fas and tumor necrosis factor α (TNF α) and these lead to the activation of the caspase cascade (Schlessinger & Ullrich, 1992). Studies have shown that binding of TNF α to TNF α receptor 1 (TNFR-1) induces nuclear factor kappa B (NF κ B) activation. NF κ B is a DNA binding protein that is involved in the activation of genes encoding proteins involved in apoptosis (Kim *et al.*, 2005). NF κ B is a member of the Rel/NF κ B protein family of transcription factors that regulate many cellular processes including apoptosis, immune and inflammatory responses, and cell growth (Liang *et al.*, 2006, Anaranda and Prefontaine, 1994, Huang *et al.*, 1997 and McKay and Cidlowski, 1999). These possess similar structural elements which include a highly conserved N-terminal Rel homology domain that contains a DNA binding sub-domain, a dimerization sub-domain; and a nuclear localization signal as shown in figure 7 (Anaranda and Prefontaine, 1994 and Bours *et al.*, 1990). The C-termini of these proteins separate the family into two classes. p65 (RelA), RelB, and c-Rel belong to class I which is characterized by the presence of a transactivating domain in the C-terminus which confers the ability to activate transcription (Blank *et al.*, 1991 and Kunsch *et al.*, 1992).

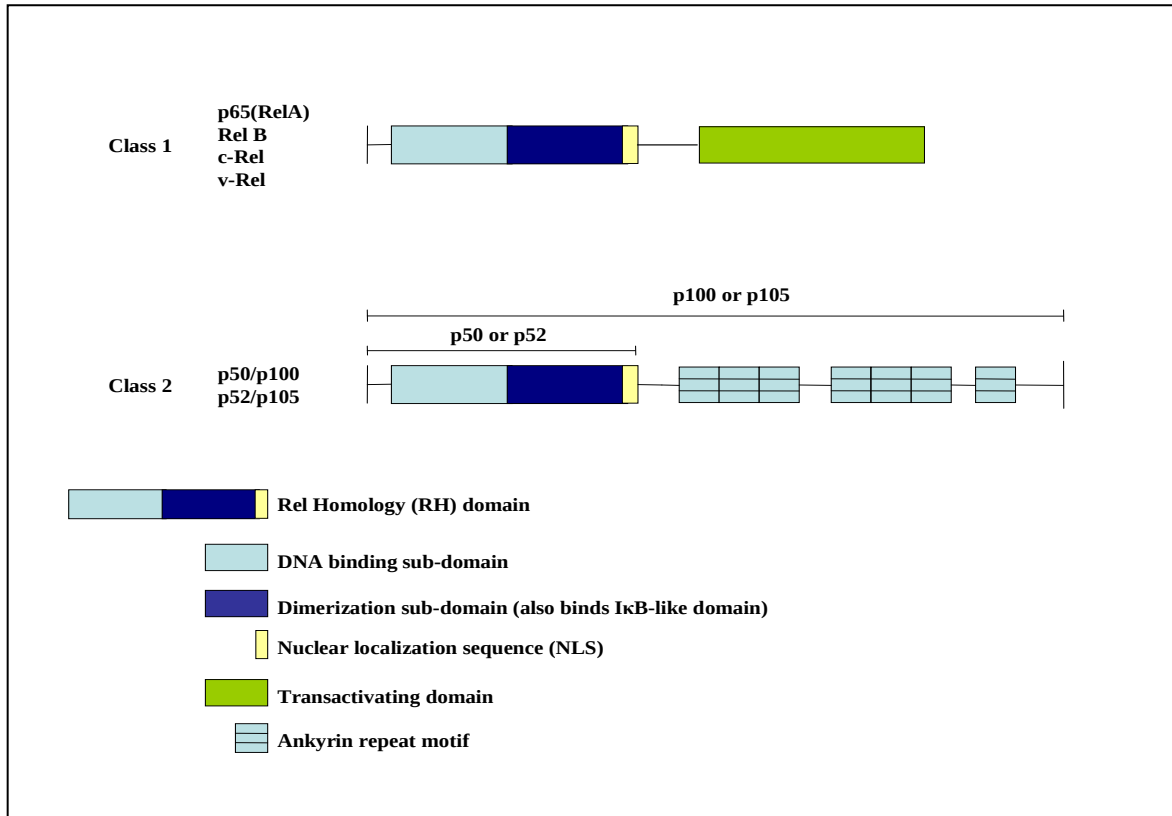


Figure 7. Schematic diagram depicting structural elements of the Rel/NFκB protein family members.

Class 2 of NFκB transcription factors include p100 and p105 and are characterized by long C-termini which are composed of multiple copies of 30-33 amino acid ankyrin repeats figures 7 & 8 (Scheinman *et al.*, and Binizzi *et al.*, 2004). The ankyrin repeats are believed to be responsible for the cytoplasmic retention of the NFκB subunits and therefore inhibition of NFκB activity (Anuranda and Prefontaine, 1994 and Bours *et al.*, 1990, Scheinman *et al.*, 1993 and Bonizzi *et al.*, 2004). Consistent with this role, the C-terminal domain of Class 2 NFκB transcription factors is structurally similar to proteins termed inhibitors of NFκB (IκB's) that are known to interact with Class 1 NFκB transcription factors, thus inhibiting their activity. The Class 2 NFκB transcription factors are activated through proteolytic cleavage which results in the

generation of p50 and p52 from the N-terminus, as well as I κ B- γ from the C-terminus (figures 7 & 8, Grimm and Baeuerle, 1995 and Liou H-C, 2002). p49, a protein that is highly related to p50 and functions in a similar manner to p50 is also derived from the p100 gene through alternative splicing (Schmid *et al.*, 1991). Hence, p100 is also sometimes referred to as the p100/49. However, p49 has not been as extensively studied as its p50 relative.

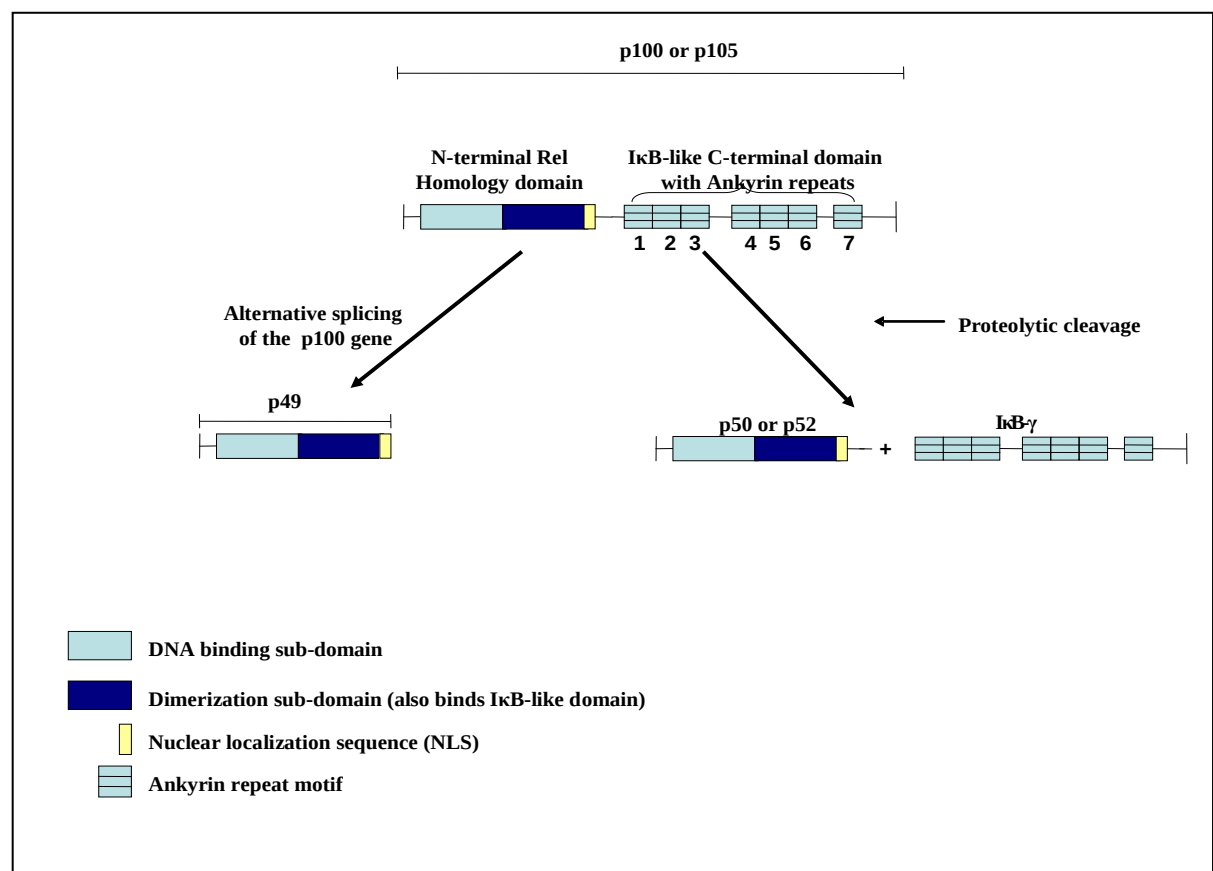


Figure 8. Schematic overview of the structure and generation of Class 2 NF κ B subunits.

The NF κ B subunits can interact with one another to form a diversity of homo- or hetero-dimers, which enables NF κ B transcription factors to regulate distinct, but overlapping sets of genes (Liou H-C, 2002, Grimm and Baeuerle, 1993 and McKay

and Cidlowski, 1999). The most abundant NFκB complex in most cells is the p50-p65 hetero-dimer (Ganchi *et al.*, 1993 and Duckett *et al.*, 1993). This binding occurs via interaction between the dimerization sub-domains of the NFκB subunits, resulting in the formation of an active NFκB heterodimer (Duckett *et al.*, 1993 and Huang *et al.*, 1997).

1.4.3 Signal transduction through NFκB

In the classical signalling pathway, the NFκB dimer is normally found in an inactive form bound to its inhibitory proteins IκBs (Ghosh *et al.*, 1998 and Gosh and Budde, 2000). Activation of signal transduction by external stimuli result in activation of the IκB kinases (IKKs) on two conserved serine residues (Bonnet *et al.*, 2006), which then results in phosphorylation of the IκBs thus leading to the release of dimeric NFκB and its translocation to the nucleus, where it activates gene transcription (Orange and Geha, 2003; Vestergaard *et al.*, 2005 and Gosh and Karin, 2002) shown below on figure 9. The phosphorylation of IκB also results in its ubiquitinylation and proteosomal degradation (Liang *et al.*, 2006, Bonizzi *et al.*, 2004 and Iwai *et al.*, 2005). Regulation of the interaction between IκB and NFκB complexes is central to regulation of NFκB activity.

In the non-classical pathway, p100 or p105 is complexed with any of the Class 1 (Rel) subunits (e.g. p65). Phosphorylation of the C-terminal IκB-like domain by IKK results in the ubiquitinylation and degradation of the C-terminal IκB-like domain, which generates an active p50/p52-p65 NFκB dimer (Duckett *et al.*, 1993, Ray and Prefontaine, 1994 and Blank *et al.*, 1991).

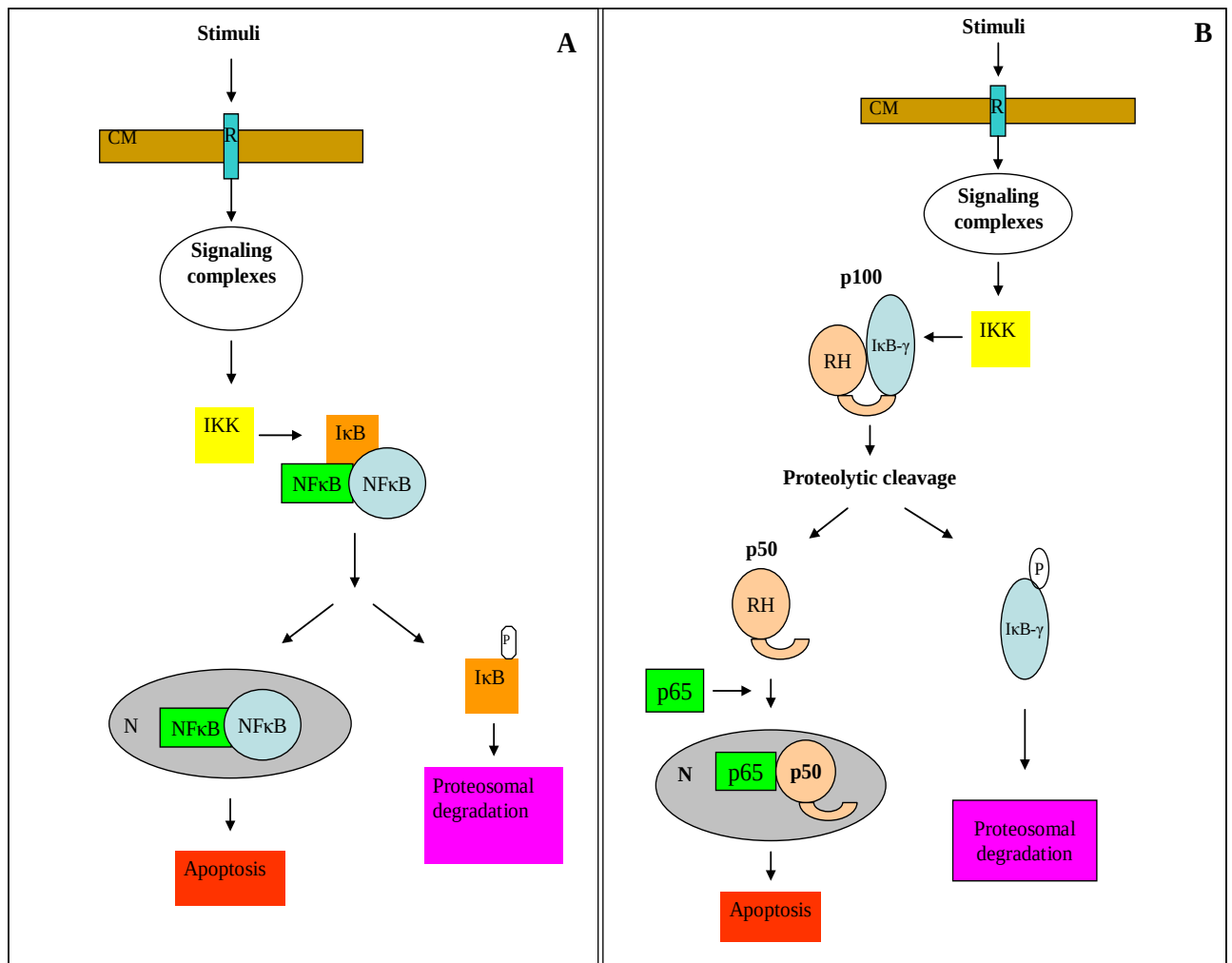


Figure 9. The schematic diagram indicating the role of IκBs in NFκB signalling pathway and apoptosis. (A) The classical and (B) The non-classical NFκB signalling pathways. CM stands for cell membrane, R stands for receptor and N stands for nucleus. RH stands for Rel homology domain and P stands for the phosphate group.

1.5 NFκB and PDK4: Roles in apoptosis and Type II diabetes

The role of NFκB in apoptosis has been extensively demonstrated in several studies (Lui *et al.*, 1996, Karin and Lin, 2002, Ghosh and Karin, 2002 and Kim *et al.*, 2005)

where they showed that inhibition of NF κ B protects cells from apoptosis and that overexpression of NF κ B results in induction of apoptosis. Another interesting function of NF κ B was demonstrated by Chen (2005), where he indicated a role for NF κ B in insulin resistance and diabetes. Since the role of PDK4 in insulin resistance and diabetes has been extensively demonstrated (Huang *et al.*, 2002 and Majer *et al.*, 1998), and the bioinformatics analysis that we performed suggested possible interaction between PDK4 and I κ B-like p100, we became interested in exploring the relationship between PDK4 and apoptosis.

In addition to the work by Chen *et al* (2005), several studies have also suggested a link between apoptosis and diabetes. For example, studies by Mandrup-Polsen *et al* (2003) demonstrated that apoptosis is important in pancreatic β cell failure that accompanies T2D. Further, glycogen synthase kinase-3 (GSK3) an enzyme that has been shown to be important in diabetes was reported to induce apoptosis when over expressed in mammalian cells (Pap and Cooper, 1998 and Cross *et al.*, 1995). In addition, insulin, a hormone that protects against diabetes and regulates expression of PDK4, is known to prevent apoptosis by activating the phosphatidylinositol kinase 3 pathway (Whitehead *et al.*, 2000; Baumann and Saltiel, 2001). All these factors led us to the hypothesis that overexpression of PDK4 may play a role in induction of apoptosis. Since our bioinformatics analysis suggested an interaction between PDK4 and I κ B-like p100, we postulated that PDK4 may compete with other NF κ B subunits for the binding site in I κ B. This may enable the formation of functional NF κ B dimers that are able to translocate to the nucleus and activate pro-apoptotic genes.

1.6 Objectives of the study

Pyruvate dehydrogenase kinase 4 (PDK4) has emerged as an interesting candidate in diabetes therapy. Due to high increase in diabetes complications and death numbers we aim at understanding PDK4 regulation by other proteins. The study will assist in determining whether targeting the interaction of PDK4 with other proteins can be used in the treatment of diabetes or other physiological complications that involve PDK4.

Our main objective was to study regulation of PDK4 through protein-protein interactions. This involved the use of bioinformatics tools to reveal proteins that potentially interact with PDK4 through protein-protein interactions. Bioinformatics was also used to identify the protein binding consensus sequence of the identified proteins in human PDK4 (hPDK4) protein sequence. Furthermore, the potential protein binding region on hPDK4 structure was also identified. Studies were also performed in mammalian cell culture to begin to uncover the relationship between PDK4 and its identified interacting partners.

CHAPTER 2

2 Materials and Methods

To begin our study we sought to use bioinformatics tools to determine if there are any proteins that may interact with PDK4. Since we are interested in the regulation of PDK4 by other proteins especially that may interact with it through protein-protein interaction, bioinformatics analysis was applied.

2.1 Bioinformatics analysis

Bioinformatics tools were used to analyse potential protein-protein interactions between PDK4 and other proteins.

2.1.1 Analysis of protein-protein interaction

The interweaver database (<http://interweaver.i2r.a-star.edu.sg/>) was used to identify proteins that would potentially interact with hPDK4 (Zhang and Ng, 2004). This tool uses a domain-based approach to search for domain-domain interactions (Ng *et al.*, 2003) in the database by using computationally derived domain fusion events (Marcotte *et al.*, 1999). These allow identification of proteins with domains that putatively interact with a domain in the source protein in this case hPDK4. The national centre for biotechnology information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>) was used to retrieve the hPDK4 amino acid sequence and convert it to a FASTA format. This was then used in the interweaver instrument to search proteins with domains that can potentially interact with hPDK4.

2.1.2 Consensus sequence identification

The primary sequence of hPDK4 was aligned with the amino acid sequences of NFκB subunits that are known to be important in dimerization. For this purpose, a sequence alignment program (ClustalW) was used. This program was also used to explore whether these amino acids are also conserved in the other PDK isoforms from different species.

2.1.3 Structural localization of the hypothetical protein binding region in hPDK4

2.1.3.1 Modelling of hPDK4

The structure of hPDK4 was modelled based on the coordinates of the published structure of rat PDK2 (1JM6 Pdb code, Steussy *et al.*, 2001). Briefly, the primary protein sequence of hPDK4 was aligned with that of rat PDK2 using Clustal W, version 1.83 (Pearson, 1990). A molecular graphics program (Swiss Pdb Viewer, Guex, N, 1996) was then used to build the hPDK4 structure. The quality of the modelled structure was verified using the WHATIF program (Vriend, 1990) and Rachamandran Plot.

2.1.3.2 Localization of the hypothetical protein binding region in hPDK4 structure

Swiss-Pdb Viewer was used to visualize the 3-dimensional (3D) structure of the modelled hPDK4. The positions of the amino acids that are conserved between

hPDK4 and NF κ B subunits were located and visualized within the 3D structure of hPDK4.

2.2 Cellular and Molecular Analysis

To further understand the relationship between PDK4 and apoptosis at the molecular level cell culture studies were performed. Cell culturing has become a very useful and powerful technique in biological research. These studies involved the usage of HeLa, a cervical carcinoma cell line and HepG2, a hepatocellular carcinoma cell lines for apoptotic assays, RNA extraction and RT-PCR analysis. The primary role for using these two cell lines is that they are known to express PDK4 protein.

2.2.1 Culturing of cells

HeLa and HepG2 cells were purchased from Highveld Biologicals (PTY) LTD (South Africa). These were grown in T-75 flasks or 100-mm dishes at a density of 0.5×10^4 cells/cm² in Dulbecco's Modification of Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 units/ml of streptomycin.

The cells were grown by incubation at 37°C in a 5% CO₂ incubator (Sanyo, MCO-15AC, Japan). The cells were then grown in serum-free medium containing 0.25% BSA for 24 hours, and then treated with C2-Ceramide (Sigma Aldrich, USA), or Linoleic acid that is conjugated to BSA at a concentration of 0.02g Linoleic acid /1g BSA (Highveld Biologicals, Gauteng), or TNF α (Sigma Aldrich, USA) at indicated concentrations and time periods.

2.2.2 Cell Viability Assay

Cell viability assays are mainly performed to accurately estimate the number of viable cells against the dead cells in mammalian tissue cultures. Trypan blue dye exclusion is the most common utilised test for cell viability. The basic principle of this test is that the dye is taken up by the cells that have lost their membrane permeability barrier and thus are considered nonviable.

For this study, cell viability was determined by trypan blue exclusion method (Kim and Kim, (2003). Cells were detached with trypsin/EDTA and diluted 1:1 with medium supplemented with 10% fetal bovine serum. The cell suspension was mixed with an equal volume of 0.4% trypan blue solution (Sigma Aldrich, USA). The cells were then applied to a hemacytometer and counted under a phase-contrast inverted Neubauer (Carl Zeiss, Germany). The unstained cells were considered to be viable.

2.2.3 DNA laddering assay

Detection of apoptosis in cells after induction of apoptosis involves different techniques depending on the experiment. DNA laddering assay was chosen for our study for the detection of apoptosis. This technique involves extraction of DNA from the cells. The DNA is then loaded on the agarose gel and visible DNA laddering occurs. The ladder is a result of the fact that apoptosis is associated with the fragmentation of chromosomal DNA into multiples of the 180 bp nucleosomal units (Gong *et al.*, 1994).

DNA laddering assays were performed using a Suicide Track DNA Laddering kit that was purchased from Calbiochem (USA). Briefly, cells were trypsinized and washed with PBS. The cell suspension of 0.5×10^6 was then transferred to a microcentrifuge tube and spun down. DNA was extracted from the resuspended pellet according to manufacturer's instructions. DNA was quantified by measuring absorbance at 260 nm. DNA laddering was visualized by resolving the DNA in an agarose gel which was stained in ethidium Bromide.

2.2.4 RNA Extraction

RNA extraction is the technique whereby total RNA is extracted from the tissues or cell line samples. The most easiest and common RNA extraction method used involves the use of TRIzolTM LS REAGENT. This method utilizes the four phases which includes disruption, homogenisation, phase separation and RNA precipitation. The cell wall and membrane of cells are disrupted followed by homogenisation and RNA precipitation.

RNA was isolated (detailed method in Appendix 1) from HeLa and HepG2 cells using the TRIzolTM LS REAGENT according to manufacturer's instructions (Sigma Aldrich, USA). RNA was quantified by measuring absorbance at 260 nm and also using a Nanodrop (Nanodrop technologies, USA). The accuracy of quantification and the integrity of the RNA were confirmed by agarose gel electrophoreses.

2.2.5 Real Time Polymerase Chain Reaction (RT-PCR)

Real-time PCR has become a very powerful technique for evaluating gene expression at the mRNA level. The evolution of PCR to the existence of Real-time PCR allows science to be used to compare gene expression under different condition e.g. early development, late development, normal cells and cancerous cells, in cells under apoptotic stimuli and in the absence of.

The RT-PCR system is based on the fluorescence reporter. RT-PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e. Real Time) as opposed to the end point detection. This signal increases in direct proportion to the amount of PCR product formed after every cycle. The cycle at which this occurs is called the threshold cycle (C_t) which is defined as the cycle at which the fluorescence emission exceeds the fixed threshold. SYBR Green is one of the fluorescence reporter used in Real Time PCR. Its chemistry provides the simplest format for detecting and quantitating PCR products in real time reactions. This dye binds the minor grooves of a double stranded DNA. When SYBR Green dye binds to a double stranded DNA, the intensity of the fluorescence emission increases. As more double stranded amplicons are produced, SYBR Green dye signal will increase. Thus, as a PCR product accumulates, the fluorescence increases.

The RT –PCR reaction was performed using iScript One Step RT-PCR kit (Bio-Rad, USA) according to the manufacturer's instructions. A final reaction containing 2X SYBR Green RT-PCR reaction mix, 10 μ M of the both the forward and reverse primers and 100ng mRNA along with the enzyme was used. The reaction protocol was as follows: cDNA synthesis: 50C for 10 min, enzyme inactivation: 95°C for 5

min and followed by PCR cycle: 94°C for 30 sec, 58° for 30sec, 72°C for 1 min 30sec, followed by a plate read and cycle repeat of PCR for 29 cycle and ended with 72°C for 10 min. Melting curve analysis was as follows: 95°C for 1 min and 55°C for 1 min with a temperature increase of 0.5°C for every 10 sec. The results were visualized on 1% agarose gel with ethidium bromide and analysed using Mini Opticon Real Time PCR system (Bio-Rad MJ Personal Thermal Cycler).

2.2.6 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a three step technique that allow for amplification of minute DNA quantity into a significant quantity of the fragment of interest through repetitive cycle of DNA synthesis. The gene of interest is amplified using primers that are complementary to the sequence of interest. The three steps include denaturation, annealing and elongations. Denaturation step involves the denaturation of the DNA double strand by high temperatures. Annealing involves annealing of the forward and reverse primers to the template DNA. The last step extension require molecules such as *Taq* polymerase, MgCl₂, dNTPs and PCR buffer to synthesize the new strand of DNA. DNA polymerase is used to synthesise a double stranded DNA by complementary base pairing with DNA template.

The PDK4 gene was amplified from the cDNA using specific primers for the PDK4 gene. These specific primers for the PDK4 gene were designed using the DNA man program and sent to Inqaba Biotech (Pretoria, South Africa) for synthesis, forward full length primer (FF) 5'- GATACTCGAGCGATGAAGGCGGCC -3'; forward short length primer (FS) 5'- CGGTCTCGAGAGAAAATGCATGTGAAAG -3' and

reverse primer 5' – TTCCCGGGGGTAAAGGCGGCCCCG -3'. The 25 µl PCR reaction mixture was prepared as shown in a table below.

Table 1. PCR reaction mixture

Reagent	Final Concentration	µl used
10X PCR Buffer	1X	2.5
MgCl ₂ (25 mM)	1.5 mM	1.5
dNTPs (2.5 mM each)	200 µM of each	2
Forward primer (10 µM)	10 µM	1
Reverse primer (10 µM)	10 µM	1
Template DNA	100 ng	3
Taq polymerase (5 U/µl)	0.5 U	0.1
ddH ₂ O		13.9
Total volume		25

The samples were prepared in the PCR tubes and the lids closed very tightly before being subjected to the Mastercycler PCR machine (Eppendorf, Bio-Rad). The first step of the PCR is run once and the following three cycles, denaturation, annealing and extension are repeated 30 times and followed by the final extension step. The PCR conditions are shown below.

Table 2. PCR conditions

Steps	Temperature and time
Denaturation	94°C for 2 minutes
Denaturation	94°C for 30 seconds
Annealing	58°C for 30 seconds
Extension	72°C for 1 minute 30 seconds
Elongation	72°C for 10 minutes

} 30 cycles

The products were either stored at 4°C for further use or run on 1% electrophoresis to determine the correct molecular size.

2.3 Cloning of PCR products in pGEM-T-Easy Vector

Cloning is the technique whereby the DNA fragment of interest is ligated to a plasmid DNA vector which has the capacity to self replicate. This allows the fragment of interest to be replicated together with the vector thus resulting in many copies of the DNA of interest. pGEM-T-Easy vector is normally used for the purpose of cloning. This vector allows the cloning of PCR products without purifying it. LigaFast cloning system from Promega was used to clone PCR products containing the gene of interest PDK4 in pGEM-T-Easy vector. The cloning was performed to make a clone that will be used to plot the Real Time PCR standard curve. The detailed schematic of pGEM-T-Easy vector is shown in Appendix 3.

The cloning process involves ligation and transformation steps discussed below.

2.3.1 DNA ligations

DNA ligation technique involves the construction of recombinant DNA molecules following the ligation between the vector and DNA fragment of interest using DNA ligase usually T4 DNA ligase.

The PCR products were cloned into the pGEM-T-Easy vector using the T overhangs on it and the A overhangs of the inserts added during the PCR. T4 DNA ligase from fermentas was used for the ligation reactions. 3 µl of PCR product was mixed with 1 µl of 10X ligation buffer, 1 µl of 10 ng of pGEM-T-Easy vector, 1 µl of T4 DNA ligase and 4 µl of sterile distilled water to a final volume of 10 µl. The mixture was prepared in an eppendorf tube, tapped to mix and briefly microfuged. The ligations were incubated at 22°C for one hour.

2.3.2 Transformations

Transformation is the technique that involves genetic alteration of the cells resulting from the uptake and expression of foreign plasmid DNA. This technique allows for the selection and propagation of the selected plasmid DNA. Competent bacterial cells are used to take up the plasmid DNA followed by positive selection by growth on medium containing an appropriate antibiotic corresponding to the resistant genes in the plasmid DNA.

The controls used in this technique included the negative control where the reaction mixture is without the clone and positive controls without PCR product or the vector.

Escherichia coli JM109 was grown overnight in 5 ml Luria broth containing 100 µg/ml ampicillin at 37°C. The culture was then inoculated into a 100 ml Psi broth also containing ampicillin and grown with vigorous aeration at 37 until it reached an

O.D₅₅₀ of between 0.4-0.6. The flask was then transferred to ice and incubated for 15 minutes. The cells were centrifuged at 5 000 rpm for 5 minutes in a Sorvall SS-34 rotor. The supernatant was discarded and 40 ml of TfbI buffer which is 0.4 volume of the original volume of Psi broth. The pellet was gently resuspended and incubated in ice for 15 minutes. The mixture was again centrifuged at 5 000 rpm for 5 minutes in a Sorvall SS-34 rotor. The supernatant again was discarded and 4 ml of TfbII buffer which is 0.04 volume of the original volume of Psi broth was added. The pellet was resuspended in TfbII buffer and incubated in ice for 15 minutes. The competent cells were then aliquoted into amounts of 100 µl per eppendorf tubes and some stored at -70°C and some used immediately. 10 µl of the prepared ligations was added to a 100 µl of competent cells. This was incubated for 20-30 minutes on ice to allow diffusion. The cells were immediately heat shocked for 90 seconds at 42°C. They were then incubated for 5 minutes on ice. 500 µl of LB was added to the cells and they were incubated at 37°C for one hour to allow phenotypic expression. 100 µl of the cells was then plated on ampicillin containing agar plates which were incubated at 37°C overnight.

2.3.3 Plasmid DNA extraction

Plasmid DNA extraction method which is also known as the alkaline lysis is the technique that involves the extraction of the plasmid DNA from bacterial cells such as *Escherichia coli* by alkaline solutions.

In order to get high quality plasmid DNA for further experiments such as restriction digestion plasmid DNA was extracted using Lysozyme boiling method described below.

Few transformants both white and blue were isolated from the plate and inoculated in 5 ml of LB containing ampicillin and grown at 37°C overnight. The cultures were then centrifuged at a maximum speed of 13 000 rpm for 2 minutes. The supernatant was discarded and the pellet was resuspended in 400 µl of STET buffer. 10 µl of 10 mg/ml lysozyme was added and the cells incubated for 20 minutes at 37°C. The mixture was incubated in boiling water for 60 seconds and immediately put in ice for 10 minutes. These were then centrifuged at 13 000 rpm for 15 minutes and the supernatant was transferred to new eppendorf tubes. 400 µl of cold isopropanol was added and the tubes mixed several times by inversion. The tubes were spun at maximum speed for 10 minutes. The supernatant was carefully sucked out using the pipette and the pellet was washed in 1 ml of cold 70% ethanol. The pellets were centrifuged at maximum speed for 5 minutes and the supernatant carefully removed as much as possible. The pellets were then dried in a vacuum dryer for 15 minutes. They were then resuspended in 50 µl of 5 mM Tris-HCl buffer (pH 8.5) containing RNase.

2.3.4 Plasmid DNA digestion

Restriction digestion is the method used to determine the presence of the insert by determining the size of the insert. Restriction endonucleases are normally used in the method above to digest the clones. These enzymes have the ability to recognise and bind to the target specific DNA sequence. These target DNA sequences are specific for each restriction enzyme and therefore the cleaved sequence will generate different cohesive ends corresponding to each restriction enzyme. These ends are very important in the ligation process as they allow binding to the vector due to compatibility with the ends on the vector resulting from digestion with the same enzyme.

Restriction analysis of the plasmid DNA was carried out using the three endonucleases from Fermentas. Xho I was used to linearize the clones whereas EcoRI was used to release the insert. To confirm whether our inserts are the right sizes Xho I and Not I were used. DNA from each clone was mixed with appropriate restriction buffers as specified by the manufacturer.

The restriction enzymes were added and sterile water used to make up to a final volume. The mixture was tapped and microfuged before incubating overnight at appropriate temperatures. Incubation temperatures were specific to each endonuclease as specified by the manufacturer. The restriction digests are shown on tables below.

Table 3. Restriction digestion for EcoRI and Xho I

	EcoRI	Xho I
Buffer 10X	1.0 µl	1.0 µl
pGEM-T-Easy clone	3.0 µl	3.0 µl
Restriction enzyme	1.0 µl	1.0 µl
Sterile distilled water	5.0 µl	5.0 µl
Total volume	10 µl	10 µl

These digestions were performed separately for each restriction endonuclease.

Table 4. Double digestion for Xho I and Not I

	Xho I	Not I
Buffer 10X	1.0 µl	-
pGEM-T-Easy clone	3.0 µl	-
Restriction enzyme	1.0 µl	1.0 µl
Sterile distilled water	4.0 µl	-
Total volume	10 µl	-

The double digestions were performed at the same time with the buffer that is suitable for both restriction endonucleases.

APPENDIX 1

1.0 Methods

1.1 Cell Lines

HeLa and HepG2 cells were purchased from Highveld Biologicals (PTY) LTD (South Africa). The two cell lines were cultured according to the manufacturer's instructions.

1.2 Culturing of cells

1. The cells were allowed to grow until they formed a monolayer in the flask and were about 85-95% confluence.
2. Confluent cells were trypsinized using trypsin/EDTA from Highveld Biologicals.
3. The cells are then washed twice with 5 ml phosphate buffered saline (PBS).
4. One ml of trypsin was added to the flask and incubated at 37°C for less than 5 minutes to detach the cells from the surface of the flask.
5. Then 5 – 10 ml of medium was added and the mixture was transferred to 15 ml centrifuge tubes and centrifuged at 3000 rpm for 5 minutes. (This step is very important as it removes the trypsin).
6. After centrifugation, the supernatant containing trypsin was sucked out.
7. Then 10 ml of 10% FBS medium was added and the pellet was gently resuspended with a pipette and the cells were split equally into flasks and stocks made for future use.
8. Stocks were made by adding 1 ml of 10% freezing media to the pellet and then resuspended very gently.

9. The mixture was then transferred to the cryovial tubes which are suitable for extreme cold temperature.
10. The tubes were sealed tight and put in ice for 30 minutes before being put at -20°C for overnight.
11. The next day the tubes were transferred to a -70°C freezer for storage until further use.

1.3 Induction of apoptosis

1. Cells were seeded in 100 mm x 20 mm Petri dishes at a density of $1 - 5 \times 10^6$ cells/ml.
2. They were cultured at the above mentioned conditions for 24 hours before treating them with the three known inducers of apoptosis ceramide, TNF α and Linoleic acid.
3. Washed twice with PBS and treated with two differing concentration of each inducer of apoptosis at three different time intervals.
4. The concentrations were as follows: 20 μ M and 40 μ M of ceramide, 25 ng/ml and 50 ng/ml of TNF α and 300 μ M and 1 mM of Linoleic acid all for 24, 48 and 72 hours.
5. The control was included for each time interval where cells were treated with serum free media.
6. After each treatment the cells were harvested by trypsinization and centrifuged at 3000 rpm for 5 minutes before performing cell viability assay.

1.4 DNA extraction

1. Cells were trypsinized briefly and washed with PBS.

2. The cell suspension of 0.5×10^6 was then transferred to a microcentrifuge tube and spun down.
3. The pellet was then resuspended gently in 55 μ l of solution 1.
4. After adding 20 μ l of solution 2 the sample was then incubated at 37°C for one hour. 25 μ l of solution 3 was added to the mixture, mixed gently and then the sample was incubated for another hour at 50°C.
5. The sample was then resuspended in 500 μ l of resuspension buffer and mixed.
6. To precipitate DNA 2 μ l of pellet paint and 60 μ l of 3M sodium acetate was added to the sample.
7. Also 662 μ l of isopropanol was added and the sample mixed by inversion several times and allowed to stand at the room temperature for 2 minutes.
8. The sample was then centrifuged at 13 000 rpm for 5 minutes.
9. A pink pellet was visible in the bottom of the tube indicating the presence of the DNA.
10. The supernatant was then removed with a pipet tip and the pellet rinsed with 500 μ l of 70% ethanol.
11. The sample was again centrifuged at 13 000 rpm for 5 minutes.
12. The supernatant was removed and pellet rinsed with 500 μ l of 100% ethanol.
13. The sample was again centrifuged at 13 000 rpm for 5 minutes and the supernatant removed with a pipet tip.
14. The DNA pellet was allowed to air dry on the benchtop for a few minutes at room temperature.
15. Then the pellet was then resuspended in 50 μ l of resuspension buffer and stored at -20°C for further use.

1.5 RNA extraction

1. The cells were seeded at a density of 0.5×10^6 on 100mm Petri dishes for two days.
2. Washed with PBS and treated with serum free medium for 24 hours.
3. Then treated with TNF α (50ng/ml), C2-Ceramide (50 μ M) and Linoleic acid (300 μ M) for 24, 48 and 72 hours.
4. The cells were then harvested by trypsinization and then media was added to neutralise it.
5. Centrifuged at 3000 rpm for 5 minutes before resuspending them in 1 ml of TRIzol™ LS REAGENT.
6. The sample was allowed to stand for 5 minutes at room temperature.
7. Then 0.2 ml of chloroform per 1 ml TRIzol™ LS REAGENT was added and the tube shaken vigorously for 15 seconds and allowed to stand for 2-15 minutes at room temperature.
8. The sample was then centrifuged at 10 000 rpm for 15 minutes at 4°C and the mixture separated into three phases: a colorless upper aqueous phase (RNA), an interphase (DNA) and a red organic phase (proteins).
9. The colorless aqueous phase was transferred into a fresh eppendorf tube and 0.5 ml isopropanol per ml of TRIzol™ LS REAGENT was added and mixed.
10. The sample was then allowed to stand for 5-10 minutes at room temperature.
11. The samples were then centrifuged at 10000 rpm for 10 minutes at 4°C to collect the RNA pellet at the bottom of the tube.
12. The supernatant is then removed and the pellet is washed with 1 ml of 75% ethanol per 1 ml TRIzol™ LS REAGENT.
13. The sample is then vortexed and centrifuged at 8 500 rpm for 5 minutes at 4°C.

14. The pellet was then briefly air-dried for 5-10 minutes (do not use speed vac).
15. Then 100µl of RNase free water was added and the sample warmed at 55°C for 10-15 minutes.
16. The sample was then stored at -20°C for further use.

1.6 Agarose gel

Agarose gel electrophoresis is a method used for analyzing nucleic acids in terms of their sizes and conformations. This technique separates DNA fragments by migration on the gel based on the size and shape of the molecule, charge of the current and the resistance of the medium. Since the DNA molecule is negatively charged, when loaded on the gel it is normally loaded at the cathode end of the gel and moves towards the anode.

The samples (DNA fragments) were quantified and visualized on 1% agarose gel. The visualization of samples was achieved by staining with ethidium bromide (EtBr) which intercalates between the bases of the DNA. EtBr illuminates the gel when viewed under the UV light transilluminator.

1.6.1 Preparation of agarose gel

1. The gel was prepared by melting 1 gram of agarose in 100 ml of 1XTBE buffer using the microwave oven.
2. It was allowed to stand until it reaches 50°C temperature before 6 µl of EtBr was added and mixed evenly.
3. The gel was then poured into the gel tray and allowed to polymerize for 20 minutes after fitting the comb in the right place.
4. The comb was then removed and the gel subjected to an electrophoresis tank containing 1X TBE buffer enough to cover the gel.
5. Then 5-10 µl DNA samples were mixed with 2µl bromophenol blue dye and loaded onto the wells using a pipet.
6. Bromophenol blue is a loading dye that acts as a marker to indicate when to stop the electrophoresis.
7. A 1000 base pair molecular weight marker used was 1000 base pairs from Fermentas, South Africa.
8. The gel was subjected to electrophoresis at 100 volts using Consort E502 power system.
9. After the solvent front from the marker reached the bottom of the gel, the gels were photographed with ChemiDoc XRS system (Bio-Rad, Italy).

APPENDIX 2

2.1. Tissue Culture media preparations

10% FBS media (100ml)

10 ml FBS

1 ml streptomycin/penicillin (100 units/ml of each)

89 ml serum (DMEM)

Filter sterilize

Freezing media (10 ml)

9 ml (10 or 20% FBS media)

1 ml autoclaved glycerol (100%)

0.4% trypan blue solution (100 ml)

0.4g of trypan blue powder

Fill to 100 ml with sterile water

2.2 Media Preparations

Luria Broth (LB)

1 % tryptone

0.5 % yeast extract

0.5 % NaCl

Luria Agar (LA)

1 % tryptone

0.5 % yeast extract

0.5 % NaCl

1.5 % technical agar

Psi Broth

1 % tryptone

0.5 % yeast extract

0.5 % NaCl

0.5 g MgSO₄

20% glucose solution (100 ml)

20g glucose

Fill to 100 ml sterile water

Autoclave

2.3 Plasmid Preparation solutions

STET buffer

8 % sucrose

0.1 % Triton X-100

50 mM Tris-HCl (pH 8.0)

50 mM EDTA

TE buffer

10 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

2.4 Agarose gel electrophoresis solutions

5X TBE

13.5g Tris

6.85g boric acid

5 ml 0.5 M EDTA (pH 8.0)

250 ml distilled water

Autoclaved

0.5 % TBE

50 ml 5X TBE

450 ml distilled water

1 % Agarose

1 g agarose

100 ml 5X TBE

Autoclave

Tracking dye

30 % glycerol in TE

0.025 % bromophenol blue

Ethidium bromide

10 mg/ml in sterile distilled water

2.5 Bacterial transformation solutions

TfbI

30 mM KOAc

100 mM RbCl

10 mM CaCl₂

50 mM MnCl₂

15 % glycerol

pH 5.8 with acetic acid

TfbII

10 mM MOPS buffer

10 mM RbCl

75 mM CaCl₂

15 % glycerol

pH 5.8 with acetic acid

APPENDIX 3

3.1. Vectors

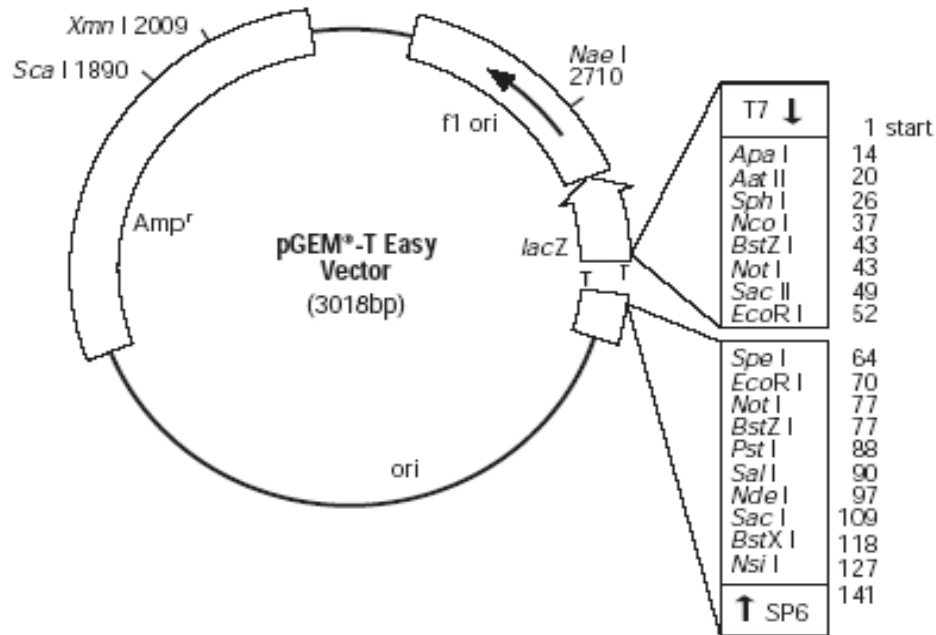


Figure 10. pGEM-T Easy vector map

The pGEM-T Easy vector is made up of 3018 bp with the T7 and SP6 RNA polymerase initiation sites at position 1 and 141 respectively. The T7 RNA polymerase promoter region occurs between 3002 and 3006 whereas SP6 promoter region occurs at positions 136 and 158.

APPENDIX 4

4.1 List of reagents and companies

Acetic acid	Merck (Germany)
Agar	Merck (Germany)
Agarose	Merck (Germany)
Boric acid	Merck (Germany)
Bromophenol Blue	Merck (Germany)
BSA	Highveld Biologicals (SA)
C2-Ceramide	Sigma Aldrich (USA)
CaCl ₂	Merck (Germany)
Chloroform	Merck (Germany)
DMEM	Highveld Biologicals (SA)
EDTA	Sigma Aldrich (USA)
Ethanol	Merck (Germany)
FBS	Highveld Biologicals (SA)
Glycerol	Sigma Aldrich (USA)
HCl	Merck (Germany)
Isopropanol	Merck (Germany)
KOAc	Merck (Germany)
LA	Sigma Aldrich (USA)
LB	Sigma Aldrich (USA)
Linoleic acid	Sigma Aldrich (USA)
Lysozyme	Sigma Aldrich (USA)
MgSO ₄	Sigma Aldrich (USA)
MnCl ₂	Merck (Germany)

MOPS	Merck (Germany)
NaCl	Merck (Germany)
PBS	Highveld Biologicals (SA)
Penicillin	Sigma Aldrich (USA)
RbCl	Merck (Germany)
Streptomycin	Sigma Aldrich (USA)
Sucrose	Sigma Aldrich (USA)
TBE	Inqaba Biotechnologies (SA)
TNF α	Sigma Aldrich (USA)
TRI Reagent	Sigma Aldrich (USA)
Tris	Sigma Aldrich (USA)
Triton-X-100	Sigma Aldrich (USA)
Trypan blue dye	Sigma Aldrich (USA)
Trypsin	Sigma Aldrich (USA)
Tryptone	Merck (Germany)
Yeast extract	Merck (Germany)

4.2 Kits

iScript One Step RT-PCR kit	Bio-Rad (USA)
Suicide Track DNA Laddering kit	Calbiochem (USA)
PCR kit	Promega
pGEM-T-Easy kit	Promega
Gel Purification kit	Promega

CHAPTER 3

3 Results

Since we hypothesized that PDK4 is regulated through protein-protein interactions, bioinformatics tools were used to determine if there are any proteins that may interact with PDK4 through protein-protein interaction. The results of these studies are shown below.

3.1 Bioinformatics analysis of proteins that potentially interact with PDK4

3.1.1 Identification of proteins that potentially interact with PDK4

Bioinformatics tools were used to analyse potential protein-protein interaction between PDK4 and other proteins. To this end, the interweaver database (<http://interweaver.i2r.a-star.edu.sg/>) was used to identify proteins that would potentially interact with hPDK4 (as described in materials and methods). This exercise resulted in identification of a number of domains that have the potential to interact with PDK4. The list of these domains is shown on **Table 5**.

Table 5. Domains that are predicted to interact with hPDK4

Domains that potentially interact with hPDK4		
ATPase family associated with various cellular activities (AAA)	Kinesin motor domain	Helix-hairpin-helix motif
Helix-loop-helix DNA-binding domain	Ubiquitin family	Zinc finger C-x8-C-x5-C-x3-H type (and similar)
Hsp70 protein	DEAD/DEAH box helicase	Proliferating cell nuclear antigen, N-terminal domain
Kunitz/Bovine pancreatic trypsin inhibitor domain	Helicase conserved C-terminal domain	HhH-GPD superfamily base excision DNA repair protein
SH2 domain	Ribosomal protein S2	Cell division protein
SH3 domain	WW domain	XPG N-terminal domain
TNFR/NGFR cysteine-rich region	WD domain, G-beta repeat	PAC motif
Immunoglobulin domain	Ribosomal protein S8	XPG I-region
Myosin head (motor domain)	Ribosomal protein S13/S18	PAS domain
Protein kinase domain	MutS domain V	GrpE
RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)	FHA domain	F-actin capping protein, beta subunit
Thrombospondin type 1 domain	AMP-binding enzyme	DNA mismatch repair protein, C-terminal domain
Zinc finger, C2H2 type	HMG (high mobility group) box	Casein kinase II regulatory subunit
Zinc finger, C3HC4 type (RING finger)	TPR Domain	NNMT/PNMT/TEMT family
Ligand-binding domain of nuclear hormone receptor	DNA gyrase/topoisomerase IV, subunit A	mRNA capping enzyme, catalytic domain
Zinc finger, C4 type (two domains)	Death domain	LSM domain
TCP-1/cpn60 chaperonin family	SAM domain (Sterile alpha motif)	Skp1 family, dimerisation domain
Carboxylesterase	HRDC domain	V-type ATPase 116kDa subunit family
Calcineurin-like phosphoesterase	Ribosomal protein L13	GAF domain
Cyclophilin type peptidyl-prolyl cis-trans isomerase	Acetyltransferase (GNAT) family	MutS domain I
Hsp90 protein	IQ calmodulin-binding motif	Toprim domain
DNA gyrase B	RasGEF domain	Putative snoRNA binding domain
ATP:guanido	Guanine nucleotide	DIL domain

phosphotransferase, C-terminal catalytic domain	exchange factor for Ras-like GTPases; N-terminal motif	
Glucocorticoid receptor	Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase	ATP:guanido phosphotransferase, N-terminal domain
Progesterone receptor	Myosin N-terminal SH3-like domain	Proliferating cell nuclear antigen, C-terminal domain
Coatmer WD associated region	Cdc37 family	MH2 domain
Helicase associated domain (HA2)	Peptidase C1-like family	Endonuclease/Exonuclease/phosphatase family
MutS domain II	BAR domain	mRNA capping enzyme, C-terminal domain
MutS family domain IV	Tubulin-tyrosine ligase family	Skp1 family, tetramerisation domain
MutS domain III	MH1 domain	SNARE domain
Protein of unknown function (DUF933)	RWD domain	Coatmer (COPI) alpha subunit C-terminus
CW-type Zinc Finger	HD domain	

The death domain (DD, highlighted in yellow) was among the identified potential interacting partners for hPDK4. The death domain family of proteins is known to play an important role in apoptosis/ programmed cell death (Desaghner and Martinou, 2000 and Kannan and Jain, 2000). The interweaver database was then used to identify the death domain proteins that are predicted to interact with PDK4. An excerpt of the results from this exercise is shown in **Table 6**.

Table 6. Four of the 24 DD proteins that were predicted to be potential interacting partners for hPDK4.

Protein name	Accession number	Function(s)
1. Death domain containing protein CRADD	P78560	(1). Apoptotic adaptor molecule specific for caspase-2 and fas/tnf receptor-interacting protein rip (2) In the presence of rip and tradd, raidd recruits caspase-2 to the tnfr-1 signalling complex
2. FAS-associating death domain containing protein (FADD)	P13158	(1) It interacts with the death domain on Fas and initiates apoptosis (2) Apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to the activated Fas (CD95) or TNFR-1 receptor (3) Active caspase-8 initiates the subsequent cascade of caspases (aspartate-specific cysteine proteases) mediating apoptosis.
3. Nuclear factor NF-kappa-B p105 subunit	P19838	Have dual functions such as cytoplasmic retention of attached NFκB proteins and generation of p52 by a cotranslational processing p52 binds to the kappa-B consensus sequence 5'-GGRNNYYCC-3', located in the enhancer region of genes involved in immune response and acute phase reactions.
4. Nuclear factor NF-kappa-B p100/p49 subunit	Q00653	Dual functions such as cytoplasmic retention of attached NFκB proteins and generation of p50 by a cotranslational processing.

24 death domain containing proteins were identified as potential partners for hPDK4. Of these 24 proteins, we showed 4 in table 6. These proteins are known to play a crucial role in apoptosis. This suggested a relationship between PDK4 and apoptosis. In addition, several scientific publications have linked diabetes to apoptosis (Chen, 2005 and Mandrup-Poulsen, 2003). We therefore hypothesised that PDK4 could be a link between diabetes and apoptosis, and thus propose to investigate the relationship between PDK4 and apoptosis. To explore this relationship, we focussed on one of the DD proteins, i.e. p100/p49.

3.1.2 Identification of the sequences in hPDK4 that have the potential to interact with NFκB subunits sequence that allows dimerization

The p100/p49 subunit and other NFκB subunits have been shown to use conserved amino acids to interact with each other in-order to form homo or heterodimers (McKay and Cidlowski, 1999 and Huang *et al.*, 1997). These amino acids are indicated in **Figure 11 below**.

(a)	p50	245	ASNLKIVRMD	RTAGCVTGGE	EIYLLCDKVQ	KDDIQIRFYE	EEENGGVWEG	FGDFSPTDVH	304
	p65	191	TAEIKICRVN	RNSGSLGGD	EIYLLCDKVQ	KEDIEVYFTG	PG.....WEA	RGSFSQADVH	245
	cRel		TAEIRICRVN	KNCGSVKGGD	EIYLLCDKVQ	KDDIEVRFVL	DN.....WEA	KGSFSQADVH	
	RelB		TSELRICRIN	KESGPCTGGE	ELYLLCDKVQ	KEDISVVFST	AS.....WEG	RADFSQADVH	
	p52		ASNLKISRMD	KTAGSVRGGD	EYIYLLCDKVQ	KDDIEVRFYE	DDENG..WQA	FGDFSPTDVH	
	p50	305	RQFAIVFKTP	KYKDVNITKP	ASVFVQLRRK	SDLETSEPKP	FLYYPEIKDK	EEVQRKRQK	363
	p65	246	RQVAIVFRTF	PYADPSLQAP	VRVSMQLRRP	SDRELSEPMF	FQYLPDITDDR	HRIEEKRRK	304
	cRel		RQVAIVFRTF	PFL.RDITEP	ITVKMQLRRP	SDQEVSEPMF	FRYLPDEKDP	YGNKAKRQR	
	RelB		RQIAIVFKTP	PYEDLEISEP	VTNVNVLQRL	TDGVCSEPLP	FTYLPDHDHS	YGVDKKRKR	
	p52		KQYAIIVFRTF	PYHKPKIDRP	VTVFLQLKRR	RGGDVSDSKQ	FTYYPVVEDK	EEVERKRKK	

Figure 11. Amino acids of NFκB subunits involved in the dimer formation with other subunits. Pink boxes indicate key and conserved amino acid residues that are involved in the dimerization interface; red boxes represent amino acids contributing to the dimer interface through polypeptide backbone; green boxes indicate the nonconserved residues in the dimer interface and purple box represents the nuclear localization at the c-terminus. Adapted from McKay and Cidlowski, 1999 and Huang *et al.*, 1997

To examine whether human PDK4 (hPDK4) contains the ability to interact with NFκB subunits in particular p100/p49; we used ClustalW a sequence alignment program to examine whether PDK4 contains the conserved amino acids that are important for NFκB recognition. The results of this alignment are shown below in **Figure 12**.

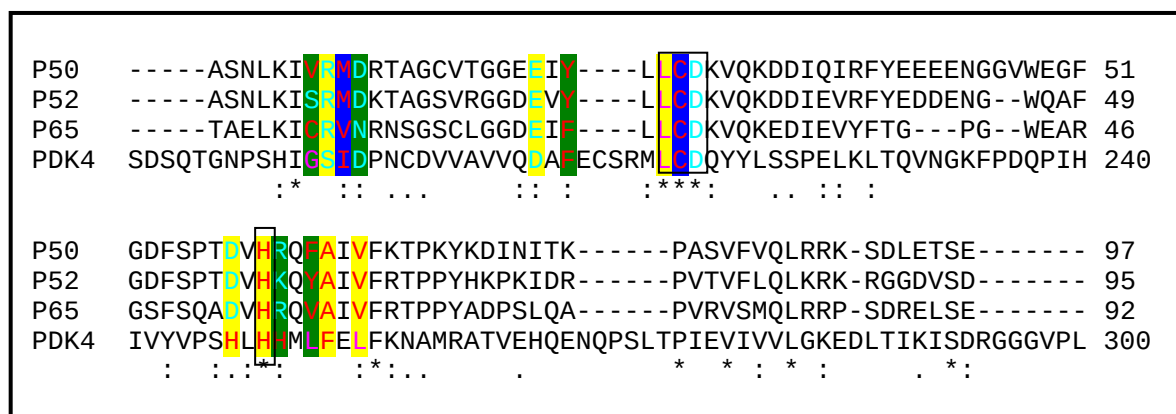


Figure 12. An alignment comparison between human PDK4 and NFκB subunits p50; p52 and p65. The sequences were aligned using clustalW program. Different colours indicate chemical properties of the specified amino acid residues. Amino acid residues in blocks indicates total conservation; yellow shading represents amino acids involved in the dimerization interface; blue shading represents amino acids contributing to the dimmer interface through polypeptide backbone and green shading demonstrates non-conserved amino acids at the dimer interface.

Three amino acids that are known to be important in NFκB subunit dimerization were conserved between NFκB subunits and hPDK4. These are lysine 214; cysteine 215 and histidine 249 (on hPDK4). NFκB amino acids corresponding to hPDK4 Lysine 214 and histidine 249 are involved in the direct dimer formation of the NFκB subunits through the side chain interactions, whereas those corresponding to cysteine 215 use

the polypeptide backbone to form the NFκB dimer. Interestingly, aspartic acid 216 also showed total conservation between hPDK4 and NFκB subunits. This amino acid has not previously been shown to play a role in the NFκB subunit dimer formation. These findings suggest that hPDK4 may potentially interact with NFκB.

3.1.3 Comparison between the amino acid sequence of human and rat PDK isoforms with NFκB dimerization sequence

The protein sequences of the other hPDKs and rat PDKs (rPDKs) isoforms were compared with the NFκB interacting amino acid sequences. This alignment was to determine any conservation of the NFκB interacting sequence within these isoforms (**Figure 13**). The results showed conservation of certain amino acids among the PDKs with NFκB subunits. The amino acids lysine (214); cysteine (215) and aspartic acid (216) which are conserved in hPDK4 were also conserved in the other human PDK isoforms as well as the rat isoforms except for a glutamic acid substitution in both human and rat PDK3. The other amino acid that is of interest is histidine which is conserved in all NFκB subunits and only human and rat PDK4 and not in the other isoforms. The other amino acids which are involved in the dimerization of the NFκB subunits were not conserved in the other PDK isoforms of both human and rat origin.

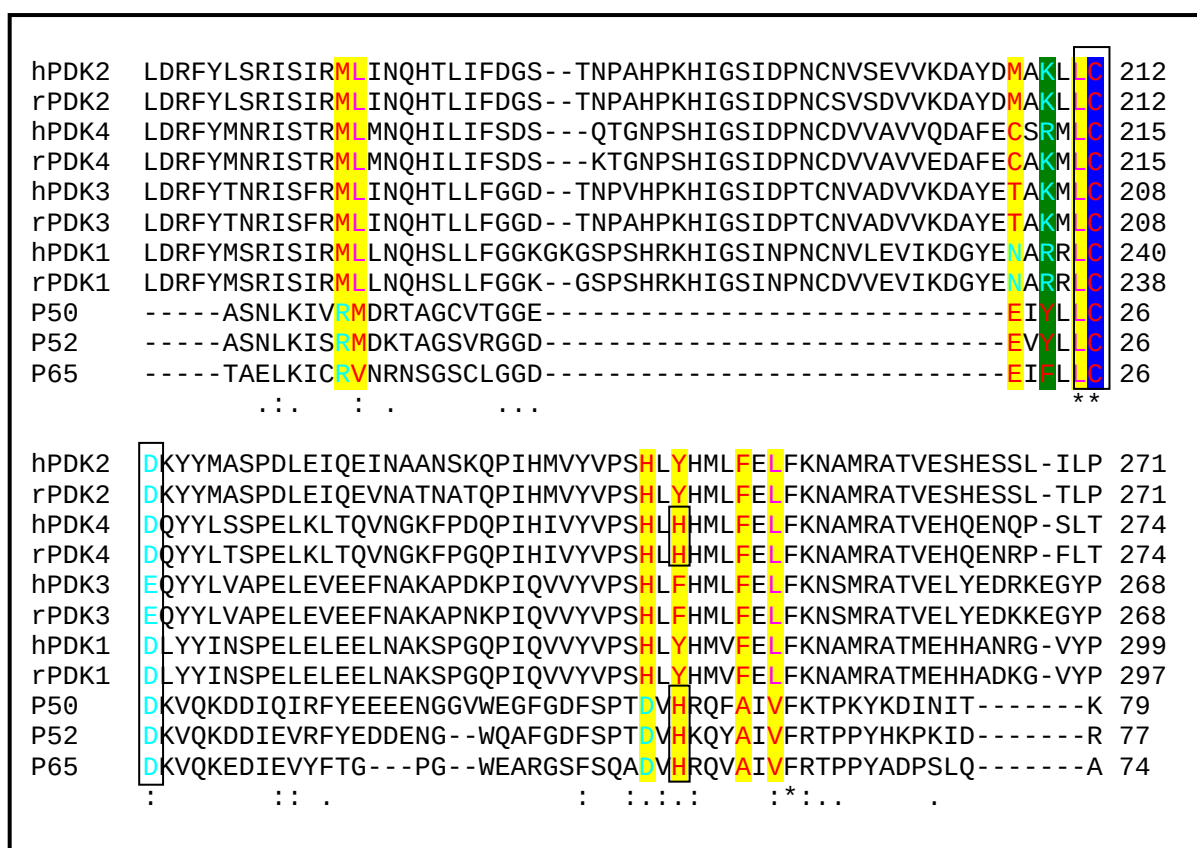


Figure 13. An alignment comparison of both human and rat PDK isoforms and nuclear factor kappa-B p50; p52 and p65 subunit. The protein sequences were aligned using clustalW program. All PDK isoforms and NFκB protein sequences were obtained from NIBI protein database. The accession numbers are as follows: human isoforms PDK4 (AH40239); PDK3 (XP_216091); PDK2 (NP_002602); PDK1 (NP_002601); rat isoforms: PDK4 (NP_446003); PDK3 (XP_216091); PDK2 (NP_110499); PDK1 (AAH89783) and NFκB subunits: p50 (P19838); p52 (Q00653) and p65 (CAA80524). Shaded area correlates to the ones above in **Figure 11 and 12**.

3.1.4 Location of a possible interaction site of NFκB subunits in hPDK4

Having identified the hPDK4 region that is similar to the dimerization sequence of NFκB subunits, we then proceeded to identify its location within the 3 dimensional structure of hPDK4. To this end, the Swiss PDB viewer was used to locate the conserved amino acids in the structure of hPDK4 that was modelled based on the coordinates of the experimentally resolved structure of rat PDK2 (CSIR report no. CSIR/PBIOTECH/RN/01/1628/B, Steussy *et al.*, 2001). The results (**Figure 14**) showed that the amino acids are situated on the region of hPDK4 which forms an interaction between hPDK4 and ADP molecule. The amino acids identified to be involved in the interaction are leucine (241); leucine (244); phenylalanine (242); histidine (236) and tyrosine (238). These residues are situated at the center of a cone-shaped cavity formed between the α -helices and β -sheets of the C-terminal domain of the protein (Steussy *et al.*, 2001). This C-terminal domain of PDK4 is known to be involved in the binding of nucleotides. In addition, this region is exposed to the outside and is solvent accessible thus suggesting interaction with other proteins is possible as well. The conserved amino acids lysine 214; cysteine 215; aspartic acid 216, and histidine 249 that have been identified to potentially play a role in interaction of hPDK4 with NFκB subunits are also located in this region. These findings suggest that PDK4 may interact with p100/p49 through these amino acids. This also strengthens our hypothesis that PDK4 may interact with p100/p49.

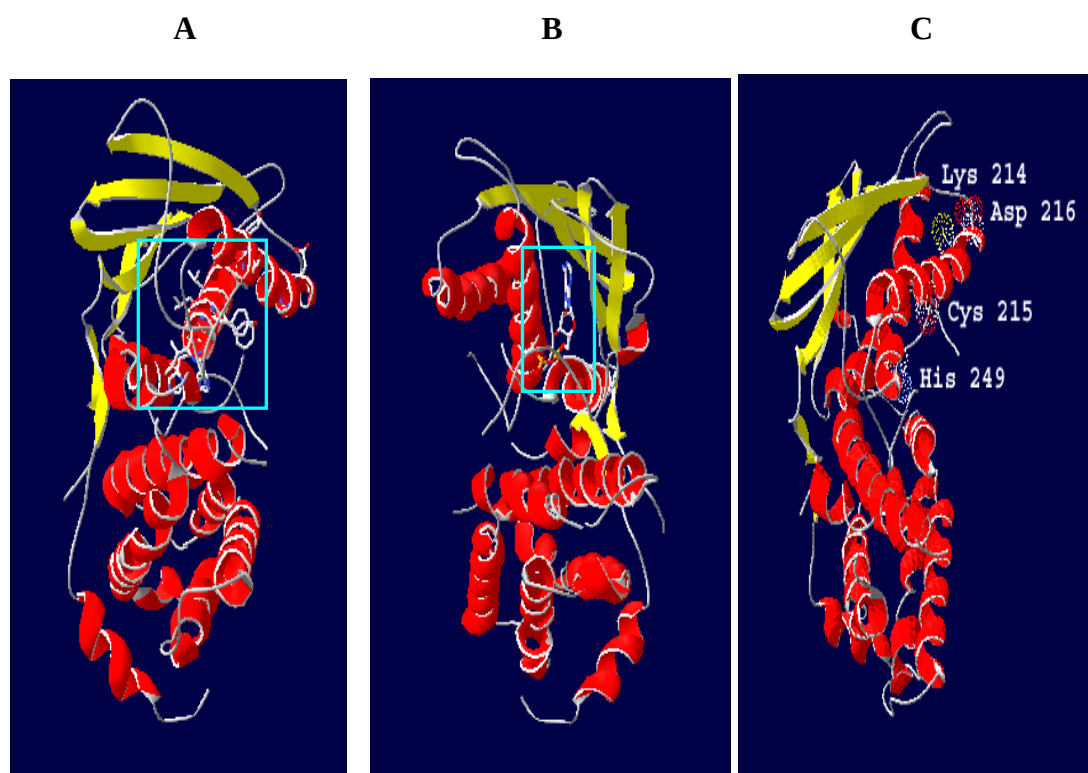


Figure 14. Rat PDK2 structures showing conserved amino acids to that of NF κ B subunits. The amino acids are situated between the α -helices (shown in yellow) and β -sheets (shown in red) of the C-terminal domain region of PDK structure. (A), Amino acids involved in the interaction between hPDK4 and ADP, these are leucine (241); leucine (244); phenylalanine (242); histidine (236) and tyrosine (238) (shown in a green large square). (B) ADP molecule (shown in a green box) bound to the C-terminal domain of the PDK structure. (C) Interacting residues of NF κ B subunits on hPDK4 structure, these are lysine 214, cysteine 215, aspartic acid 216, and histidine 249.

3.2 Examination of the relationship between hPDK4 and apoptosis

Having used computational methods to uncover the potential interaction between hPDK4 and death domain containing proteins in particular p100/p49, we then used molecular biology techniques and cell culture studies to examine the relationship between hPDK4 and apoptosis. In these experiments, HeLa cells and HepG2 cells were treated with agents that are known to induce apoptosis and these cells were then assessed for cell death, apoptosis and levels of hPDK4 mRNA.

3.2.1 Induction of cell death by TNF α , Ceramide, and Linoleic acid

TNF α , ceramide and Linoleic acid are known to induce apoptosis (Dichtl *et al.*, 2002; Kim *et al.*, 2005 and Lee *et al.*, 2005). To establish the optimal concentration and time course at which these factors induce cell death, HeLa cells and HepG2 cells were treated with different concentrations of TNF α , C2-Ceramide, and Linoleic acid over a specified time period. The trypan blue exclusion method was used to determine the degree of death induced in these cells as described in the materials and methods section. The concentrations of inducers used were 20 and 50 ng/ml for TNF α (**Figure 15 and 16**), 20 and 40 μ M for C2-Ceramide (**Figure 17 and 18**) and 300 μ M and 1 μ M for Linoleic acid (**Figure 19 and 20**). The cells were treated for 2, 4, 6, 24 and 48 hours for each concentration. The controls were included for each time intervals where cells were grown in serum free media.

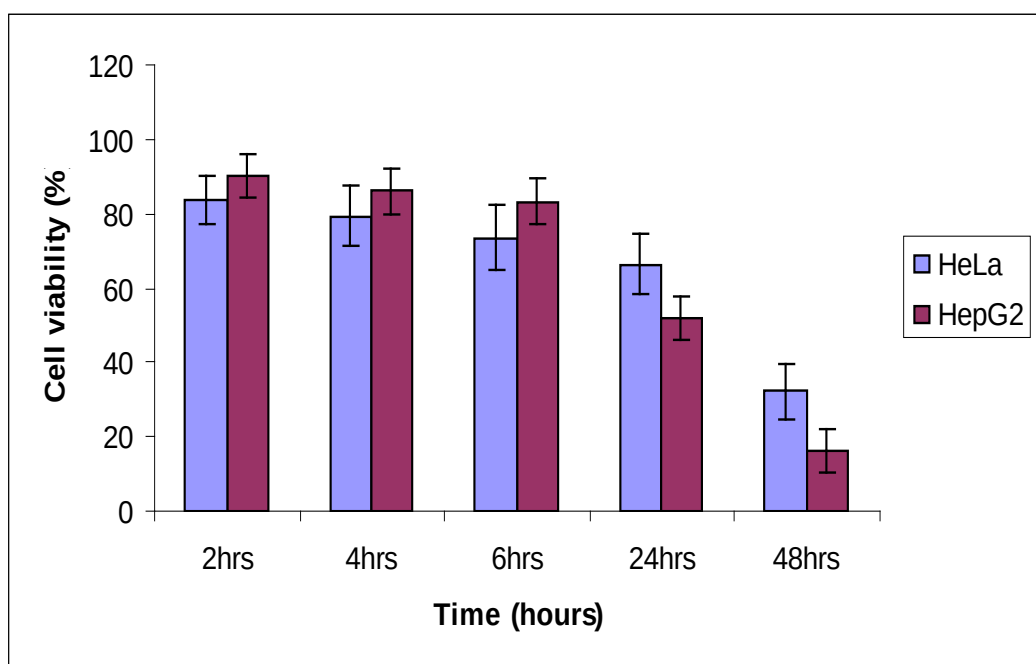


Figure 15. Cell viability assessments of HeLa and HepG2 cells that were treated with 20 ng/ml TNF α concentration for different time points. Blue bars indicate HeLa cells and the purple bars indicate Hep G2 cells.

Both HeLa and HepG2 cell viability decreased with increasing time. At 48 hours of treatment with 20 ng/ml concentration of TNF α , there was a considerable increase in cell death in both HeLa and HepG2 cells.

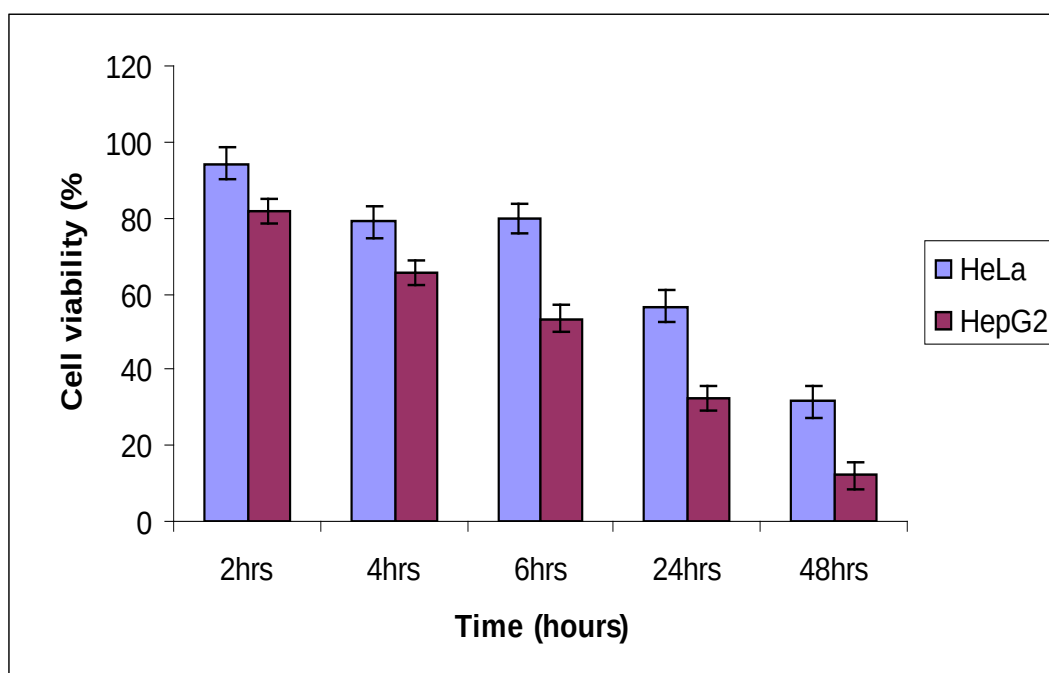


Figure 16. Cell viability assessments of HeLa and HepG2 cells treated with 50 ng/ml TNF α concentration for different time points. Blue bars indicate HeLa cells and the purple bars indicate Hep G2 cells.

Increasing TNF α concentration to a 50 ng/ml in both the cell lines resulted in massive increase in cell death. After 6 hours, cell death was about 50 % and at 48 hours there was about 10 % cell viability.

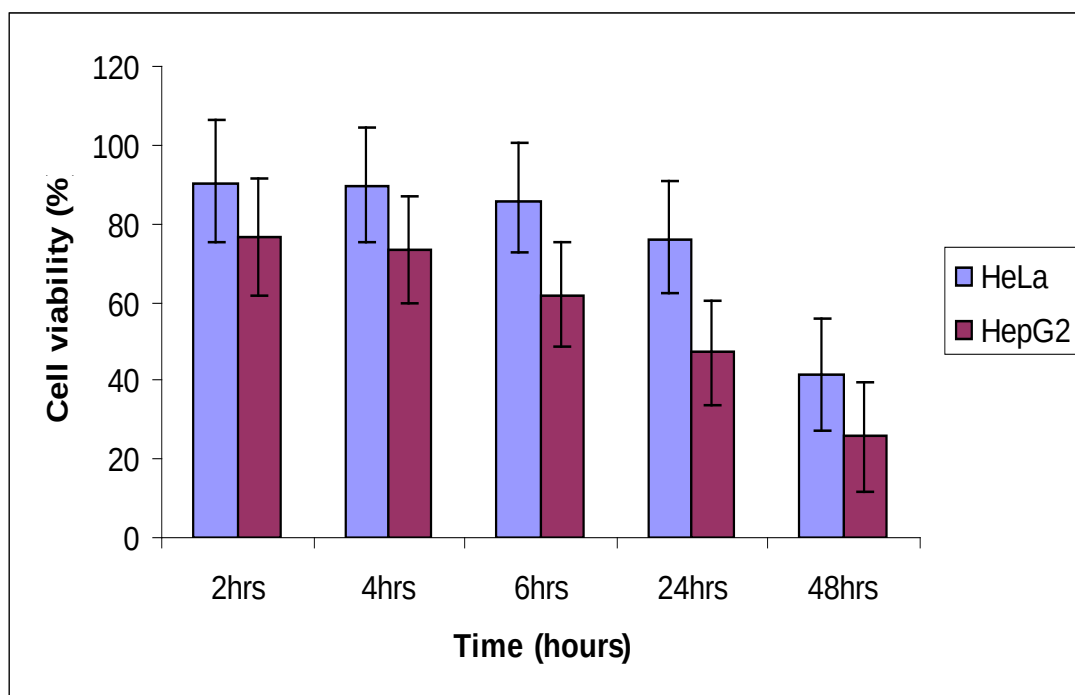


Figure 17. Cell viability assessments of HeLa and HepG2 cells treated with 20 μ M C2-Ceramide for different time points. Blue bars indicate HeLa cells and the purple bars indicate Hep G2 cells.

Lower concentrations of C2-Ceramide of 20 μ M in both HeLa and HepG2 cell lines resulted in induction of cell death. After 48 hours there was a sharp increase in cell death to about 30 % cell viability.

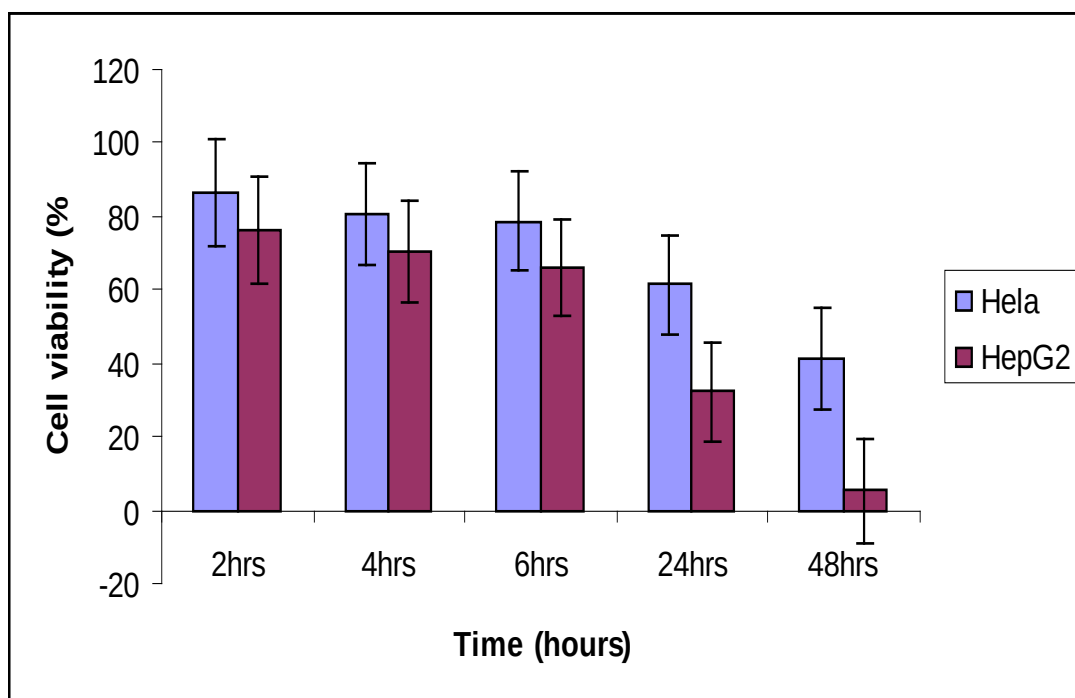


Figure 18. Cell viability assessments of HeLa and HepG2 cells treated with 40 μM C2-Ceramide for different time points. Blue bars indicate HeLa cells and the purple bars indicate HepG2 cells.

A higher concentration of C2-Ceramide of 40 μM showed decrease cell viability mostly after 24 hours. At 48 hours there were very few viable cells particularly in HepG2 cells.

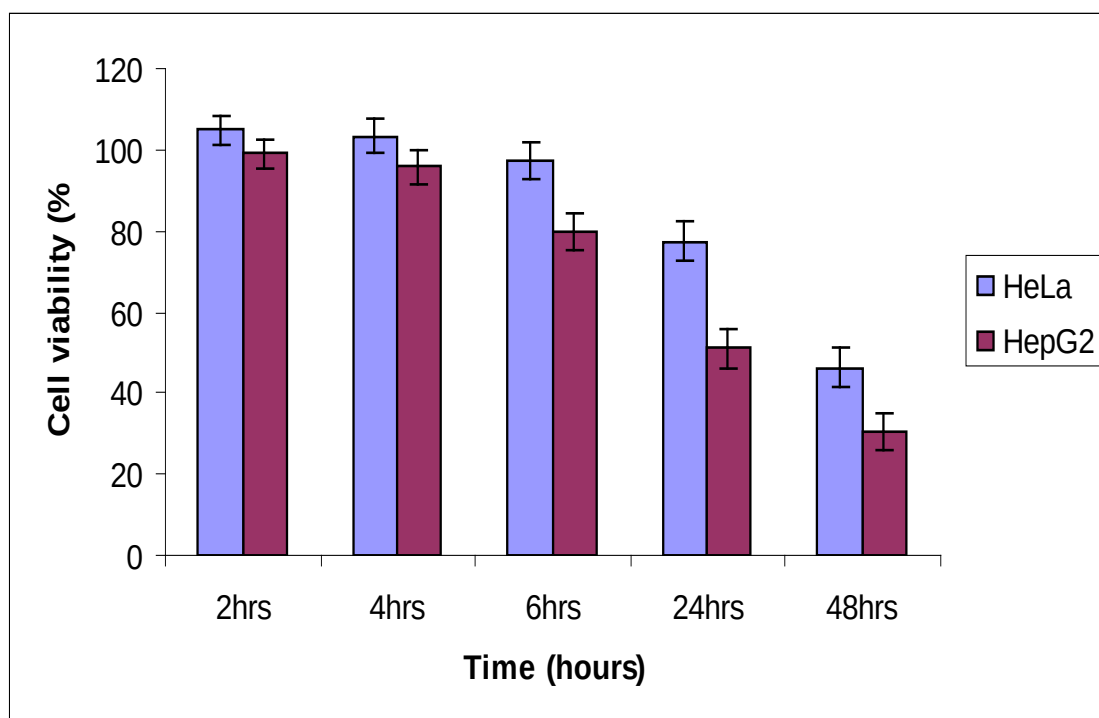


Figure 19. Cell viability assessments of HeLa and HepG2 cells were treated with 300 μ M concentration of Linoleic acid for different time points. Blue bars indicate Hela cells and the purple bars indicate Hep G2 cells.

Treating both HeLa and HepG2 cells with 300 μ M Linoleic acid also induced cell death to a higher extent in HepG2 cells when compared to HeLa cells.

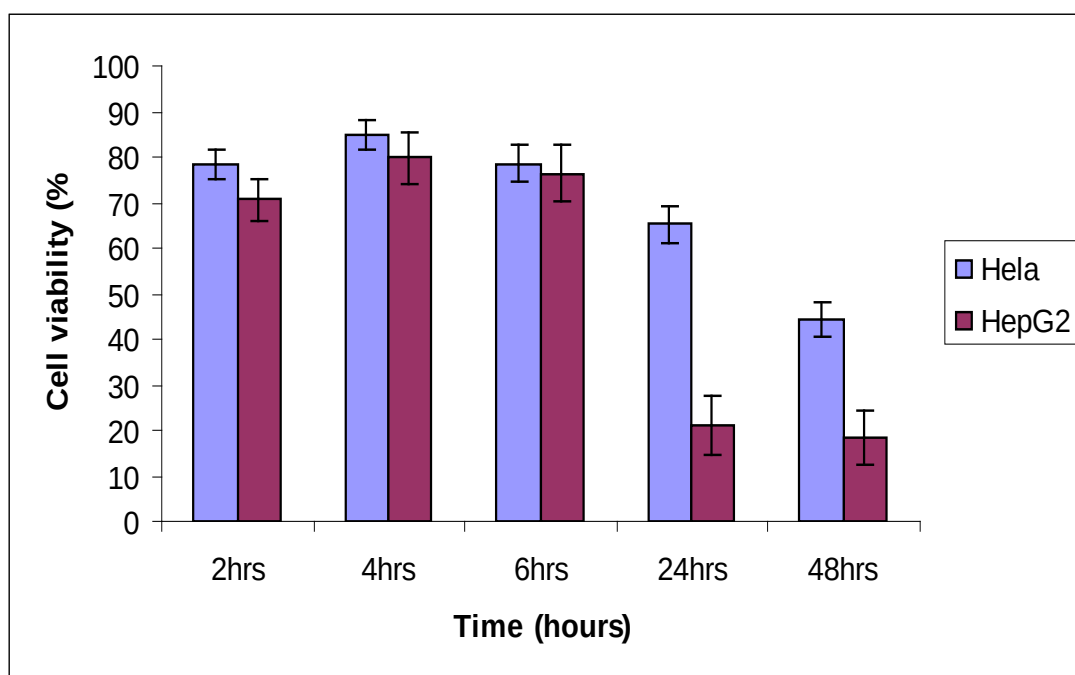


Figure 20. Cell viability assessments of HeLa and HepG2 cells treated with 1 mM Linoleic acid for different time points. Blue bars indicate HeLa cells and the purple bars indicate HepG2 cells.

Cell viability was reduced more after 24 and 48 hours in both the HeLa and HepG2 cells. 1mM Linoleic acid also induced cell death to a higher extent in HepG2 cells than in HeLa cells.

These three factors $\text{TNF}\alpha$, C2-Ceramide and Linoleic acid were shown to induce cell death at both the different concentrations used. The highest concentrations of the three demonstrated to be the optimal concentrations that resulted in induction of cell death that can be clearly measured with respect to exposure time.

3.2.2 Induction of apoptosis by TNF α , C2-Ceramide, and Linoleic acid

To assess whether apoptosis contributes in the cell death that is induced by TNF α , C2-Ceramide, and Linoleic acid, cells were treated with the optimal concentrations of these factors. Having observed that cell death is evident after 24 hours of cell treatment with these factors, cells were then treated for 24, 48 and 72 hours in order to assess apoptosis. Apoptosis was measured by using a DNA laddering assay as described in materials and methods. Results from these experiments are shown in **Figure 21**.

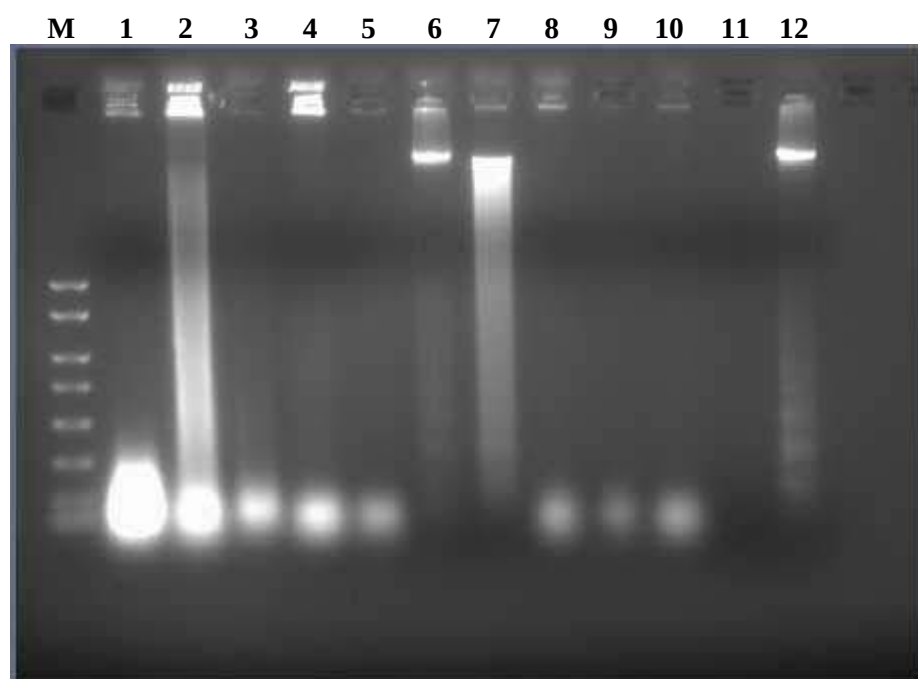


Figure 21. Induction of DNA laddering in HeLa cells that were treated with TNF α , Linoleic acid and C2-Ceramide. Lane M was a DNA marker, 1000 base pair ladder. Lanes 1; 5 and 9 were controls which were not treated, 2; 3 and 4 were cells treated with 50ng/ml TNF α at 24; 48 and 72 hours respectively. Lanes 6; 7 and 8 were treated with 40 μ M C2-Ceramide at the same above hours. Lanes 10; 11 and 12 were treated with 300 μ M Linoleic acid at the same above conditions.

Cells that were grown in serum free media were used as controls. Cells treated with TNF α showed DNA laddering for all the time intervals. C2-Ceramide induced apoptosis after 48 and 72 hours, and Linoleic acid also induced DNA laddering after 48 hours.

3.3 The effect of TNF α , C2-Ceramide, and Linoleic acid on PDK4 mRNA expression levels

3.3.1 Cloning of human PDK4 (hPDK4) into pGEM-T-Easy

RT-PCR was performed to determine the effect of TNF α , C2-Ceramide and Linoleic acid on PDK4 mRNA expression. In order to achieve this objective we had to first optimize the two set of primers designed for RT-PCR. To do that hPDK4 cloned into pENTR vector was used as a template for PCR. The PCR results (**Figure 22**) confirmed the presence of two fragments differing in about 200 base pairs. The larger fragments (**2** and **4**) full length (FF) gene was ~ 1.3 kilo base (kb) and the smaller fragments (**1** and **3**) short length (FS) gene was ~ 1.1 kb.

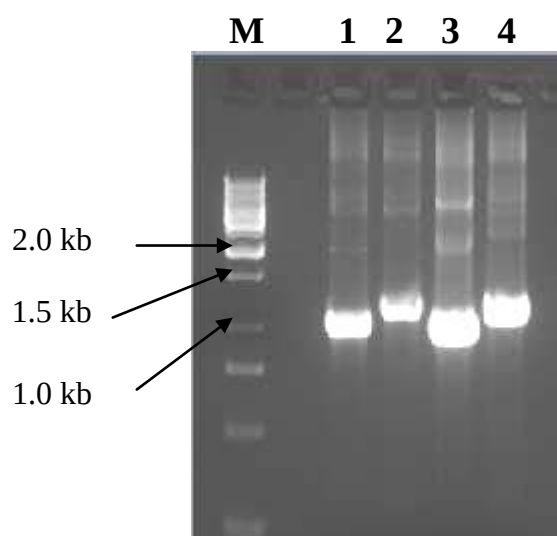


Figure 22. PCR products for hPDK4.

Lane M is a 1 kb ladder molecular weight marker from Fermentas, South Africa. Lane 1 and 3 are PCR fragments containing short length (FS) hPDK4 gene without the mitochondrial leader sequence. Lanes 2 and 4 are PCR fragments containing full length hPDK4 gene.

3.3.2 Confirmation of the inserts by restriction digestion

The PCR fragments containing the full length and short length hPDK4 were cut from the 1% agarose gel after the right sizes. The fragments were purified using Promega gel purification kit according to the manufacturer's instruction. The fragments were then ligated to 'T' overhangs of the pGEM-T-Easy vector using the 'A' overhangs on the fragments produced by the PCR BioTaq polymerase from New England Biolabs. Plasmid DNA was extracted from the positive clones and was linearized by digestion with Xho I and run on 1% agarose gel (**Figure 23**).

The results showed the expected fragments of about ~ 4.3 kb and 4.1 kb which represented the two clones with full and short length genes. The clones were further digested with Xho I and Not I and the results (**Figure 24**) confirmed the presence of the right inserts.

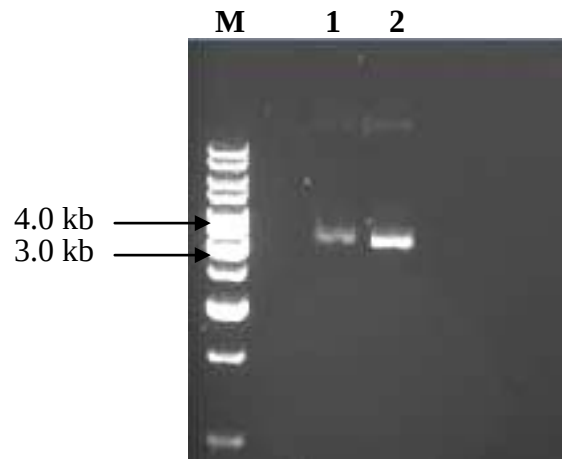


Figure 23. Restriction analysis of the clones.

Lane M is a 1 kb ladder molecular weight marker from Fermentas. Lane 1 is a restriction digested clone containing a full length insert with a size of ~ 4.3 kb. Lane 2 is a clone containing a short length insert with a size of ~ 4.1 kb. Both the clones were linearized with Xho I restriction enzyme.

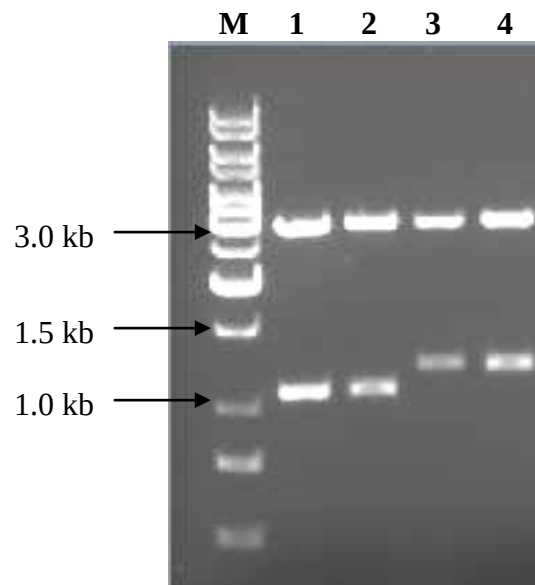


Figure 24. Restriction analysis of the inserts.

Lane M is a 1 kb DNA ladder molecular weight marker from Fermentas. Lane 1 and 2 are clones digested with Xho I and Not I to release the short length insert which is ~ 1.1 kb in size. Lane 3 and 4 clones were also digested with Xho I and Not I to release the full length insert which is ~ 1.3 kb in size. Both Xho I and Not I were used because they were incorporated in the designed two set of primers to confirm the presence of the inserts.

3.3 The effect of TNF α , C2-Ceramide and Linoleic acid on the PDK4 mRNA expression levels

3.3.1 Optimising conditions for the RT-PCR

In order to examine whether there is a relationship between PDK4 and apoptosis, HepG2 cells were treated for 24, 48 and 72 hours with TNF α , C2-Ceramide, or Linoleic acid. Real time PCR was performed to determine the level of expression of

the PDK4 mRNA after treatment with these three factors. The RNA used was extracted from these cells as described in materials and methods. The first step to be done was to optimize for the concentration of the DNA template to be used and the primers as this is the most important step in RT-PCR. Different concentrations of the DNA were used and 100ng was identified as the suitable amount. Based on the above finding, 100ng of the treated samples was run on the RT-PCR and the results are shown in **Figure 25**.

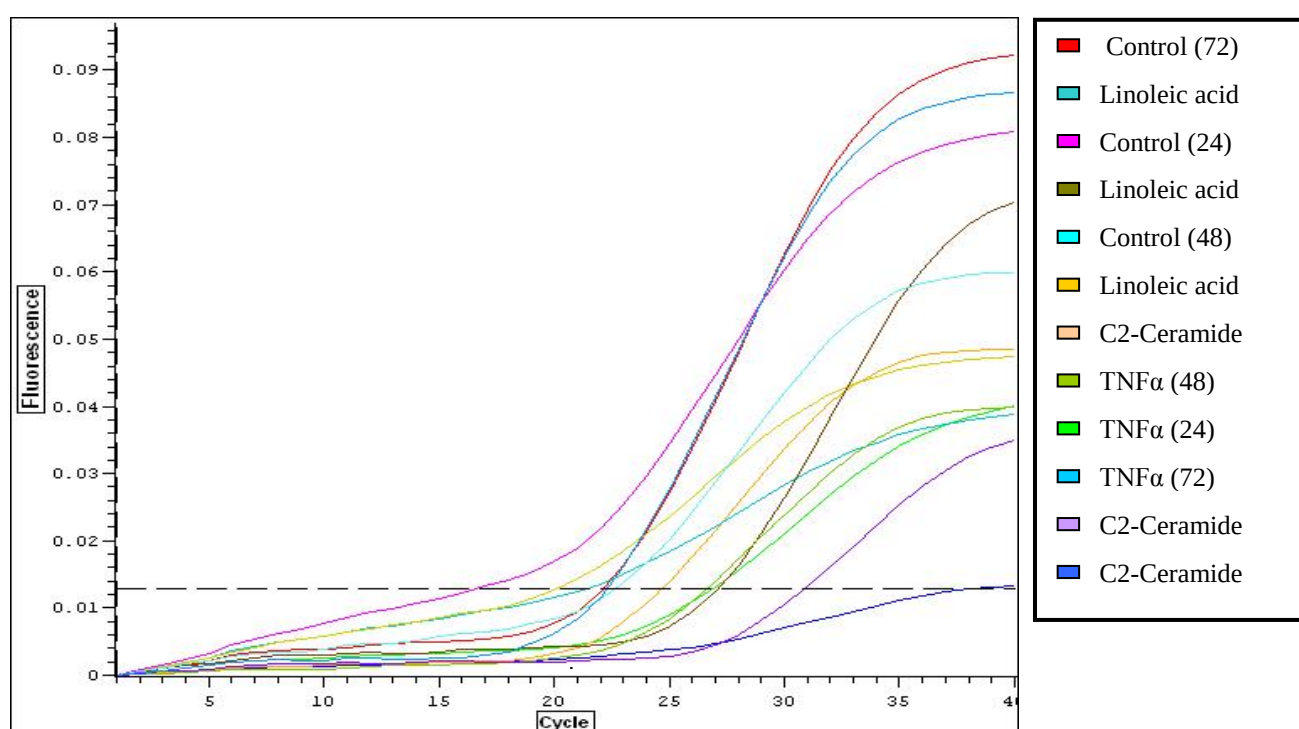


Figure 25. Amplification plot for the PCR amplification of PDK4 gene from HepG2 cells after treatment with TNF α , C2-Ceramide and Linoleic acid over a 72 hour period. Different curves represented by different colours represent PCR amplicons of PDK4 genes. Dotted lines represent the threshold cycles (Ct) of each sample above the threshold line

The PCR efficiencies of the products varied from 7 and 73% which is below the accepted range of between 90-100%. The low efficiency values may have resulted due to the secondary structure formation, concentration of the product or the quality of the primers. The efficiencies increased with increasing treatment time of each inducer. The Ct values for all the samples were below 40 (**Table 7**) and this confirmed successful amplification of the product. The efficiencies increased with increasing treatment time of each inducer. The Ct values for all the samples were below 40 and this confirmed successful amplification of the product.

Table 7. Efficiencies (%) and threshold cycle (Ct) values of the RT-PCR product

Sample	Time (hrs)	Efficiency	Ct
Control (untreated)	24	16.47	17.22
TNF α	24	3.40	15.73
C2-Ceramide	24	6.57	18.59
Linoleic acid	24	46.96	28.12
Control (untreated)	48	37.21	23.62
TNF α	48	40.47	26.91
C2-Ceramide	48	39.25	32.81
Linoleic acid	48	72.91	23.80
Control (untreated)	72	49.23	24.32
TNF α	72	43.59	27.78
C2-Ceramide	72	39.58	31.44
Linoleic acid	72	41.96	25.75

The melting curve for the treated samples was performed to determine whether the amplified gene hPDK4 is pure. The results are shown below in the melting curve in **Figure 26**. All the samples showed to have the melting temperature peaks for PDK4 gene at 83°C. However, there were some smaller peaks which indicated that the product was not very pure and this might have resulted due to contamination while preparing the samples for RT-PCR.

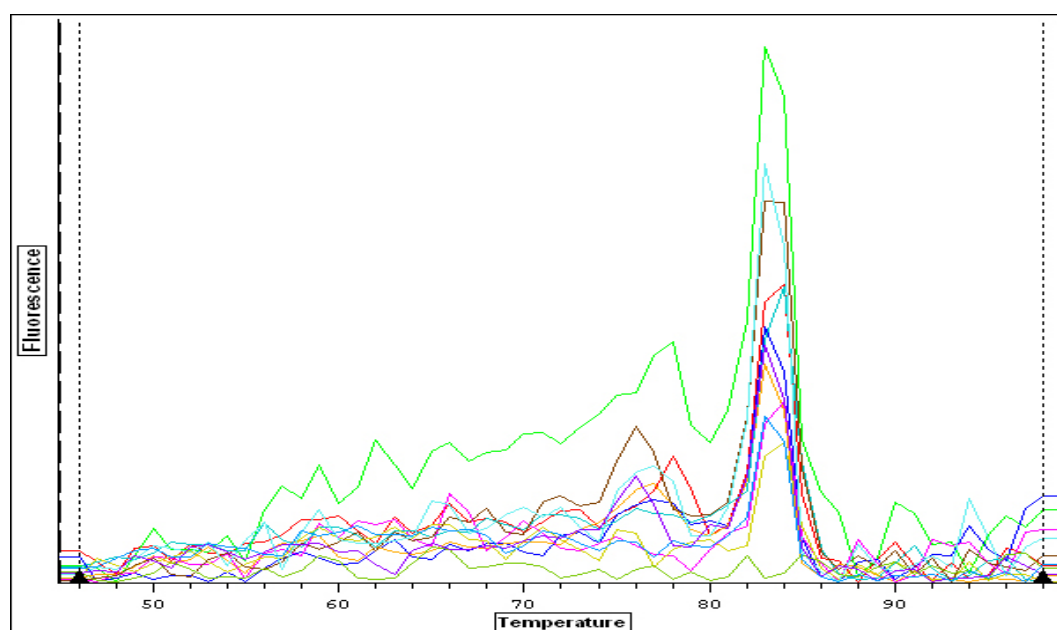


Figure 26. Melting temperature profiles of PDK4 gene after HepG2 cells treatment with $\text{TNF}\alpha$, C2-Ceramide and Linoleic acid over a 72 hour period. The melting temperature peaks for PDK4 gene were generated at 83°C.

3.3.2 Determination of the PDK4 mRNA expression levels

To further determine the effect TNF α , C2-Ceramide and Linoleic acid have on the expression levels of the PDK4 mRNA; RT-PCR was performed using the 100ng cDNA concentration of each sample identified above after optimization. The expression was performed to compare the difference in the PDK4 mRNA expression levels in the HepG2 cells after treating with TNF α , C2-Ceramide and Linoleic acid for over 72 hour period. The control which is the untreated was used to serve as the reference point as well as the negative control which contained no cDNA. The standard curve was constructed by making serial dilutions of the PDK4 clone prepared earlier by cloning into pGEM-T-easy. The standard curve (**Figure 27**) was used to compare quantitatively the expression level between the treated samples and the untreated.

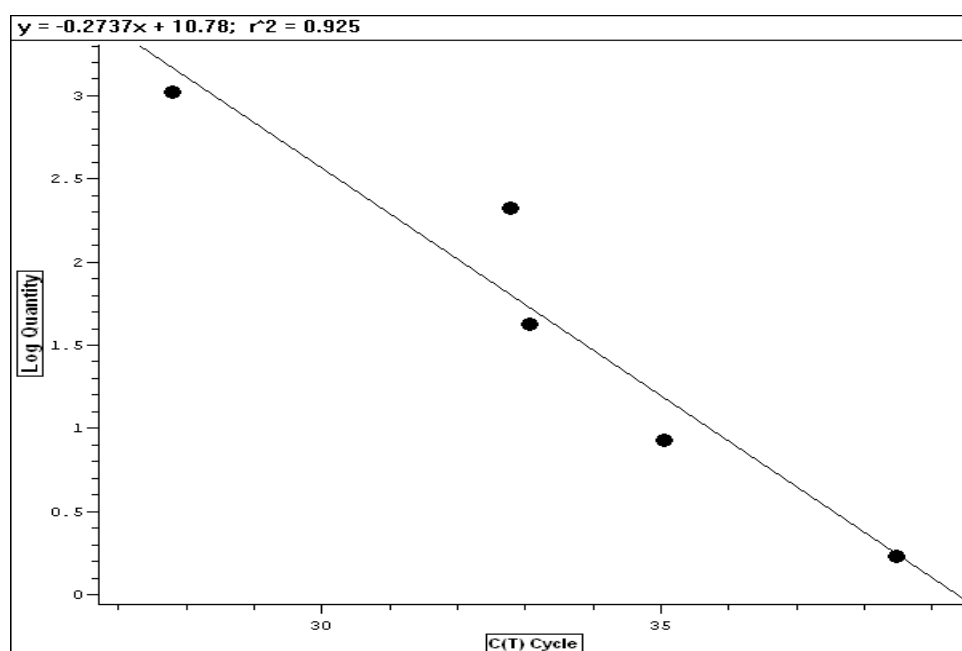


Figure 27. A standard curve for real-time quantitation of PDK4 DNA. The serial dilutions of the control DNA (1000ng to 0.05ng) was performed for the construction of standard curve. The PCR efficiency was 93% as indicated by the slope $m = -0.27$.

The PCR efficiency was obtained to be 93%. This was considered a good efficiency as it should lie between 90-100%. The slope of the graph showed a perfect doubling of the template as the value obtained was -0.27 which is close to -0.30 the expected slope. The quantitative graphs representing the fluorescence measured at each cycle for each sample were plotted. The graphs were plotted separately based on the treatment time for example after 24 hours (**Figure 27**); 48 hours (**Figure 29**) and 72 hours (**Figure 30**).

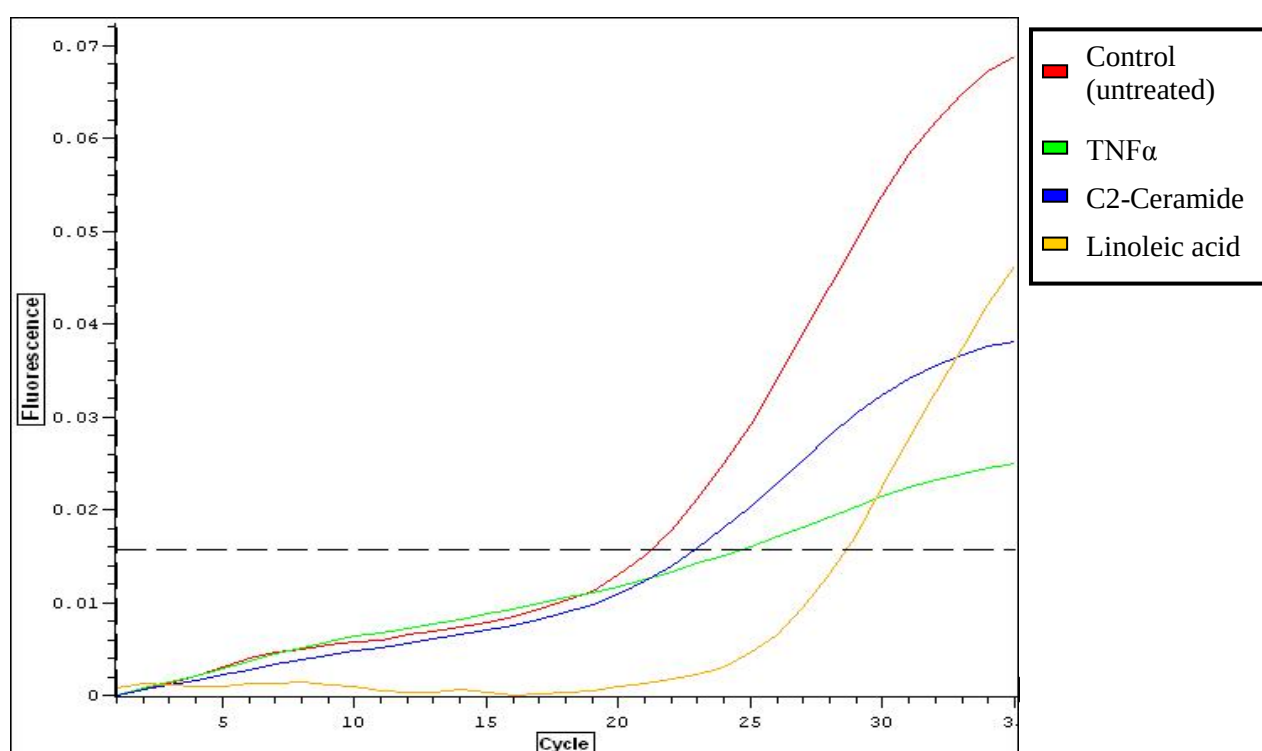


Figure 28. Quantitative graph of PDK4 gene after 24 hour treatments with the three inducers. Red graph represents the control; blue graph represents C2-Ceramide; green represents TNF α and yellow represents Linoleic acid. Dotted lines represent the threshold cycles (Ct) of each sample above the threshold line.

A quantitation graph measures the fluorescence intensity against the threshold cycle. This is a representation of amount of products produced within few PCR cycles to

reach the threshold which means samples with more templates initially will require fewer cycles to reach threshold than samples with less template. The threshold cycle (Ct) of a sample is defined as the cycle at which the sample's fluorescence trace crosses the threshold line. The results demonstrate the control graph to appear first in all the three experiments (**Figure 28; Figure 29 and Figure 30**) indicating there was more template and thus more products were produced.

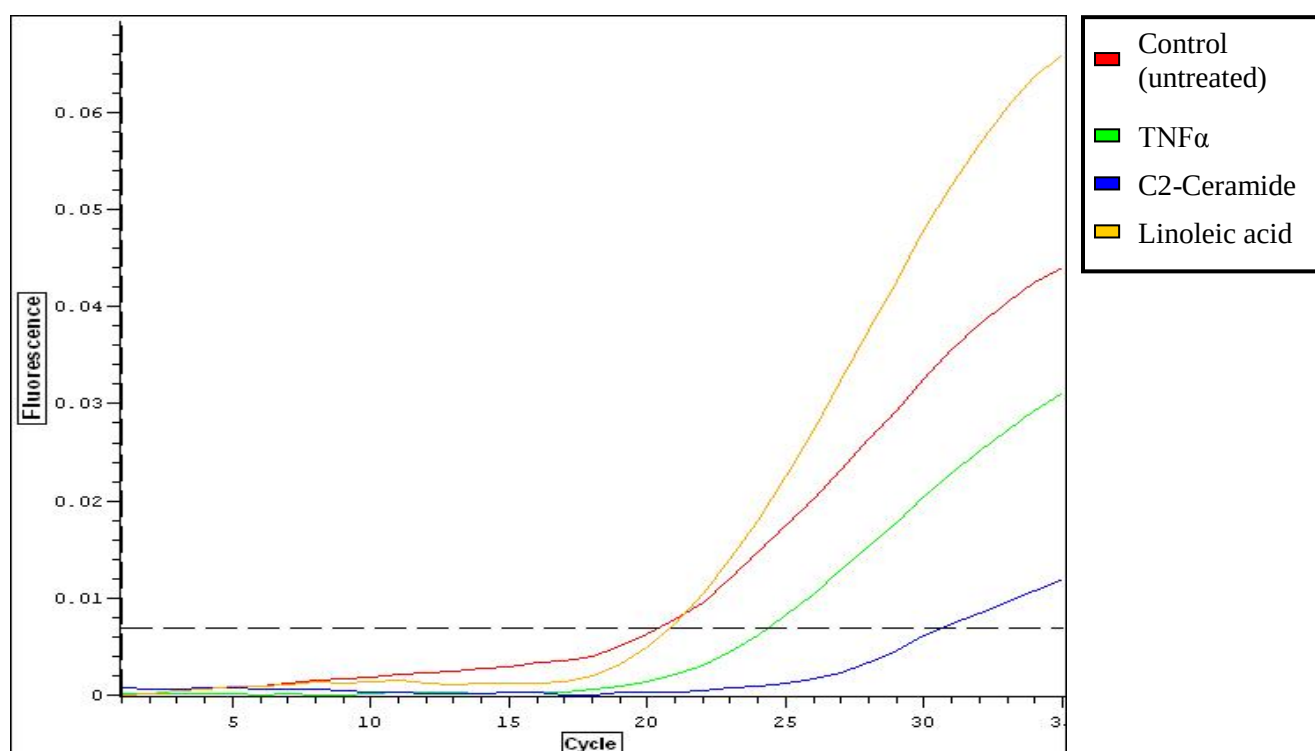


Figure 29. Quantitative graph of PDK4 gene after 48 hour treatments with the three inducers. Red graph represents the control; blue graph represents C2-Ceramide; green represents TNF α and yellow represents Linoleic acid. Dotted lines represent the threshold cycles (Ct) of each sample above the threshold line.

Both TNF α and C2-Ceramide showed a similar downregulating effect on PDK4 mRNA in all the three treatments. Linoleic acid did show a downregulation of PDK4 mRNA but it was different from the two (**Figure 29 and Figure 30**).

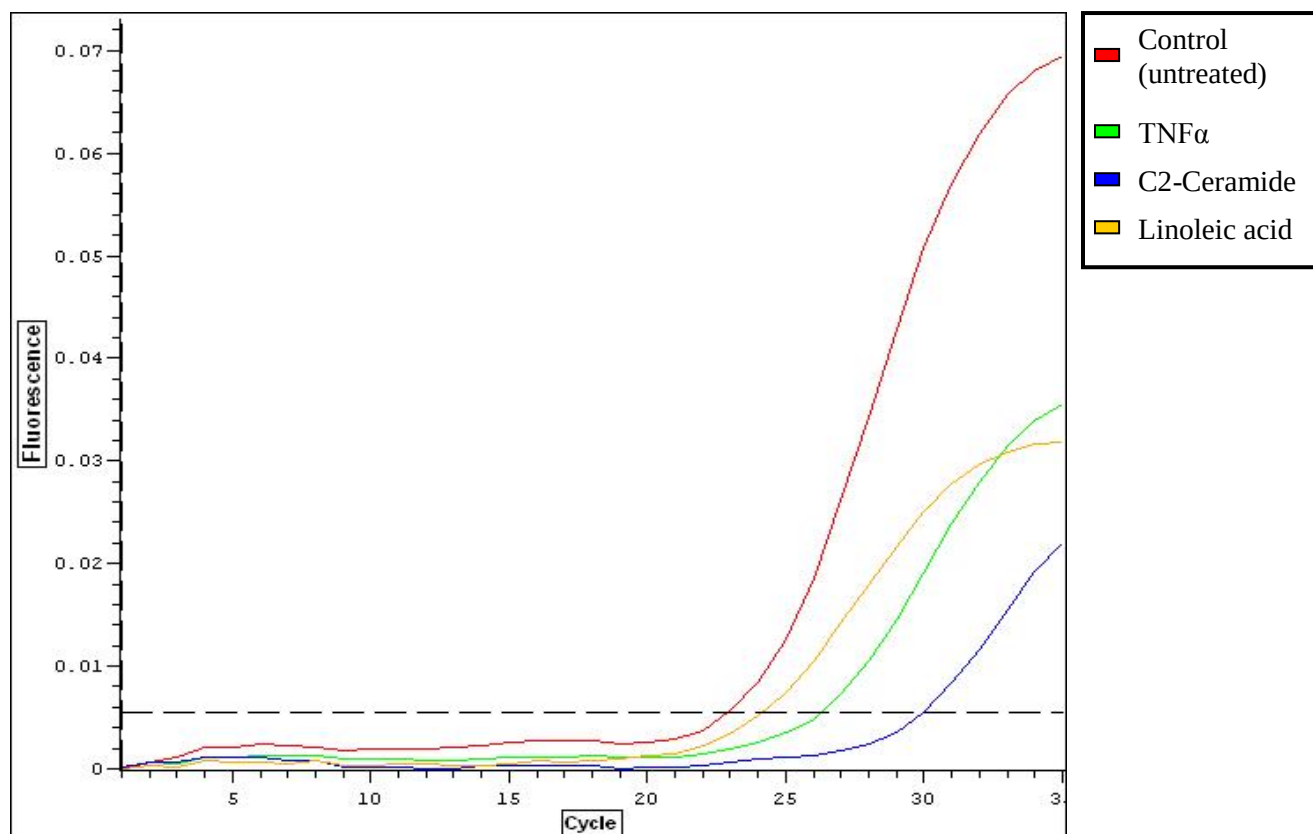


Figure 30. Quantitative graph of PDK4 gene after 72 hour treatments with the three inducers. Red graph represents the control; blue graph represents C2-Ceramide; green represents TNF α and yellow represents Linoleic acid. Dotted lines represent the threshold cycles (Ct) of each sample above the threshold line.

3.3.3 Relative quantitative expression levels of PDK4 mRNA

Relative expression was performed to compare the difference in the PDK4 mRNA expression levels in the HepG2 cells after treating with TNF α , C2-Ceramide and Linoleic acid. The control which is the untreated was used as the reference point. The standard curve was used to quantify and compare the mRNA expression levels between the treated samples and the untreated which is the control. The comparison of the PDK4 mRNA expression between the control and the three factors known to

induce apoptosis was conducted under three different time intervals of 24, 48 and 72 hours (**Table 8; 9 and 10**). The same concentration of 100ng DNA of each sample was added.

To determine the relative expression level of the PDK4 mRNA in absolute terms a formula was used, Relative Expression Level = $2^{-\Delta Ct}$. The ΔCt is the difference in the Ct values for the different inducers compared (**Table 8; 9 and 10**). This formula compared the expression of PDK4 mRNA when treated with TNF α , C2-Ceramide and Linoleic acid on HepG2 cells. By using the above formula the reference mRNA will be represented by 1 which as a result will serve as the base from which an increase or a decrease in expression can be determined. After 24 hour treatment (**Table 8**) it was found that there was a slight decrease in all the three inducers. Linoleic acid already showed a significant 10 fold decrease at this time interval compared to others. Although after 48 and 72 hours, Linoleic acid (**Table 9 and 10**) demonstrated a drastic increase in expression with increasing time well below the control indicating downregulation. TNF α indicated a steady but constant 3 fold decrease in PDK4 expression level. The three factors indicated a clear decrease in the PDK4 mRNA expression levels compared to the control.

Table 8. Relative quantitative expression of PDK4 after 24 hour treatment

Sample	Efficiency (%)	Threshold cycle (Ct)	Relative expression level ¹	Concentration (ng)
Control (untreated)	16.47	17.22		1644
TNF α	3.40	15.73	1.5	3190
C2-Ceramide	6.57	18.59	- 1.4	898.9
Linoleic acid	46.96	28.12	- 10	13.25

1. The relative expression level of PDK4 mRNA was determined using the following formula: Relative expression level = $2^{-\Delta C_t}$

The 24 hours treatment results revealed TNF α to have reached the lowest threshold cycle (**Table 8**) first before the control. These results (**Figure 31**) demonstrated an increase in PDK4 mRNA levels when treated with TNF and a decrease when treated with C2-Ceramide and Linoleic acid.

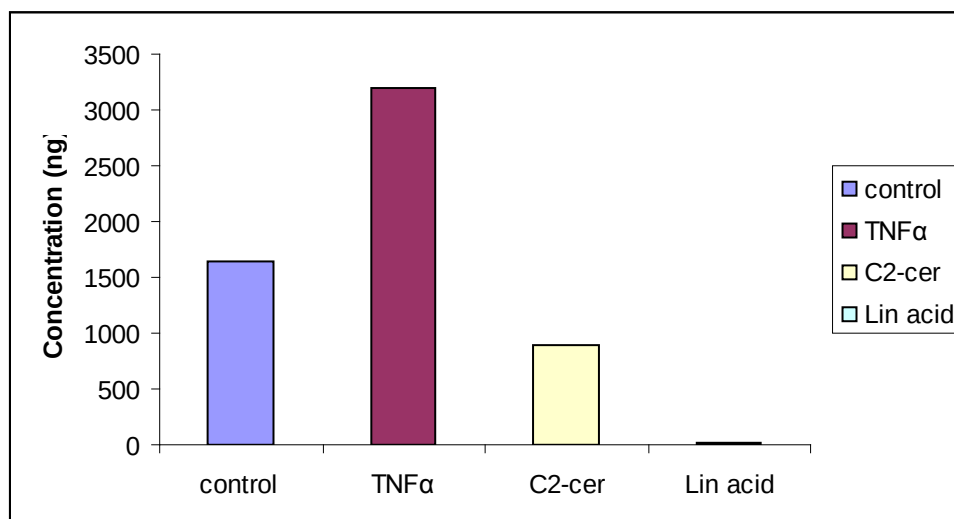


Figure 31. Quantitative comparison of the PDK4 gene between the control (untreated) and the three inducers of apoptosis after treating for 24 hours.

Table 9. Relative quantitative expression of PDK4 after 48 hour treatment

Sample	Efficiency (%)	Threshold cycle (Ct)	Relative expression level ¹	Concentration (ng)
Control (untreated)	37.21	23.62		97.11
TNF α	40.47	26.91	- 3.29	22.63
C2-Ceramide	39.25	32.81	- 9.19	1.665
Linoleic acid	72.91	23.8	- 0.18	89.8

1. The relative expression level of PDK4 mRNA was determined using the following formula: Relative expression level = $2^{-\Delta C_t}$

The TNF α results were not consistent with the treatment after 48 and 72 hours (**Figure 32 and 33**) as there was instead a decrease in the PDK4 mRNA levels. This strongly suggested that TNF α is downregulating PDK4 mRNA. C2-Ceramide also indicated to have a strong effect (**Figure 32**) on PDK4 mRNA as it showed to decrease further with increasing treatment time.

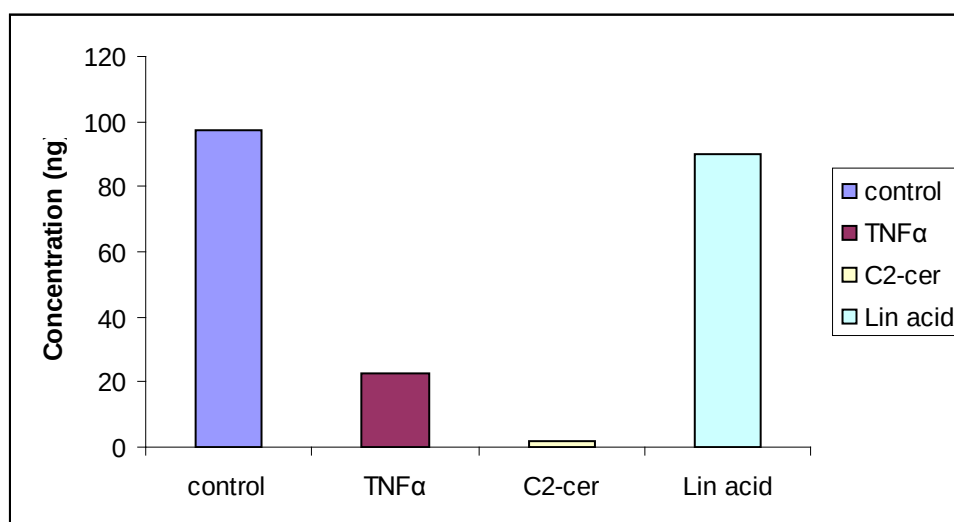


Figure 32. Quantitative comparison of the PDK4 gene between the control and the three inducers of apoptosis after 48 hour treatment.

Table 10. Relative quantitative expression of PDK4 after 72 hour treatment

Sample	Efficiency (%)	Threshold cycle (Ct)	Relative expression level ¹	Concentration (ng)
Control (untreated)	49.23	24.32		71.15
TNF α	43.59	27.78	- 3.5	15.45
C2-Ceramide	39.58	31.44	- 7.12	3.059
Linoleic acid	41.96	25.75	-1.4	37.82

1. The relative expression level of PDK4 mRNA was determined using the following

formula: Relative expression level = $2^{-\Delta Ct}$

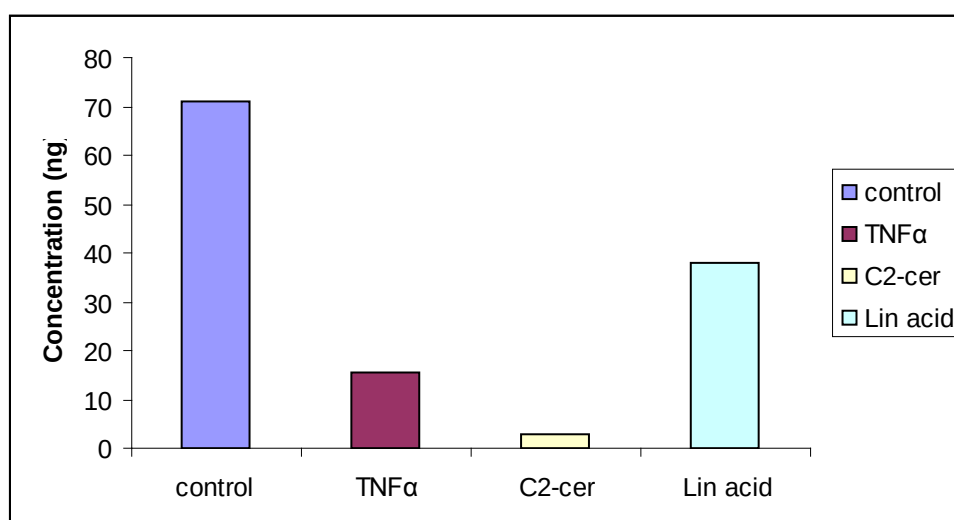


Figure 33. Quantitative comparison of the PDK4 gene between the control and the three inducers of apoptosis after 72 hour treatment.

Linoleic acid effect was not as effective as TNF α and C2-Ceramide. It did show suppression of the PDK4 mRNA but it was less compared with the other two factors. All the three known inducers of apoptosis, TNF α , C2-Ceramide and Linoleic acid demonstrated to a decrease in the PDK4 mRNA levels which means the three factors suppresses PDK4 expression levels.

CHAPTER 4

4 Discussion

4.1 Bioinformatics analysis of proteins that potentially interact with PDK4

Understanding the regulation of PDK4 is important since it provides more opportunities for intervention in the treatment of type II diabetes which currently affects 6% of the world population and is reported to be growing at a rapid rate (Owen and Hattersley, 2001 and Bailey, 2000). Currently, the regulation of PDK4 has been studied primarily at the level of gene expression and interaction of the PDK protein with allosteric factors. Studies by Popov *et al.*, (1993) indicated that PDK4 activity is also regulated by the interaction with its protein substrate pyruvate dehydrogenase complex (PDC). This, together with that are the many roles that protein-protein interaction is known to play in regulating target protein activity and cellular homeostasis (Sugden and Holness, 2003), inspired us to examine whether PDK4 interacts with other proteins as well. To that end, the Interweaver database was used to uncover proteins that have domains predicted to interact with hPDK4. This revealed an extensive number of domains from a variety of protein families that could potentially interact with hPDK4 (**Table 5**) as well as specific proteins that could be involved (**Table 6**). The death domain (DD) protein is a highly conserved region of ~ 80 amino acids which is found in proteins that are involved in apoptosis (Schlessinger and Ullrich, 1992). The major role of this domain is to connect together the proteins involved in apoptosis through hydrophilic intractions (Aravind *et al.*, 1999 and Harper *et al.*, 2003).

The findings mentioned above suggested a role for PDK4 in apoptosis. This was very interesting since PDK4 was primarily associated with regulation of glucose clearance and diabetes, and not apoptosis. A subsequent search of published literature revealed two papers that suggested a role for PDK in apoptosis. Work reported by Roche and Hiromasa (2007) indicated that inhibition of PDK activity by pan-PDK inhibitors resulted in activation of PDC and induction of apoptosis in cancer cells that selectively convert glucose to lactate. However, it is not clear from this study whether this effect is PDK isoform specific since the inhibitors did not differentiate between the different PDK isoforms. However, results reported by Kim *et al.*, (2006) demonstrated that over-expression of PDK1 rescued cells from hypoxia-induced apoptosis. Whether PDK4 also plays the same role has not previously been demonstrated.

The possible mechanism for involvement of PDK in apoptosis has not been defined. Interweaver database indicated that p100/p49, a subunit of NFκB was among the DD containing proteins identified to have the potential to interact with hPDK4 (**Table 6**). Numerous studies have provided compelling evidence that NFκB plays an important role in induction of apoptosis (Kim *et al.*, 2005; Orange and Geha, 2003 and Vestergaard *et al.*, 2005). Interestingly, NFκB has been extensively linked to the development of insulin resistance and diabetes (Nunn *et al.*, 2007; Chen *et al.*, 2005; Schmid *et al.*, 2006 and Alves *et al.*, 2005). However, interaction between NFκB and PDK4 has not been reported. To address this, bioinformatics tools were further utilized. To that end, ClustalW was used align the protein sequences of PDK4 and other NFκB subunits including p50 (representing p100/p49) that was retrieved from NCBI protein database in order to examine whether PDK4 contains sequence regions

that are known to be involved in NFκB subunit interactions. The amino acid sequences on NFκB subunits responsible for interaction with other subunits were identified by Huang *et al.*, (1997). These amino acids were classified into three categories: the conserved residues directly involved in dimerization; the non-conserved residues; and the residues contributing to the dimer interface through polypeptide backbone contacts (**Figure 11**).

The alignment of PDK4 and the NFκB subunits demonstrated conservation of certain amino acids responsible for the dimerization with other subunits. These amino acids are (using hPDK4 nomenclature) lysine 214; cysteine 215; aspartic acid 216 and histidine 249 (**Figure 12**). The total conservation of lysine 214; cysteine 215, and histidine 249, which are directly involved in the dimerization with other subunits, indicated a possibility for p100/p49 to interact with PDK4. Aspartic acid 216 was also totally conserved, although its role in NFκB dimer formation has not been previously identified, despite its location on a sequence that is known to be important in this process. Interestingly, this region is also conserved in all the other isoforms of human and rat PDK (**Figure 13**). Currently the function of these three residues in the PDKs had not been defined. What is known is that they form part of the α9 helix which forms part of the C-terminal domain. The histidine residue at position 249 that was conserved between hPDK4 and NFκB subunits (**Figure 13**) was only conserved in both rat and human PDK4 and not in any other isoforms, perhaps indicating a differentiating role for PDK4 from other PDK isoforms.

To locate the conserved amino acids on the hPDK4 structure Swiss Pdb Viewer program was used. Human PDK4 structure was modelled based on the published rat

PDK2 coordinates. The results showed the conserved amino acids to be located on the C-terminal end of hPDK4. This region is highly conserved and made of α and β sheets folded together to form a heterodimer that contains the nucleotide binding site (Steussy *et al.*, 2001). These sheets are folded in such a way that they form uniform subdomains known as N box, G1 and G2 box and the G subdomains which are the ones involved in nucleotide binding (Bowker-Kinley and Popov, 1999). The amino acid residues that are involved in interaction with NF κ B subunits were situated around this area on PDK4 structure (**Figure 14**) where PDK4 binds the ADP molecules (Steussy *et al.*, 2001 and Bao *et al.*, 2004). These nucleotides binding pockets are exposed outwards and are easily accessible. This suggests that the region is solvent accessible which makes it easy for the interaction with other proteins as well. These findings strengthen the hypothesis that PDK4 may interact with p100/p49 through these amino acids.

4.2 Examination of the relationship between hPDK4 and apoptosis

4.2.1 Induction of apoptosis by TNF α , C2-Ceramide and Linoleic acid in Hela and Hep G2 cells

The above discussed results suggest strongly that hPDK4 may have a role in apoptosis. To further explore this hypothesis, cells in tissue culture were used to examine whether there is a relationship between hPDK4 and factors that are known to induce apoptosis. These factors include TNF α , C2-Ceramide, and Linoleic acid (Mu *et al.*, 2001, Lee *et al.*, 2005, Bocca *et al.*, 2007 and Lihui *et al.*, 2007, and Haimovits-Friedman *et al.*, 1997). Consistent with previously reported work, these factors induces cell death (**Figures 14 – 19**) and apoptosis (**Figure 20**) at defined doses and timeframes in Hela and HepG2 cells.

4.2.2 The effect of TNF α , C2-Ceramide and Linoleic acid on the level of PDK4 mRNA expression

The effect of TNF α , C2-Ceramide and Linoleic acid on the PDK4 mRNA levels was investigated. For this purpose, Hep G2 cells were treated with TNF α , C2-Ceramide and Linoleic acid at dosage and time period that had been previously determined to be suitable for induction of apoptosis. Real-time PCR experiments performed on RNA extracted from these cells indicated that all these factors decreased the levels of hPDK4 mRNA to different degrees when compared to levels observed in control (untreated) cells. Linoleic acid had the least effect on PDK4 expression levels compared to the other two inducers. TNF α and C2-Ceramide had nearly similar effect on the PDK4 mRNA expression levels. The results showed by TNF α and C2-Ceramide maybe due to the fact that TNF α uses ceramide as one of its down stream signaling molecules when it induces apoptosis (Lee *et al.*, 2005 and Haimovits-Friedman *et al.*, 1997). These findings support the hypothesis that PDK4 plays a role in apoptosis.

CHAPTER 5

5 Conclusion

The goal of this research was to explore potential interaction between PDK4 and other proteins in order to further understand how the activity of PDK4 is regulated and how this might impact on the development of insulin resistance and diabetes. Analysis performed using computational methods uncovered numerous proteins that contained domains from several protein families that were predicted to interact with hPDK4. Among the families identified was the death domain containing protein family which is known to be important in apoptosis, suggesting a role for hPDK4 in apoptosis. Of particular interest to us were the subunits of NF κ B since NF κ B is also known to play a role in insulin resistance and diabetes. Further, computational analysis revealed that amino acids that are known to participate in the dimerization of NF κ B subunits are also conserved between hPDK4 and NF κ B, as well as in PDK isoforms. Further, these amino acids are located in the solvent accessible region of PDK indicating accessibility to PDK substrates. Results from these studies strongly indicate interaction between hPDK4 and NF κ B and strongly suggest a role for hPDK4 in apoptosis.

Our studies in Hela and HepG2 cells confirmed a relationship between hPDK4 and apoptosis. These studies demonstrated that treatment of these cells with TNF α , C2-Ceramide and Linoleic acid led to the induction of cell death and apoptosis, as well as reduction in levels of hPDK4 mRNA. These results suggest an anti-apoptotic role for PDK4. To further understand the underlying mechanism involved in the possible interaction between PDK4 and the subunits of NF κ B and its involvement in apoptosis

more studies are required. Over expression of hPDK4 would enable the examination of whether hPDK4 protects cells against the induction of apoptosis. Likewise, it would be interesting to explore whether inhibition of hPDK4 by RNA interference or specific inhibitors results in induction of apoptosis in mammalian cells. Immunoprecipitation experiments would also confirm whether there is interaction between hPDK4 and any of the NF κ B subunits. Site directed mutagenesis of the conserved amino acids (on hPDK4) would also demonstrate their involvement in this interaction and their role in regulating apoptosis.

We postulate that PDK4 interacts with p100 and phosphorylates it on serine residues. Phosphorylation of p100 by PDK4 prevents association of p100 with IKK and thus prevents the generation of p50 and the formation of a functional NF κ B hetero- dimer between p50 and p65. This therefore prevents induction of pro-apoptotic genes. Therefore induction of pro-apoptotic genes would require suppression of PDK4. Serine phosphorylation of signalling molecules has previously been shown to block signalling cascades and result in insulin resistance (Waraich *et al.*, 2008, Xu *et al.*, 2008, Feng *et al.*, 2008, Tremblay *et al.*, 2007, Coba *et al.*, 2004 and Takane *et al.*, 2001) as well as growth hormone resistance (Ahmed *et al.*, 2005, Shumate *et al.*, 2005, Coba *et al.*, 2004 and Takane *et al.*, 2001). Verification of this hypothesis with regard to the role of PDK4 in NF κ B mediated signal transduction would require utilization of PDK4 kinase activity assays with p100 as substrate, to demonstrate that PDK4 results in phosphorylation of p100. Further, phosphorylation sites would have to be mapped on p100 and the role of phosphorylation of these sites in signal transduction would have to be elucidated. Studies examining the impact of over-expression of PDK4 on apoptosis would add tremendous value in verifying our hypothesis. The model we are postulating is demonstrated in figure 34 below.

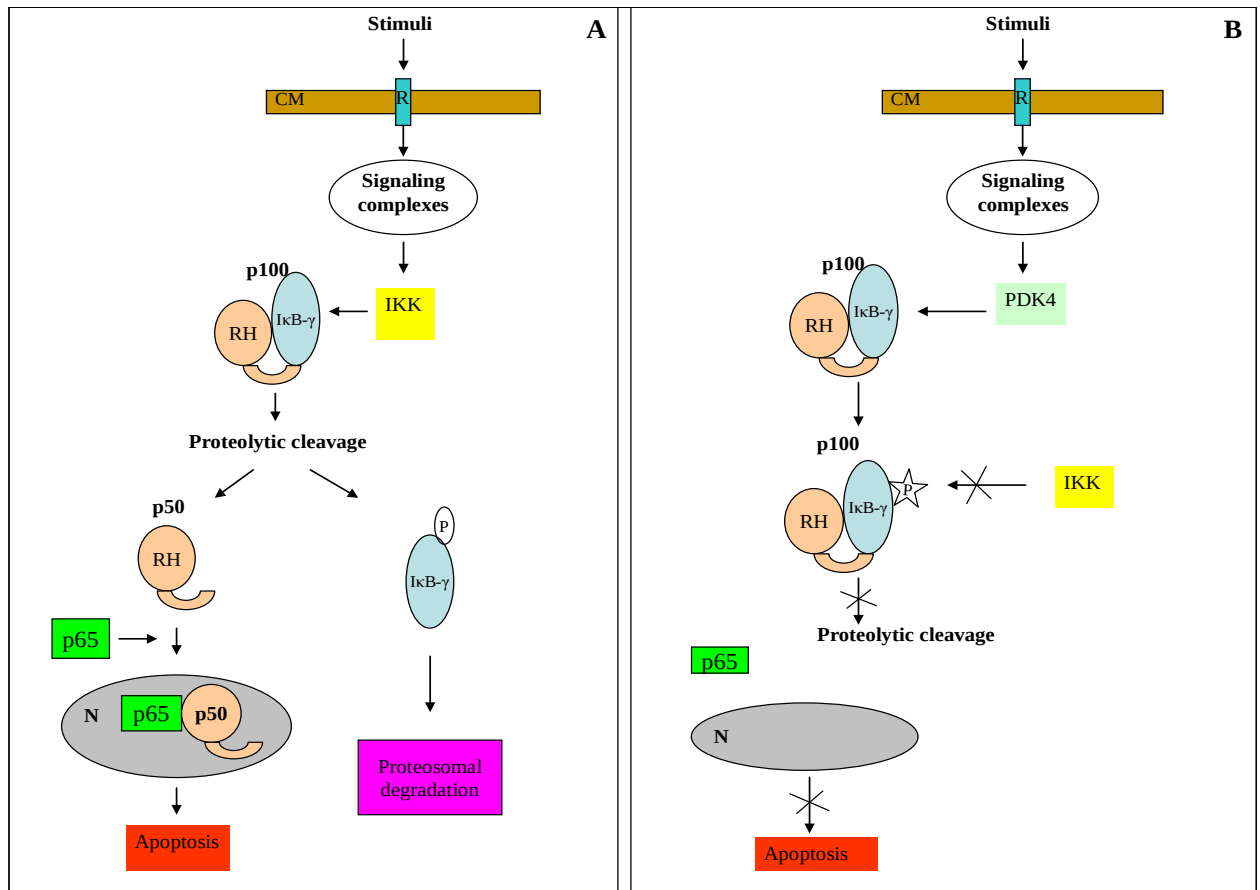


Figure 34. The schematic diagram indicating the possible role of PDK4 in preventing non-classical NFκB signalling. (A) The classical NFκB signalling pathways and (B) Blocking of the non-classical NFκB pathway by PDK4. CM stands for cell membrane, R stands for receptor and N stands for nucleus. RH stands for Rel homology domain. Oval P stands for the phosphorylation event that enables signalling and star P indicates the possible phosphorylation event that would prevent normal NFκB signalling. Crossed arrows indicate blocked events and un-crossed arrows indicate events that are permitted to occur.

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