An Investigation for Genetic Susceptibility to Type 1 Diabetes Mellitus and its Microvascular Complications

by

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To

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An Investigation for Genetic Susceptibility to Type 1 Diabetes Mellitus and its Microvascular Complications

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Abstract

Long-term exposure to diabetes mellitus is associated with metabolic abnormalities such as chronic hyperglycemia and redox imbalance. Uncontrolled hyperglycaemia and genetic factors are implicated in the pathogenesis of diabetic microvascular diseases. Genetic mutation through the genes coding for enzymes involved in glucose metabolism and immuno-regulatory mechanisms may contribute to the susceptibility to type 1 diabetes mellitus (TIDM) and its chronic microvascular diseases. Previous studies have shown that the transcription factor, nuclear factor kappa B (NFκB) and heat shock proteins (HSPs) are two redox-sensitive cellular responses of most immune and inflammatory diseases including diabetes and its late vascular complications. NFκB promotes the transcription of a wide array of proinflammatory mediators and adhesion molecules. HSPs are proposed to have a cytoprotective effect; in contrast they have the capacity to promote pathogenic processes. In this study, polymerase chain reaction (PCR)-based microsatellite analysis and restriction fragment length polymorphism (RFLP) analysis were used to genotype the genes coding for NFκB, HSP70-A2, sorbitol dehydrogenase (SORD) and protein kinase C-β (PKC-β). The A10 allele of the NFκB gene and H3 and H7 alleles of the HSP70-A2 gene were identified as risk markers of TIDM ($P< 0.01$). These alleles were not associated with microvascular complications. No evidence of associations was obtained between either PKC-β or SORD genes with TIDM and its late complications. Uncontrolled hyperglycaemia may alter the transcription mechanism of many genes, which control vascular homeostasis. The electrophoresis mobility shift assay (EMSA) was used to assess the transcription factor, heat shock factor-1 (HSF-1) and NFκB-DNA binding activity in response to a concentration of 31mM D- glucose in peripheral blood mononuclear

cells (PBMCs) from patients with TIDM with and without microvascular complications. Hyperglycaemia induced significant increases in both NFκB and HSF-1-DNA binding activities in PBMCs from patients ($p= 0.003$ and 0.017 respectively). The protein activity was more pronounced in PBMCs from patients with microvascular complications. Hyperglycaemia-induced NFκB-DNA binding activity was correlated to that of HSF-1 ($p< 0.01$). Patients with TIDM with microvascular complications demonstrated a significant increase in NFκB-DNA binding activity compared to patients with a short duration of diabetes (SD) or diabetic controls (DC) ($p= 0.003$ and $p = 0.047$ respectively).

A significant positive correlation was found between the duration of diabetes and hyperglycaemia-induced NFκB-DNA binding activity ($p=0.035$). These results suggest that hyperactive flux through the polyol pathway is relevant to hyperglycaemia-induced protein activity since the aldose reductase inhibitors (ARIs) zopolrestat and sorbinil reduced HSF-1 and NFκB-DNA binding activity in PBMCs. In conclusion, these results suggest that NFκB and HSP70-A2 genes may contribute to the genetic susceptibility to TIDM. Uncontrolled hyperglycaemia in diabetes may alter the transcription mechanism and magnify the proinflammatory responses, which accelerate the development of diabetic microvascular complications.

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award. This study was carried out in The Department of Molecular Medicine, University of Plymouth. The programme was an investigation for genetic susceptibility to Type 1 Diabetes Mellitus and its microvascular complications. Relevant scientific conferences were regularly attended at which work was often presented with a published paper and several papers prepared for publication.

Signed: Doha Hegazy

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Abbreviation

3` UTR	3` Un-Translated Region
5` UTR	5` Un-Translated Region
ACE	Angiotensin Converting Enzyme
ADA	American Diabetes Association
AGEs	Advanced Glycated End products
A I	Angiotensin I
A II	Angiotensin II
ALR2	Aldose Reductase Enzyme
APS	Ammonium PerSulphate
ARIs	Aldose Reductase Inhibitor
ASO	Allele Specific Oligonucleotide
ATP	Adenosin Tri-Phosphate
AU	Arbitrary Unit
BB	Bio-Breeding rat
BDA	British Diabetes Association
Bp	basepair
BSA	Bovine Serum Albumin
Cpm	Counts per minute
DAG	Di-Acyl-Glycerol
DC	Diabetic Controls
DCCT	Diabetes Control and Complication Trials
dd H2O	double distilled water
DERIG	Diabetes Epidemiology Research International Group
DN	Diabetic Nephropathy
DNA	Deoxy ribo- Nucleic Acid
DNU	Diabetic Neuropathy
DR	Diabetic Retinopathy
DUK	Diabetes UK
E6.1	Jurkat cell line
ECCC	European Collection of Cell Culture
ECM	Extra-Cellular Matrix
EDTA	Ethylyne-Diamine-Tetra-Acetic acid

EMSA	Electrophoresis Mobility Shift Assay
ESRD	End Stage Renal Disease
ET-1	Endothelin 1
FASEB	Federation of American Societies for Experimental Biology
FCS	Foetal Calf Serum
FPG	Fasting Plasma Glucose
FS	Fragment Size
GAD	Glutamic Acid Decarboxylase
GENEDIAB	Génétique de la Néphropathie Diabétique
GLUT	Glucose Transporter
Hep G2	Hepatic cell line
HLA	Human Leukocyte Antigen
HO	Hyperosmolar Stress
HSF-1	Heat Shock Factor 1
HSPs	Heat Shock Proteins
HUVEC	Human Umbilical Vein Endothelial Cells
IAA	Insulin Auto Antibodies
ICA	Islets Cell Auto-antibodies
ICAM	Intra Cellular Adhesion Molecule
IDDM	Insulin Dependent Diabetes Mellitus
IFN-γ	Interferon gamma
IGT	Impaired Glucose Tolerance
ILs	Interleukins
INS	Insulin Gene
IU	International Unit
JNK	C-Junction N-terminal kinases
KDa	Kilo Dalton
LDL	Low Density Lipids
LPS	Lipopoly Sacccharide
MA	Microalbuminuria
MAPK p38	Mitogen activated protein kinase p38
MAPKs	Mitogen Activated Protein Kinases
MCP-1	Monocyte Chemoatactant Protein-1
MEM	Minimum Essential Medium
MHC	Major Histocompatability Complex

MODY	Maturity Onset Diabetes in the young
mRNA	Messenger Ribo-Nucleic Acid
n	Number
N	Unstressed Cells
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Reduced form Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced form Nicotinamide Adenine Dinucleotide Phosphate
NC	Normal healthy Controls
NDDG	National Diabetes Data Group
NFκB	Nuclear Factor kappa B
NG	Normo-glycemia
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NIH	National Institute of Health
NO	Nitric Oxide
NOD	None Obese Diabetic mouse
OD	Optical Density
OGTT	Oral Glucose Tolerance Test
p	Short arm of chromosome
p.s.i	pressure specific index
PAGE	Poly-acrylamide Gel Electrophoresis
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PKC-β	Protein Kinase C- beta
PON1	Paraoxonase 1
PON2	Paraoxonase 2
q	Long arm of chromosome
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo-Nucleic Acid

ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute 1640
s.d.	Standard Deviation
SD	Patients with DM for Short Duration
SDS	Sodium Dodecyle Sulphate
SNP	Single Nucleotide Polymorphism
SORD	Sorbitol Dehydrogenase enzyme
T1DM	Type 1 Diabetes Mellitus
T1DM-Comp	Patients with T1DM with microvascular Complications
T1DM-SD	Patients with T1DM for Short Duration
T2DM	Type 2 Diabetes Mellitus
T2DM-Comp	Patients with T2DM with microvascular Complications
T2DM-SD	Patients with T2DM for Short Duration
T4PNK	T4 Poly-Nucleotide Kinase
TEMED	Tetra-Methyl-Ethylene-Diamine
UAER	Urinary Albumin Excretion Rate
UK	United Kingdom
UKPDS	UK Prospective Diabetes Study
USA	United States of America
VCAM	Vascular Cell Adhesion Molecule
VSMC	Vascular Smooth Muscle Cell
WHO	World Health Organization

Chapter 1

INTRODUCTION

1.1 Diabetes mellitus: Overview of diabetic research and genetic studies

Diabetes is the most common metabolic syndrome and is characterised by hyperglycaemia. The disease is a disorder of glucose homeostasis due to defects in insulin secretion, insulin action, or both (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998; World Health Organization (WHO), 1999). Insulin deficiency is associated with increased levels of diabetogenic hormones including epinephrine, glucagon, cortisol, and growth hormones, which promote insulin resistance (Marks et al., 1996). The National Diabetes Data Group (NDDG) and WHO differentiate diabetes into two main types: insulin dependent diabetes mellitus (most recently known as TIDM) and non- insulin dependent diabetes mellitus known as type II (TIIDM) (NDDG, 1979; WHO, 1985). Diabetes, like certain other diseases such as cancer, asthma, and hypertension, tends to run in families but does not show typical Mendelian pedigree patterns (Froguel, 1999; Gambaro et al., 2000). People with diabetes are at increased risk of progressive development of cardiovascular, peripheral and cerebro-vascular diseases (Diabetes Control and Complications Trials (DCCT), 1993).

Chronic hyperglycaemia is the main initiator of late diabetic vascular complications, which are the major cause of morbidity and mortality in patients with diabetes (DCCT, 2000; UK Prospective Diabetes Study (UKPDS) 35, 2000). The mechanisms underlying the long-term vascular abnormalities of diabetes have attracted considerable attention. There have been numerous diabetes prevention and control trials throughout the world to determine if TIDM and its late vascular complications could be prevented or delayed. DCCT and the NDDG are clinical studies conducted by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the National Institute of Health (NIH) in United States of America (USA). In the United Kingdom (UK), the UKPDS was a multi-centre study

looking at TIIDM. These studies showed that keeping blood glucose level as close to normal as possible may limit the progress of diabetic complications (DCCT, 1993; NIH, 1994; 2002; UKPDS 33 and 34, 1998; UKPDS 35, 2000).

Identification of people at risk for diabetes and its complications may help to reduce its morbidity and mortality and reduce the costs associated with treatment. Genome-wide scanning and candidate genes are general lines that help to identify the genetic causes of TIDM and its complications. Association analysis, family studies and animal models help to identify candidate genes.

Association analysis describes a comparison of the frequency of polymorphic genes in patients versus matched control populations (ethnic, sex, age, and lifestyle) and may suggest a causative role for the gene polymorphism (Cavan et al., 1992). Multiplex family linkage analysis could determine whether there is evidence for co-segregation of a susceptibility locus and a disease marker in families with multiple affected persons (Wolf et al., 1983; Hashimoto et al., 1994; Davis et al., 1994). In the UK, the largest collection of multiplex families is contained in the DUK Warren Repository formerly British Diabetic Association (BDA) (Bain et al., 1990). The transmission disequilibrium test is a family-based association test. It considers a population sample of the disease (trios of affected individuals and their parents). A significant difference between transmitted and un-transmitted alleles could confirm the associations. However, an unknown mode of inheritance of the disease (polygenic), low penetrance, inadequate family size and environmental effects may reduce the power of family studies (Cavan et al., 1992; Bennett et al., 1995). So, affected sib-pair linkage analysis could determine the proportion of shared alleles.

Animal models have enhanced understanding of the aetiology of TIDM and support the understanding of the molecular mechanisms of the disease and its complications. Animal studies have facilitated close investigation of linkage and segregation of marker genes with various diseases. The Bio breeding rat (BB) and non-obese diabetic mouse (NOD) are the main animal models of autoimmune diabetes. They have diabetogenic loci within the major histocompatibility complex (MHC), and spontaneously develop a diabetic syndrome similar to human TIDM (Yoon et al., 2001).

1.1.1 Classification of diabetes mellitus

1.1.1.1 Type I diabetes mellitus (TIDM)

TIDM is the third most prevalent chronic disease of childhood after asthma and mental retardation (Rewers et al., 1997). It is an autoimmune-inflammatory disease, leading to progressive loss of pancreatic islet β cells and decreased insulin production (DCCT, 1993; The expert committee on the diagnosis and classification of diabetes mellitus 1998; American Diabetes Association (ADA), 2002). TIDM was formerly termed juvenile diabetes because it presents mainly in children and young adult. The peak incidence is between 5-15 years and it accounts for 10% of people with diabetes worldwide (DERIG, 1990; Karvonen et al., 2000). Patients with TIDM are dependent on insulin to preserve life. Extensive exposure to diabetes contributes to the development of microvascular complications, which is associated with increased incidence of morbidity and mortality (DCCT, 1993). The disease is described as a complex trait because its pathogenesis involves both environmental and genetic factors (polygenic). Environmental triggers such as infection may activate autoimmune mechanisms in genetically susceptible individuals. TIDM belongs to a family of diseases characterised by their association with the MHC and appearance of antibodies against self-proteins (Todd, 1995; Todd et al., 2001).

TIDM can be associated with other endocrine disorders and represents an autoimmune polyendocrine syndrome in which patients develop problems in numerous glands including insulin deficiency, hypogonadism, adrenal insufficiency, and hyper or hypothyroidism (Nagamine et al., 1997). TIDM can co-exist with other autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS) and rheumatoid arthritis (RA) (Dotta et al., 1997; Todd et al., 2001).

1.1.1.2 Type II diabetes mellitus

Adult (maturity) onset diabetes is known as TIIDM. The onset is usually after the age of 30 years and is characterised by insulin resistance in peripheral tissues which is usually combined with a functional defect of β cell and insulin deficiency (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998; UKPDS 33 and 34, 1998). Obesity and environmental factors are probably involved in the pathogenesis of TIIDM. Studies have shown that TIIDM is the most common type of diabetes and accounts for 85-90% of all patients with diabetes (Zimmet, 1999; Kukreja et al., 1999).

1.1.1.3 Impaired glucose tolerance

Impaired glucose tolerance (IGT) is identified as borderline or sub-clinical diabetes. The fasting plasma glucose level (FPG) during an oral glucose tolerance test (OGTT) is above normal but below those that are defined as diabetic (fasting < 7mmol/l, after 2 hours 7-11 mmol/l) (The expert committee on the diagnosis and classification of diabetes mellitus 1998). IGT may produce symptoms of either TIDM or TIIDM. It may remain for many years or return back to normal glucose tolerance. The prevalence of hypertension and atherosclerosis increases in this class of individuals (The Expert Committee on the

Diagnosis and Classification of Diabetes Mellitus, 1998; Alberti et al., 1998).

1.1.1.4 Gestational diabetes mellitus

Any degree of glucose intolerance during pregnancy has been identified as gestational diabetes mellitus (GDM). During pregnancy the placenta may cause hormonal disturbance and reduce the insulin effect. Pregnant women at increased risk include obese women and those with a family history of diabetes. The risk for progression to IGT and TIIDM is increased in women who had GDM. Postpartum plasma glucose levels may meet the criteria of TIIDM and IGT or may return back to the normal glucose level (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998; Alberti et al., 1998).

1.1.1.5 Maturity-onset diabetes in the young

Maturity onset diabetes in the young (MODY) is an atypical form of diabetes. It presents a typical clinical course of TIIDM in young age (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998). Clinical examination, family history, an immunologic, genetic and metabolic characters could distinguish this class from TIDM.

1.1.1.6 Other types of diabetes mellitus

Genetic defects, such as in β cell function, insulin action and/or insulin receptor may contribute to the pathogenesis of diabetes. Pancreatic, endocrine, metabolic diseases, infection and trauma may lead to the development of secondary diabetes (Alberti et al., 1998; WHO, 1999).

1.2 Type I diabetes mellitus

1.2.1 A brief history of diabetes mellitus and insulin discovery

Diabetes is a Greek word meaning pipe-like due to the frequent and voluminous urination by patients accompanied with thirst. Mellitus is a Latin word for honey to differentiate the disease from diabetes insipidus, which is a result of a deficiency of antiduretic hormone with excessive sugar-free urine output. The first use of the term diabetes mellitus was accredited to the Greek physician Cappadocia in the second century AD (Bliss, 1996).

The disease was known since the writings from the earliest civilizations (Egypt, China, and India). They showed that the disease was linked to the type of diet and associated with symptoms of polyuria, polydipsia, polyphagia and loss of weight. Later, the physicians referred to it as a disease of the blood, kidneys, liver, or stomach. As early as 1788, Thomas Crawley established a link between poor pancreatic functions and diabetes (Bliss, 1996).

The pancreas is a digestive gland found behind the lower part of the stomach in the left side of the body. In 1869, Paul Langerhans discovered the existence of two systems of cells: the acinar (exocrine) cells, which secrete the digestive pancreatic enzymes and also identified cells that accounted for ~2% of total with un-determined functions and scattered in groups between the exocrine cells that were later identified as islets of Langerhans (Kloppel et al., 1997). Islets of Langerhan are classified into 4 types: 1) Alpha cells that secrete the counter-regulatory hormone glucagon. 2) Beta (β) cells, which produce insulin form 70-80% of the endocrine mass and play a central role in the regulation of carbohydrate metabolism through the action of insulin. 3) Delta cells produce

somatostatin, which is a potent inhibitor of insulin and glucagon. 4) Pancreatic polypeptide cells (PP), which may modulate the intra-islet homeostasis. The interaction of these various cell types is critical for maintaining the integrity of the tissue (Kloppel et al., 1997).

In 1889, Minkowski showed that pancreatectomy in the dog resulted in diabetes. In 1893, Hēdon confirmed that a total pancreatectomy was necessary to cause diabetes. In 1921, Banting and Best discovered insulin. Finally, the fatal disease had been controlled and Nobel prizes were awarded to Banting and Best in 1923, 1958, and 1964 (Bliss, 1996).

1.2.2 Epidemiology of TIDM

The diabetes epidemiology research international group (DERIG) and the WHO play a key role in analysing epidemiological data between countries. Reports from a number of countries have suggested that there is a worldwide increase in incidence of TIDM (3.0 % per year) and this rate is expected to be more than double over the next 25-30 years (Onkamo et al., 1999). Currently, a vast global network of registers of patients with TIDM and major international collaborations are covering incidence, prevalence, morbidity, and mortality rates of TIDM. The disease is common in the western world and is principally a disease of Caucasians (Kukreja et al., 1999). There is a range of global variation in the incidence of TIDM among children aged less than 14 years of age (WHO, 1999). It has been reported that Finland and Sardinia show the greatest incidence rates while China and South America show the lowest incidence rates (DERIG, 1988; Green et al., 1992; Karvonen et al., 2000). A great difference has been detected in the incidence between the countries around the Baltic Sea (Padaiga et al., 1997). In Kuwait as a part of the WHO research group, a higher incidence of TIDM has been observed compared with the other Gulf areas (Shaltout et al., 1995). It is supposed that ethnic and racial distribution may

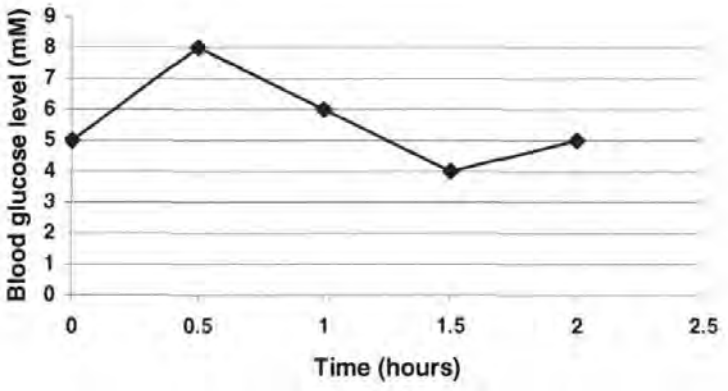
imply the involvement of both the environmental and genetic factors in that variation. Further, reports have shown seasonal trends for TIDM incidence with the highest incidence in winter and a decline during summer time (DERIG, 1988; Zimmet 1999).

1.2.3 Glucose homeostasis

Glucose is the main energy source for cells. Insulin and glucagon are the main hormonal regulators of blood glucose homeostasis (Marks et al., 1996; Kloppel et al., 1997). Insulin is an anabolic hormone, which increases the storage of glucose, fatty acids and amino acids in cells and tissues (elevated in the post-prandial state) while glucagon is a catabolic hormone, which mobilizes glucose, fatty acids and amino acids from stores to blood (elevated during fasting). After a typical meal, the blood glucose level rises from a fasting level of 80-100mg/dL (~5.5 mM) to a level of 120-140 mg/dL (8mM) within 30-60 minutes then reverts back to the fasting level within 2 hours (Figure 1.1 A). High glucose levels in blood stimulate insulin secretion that is degraded within minutes in the liver and kidneys (Marks et al., 1996).

The initial step of the glucose-insulin pathway is the attachment of insulin to the insulin receptor on the β cell membrane. The glucose transporter (Glut) facilitates the transport of glucose into the cell. Glucose is then phosphorylated with an adenosine-tri-phosphate (ATP)-dependent glucokinase, which is the key regulatory enzyme of glycolysis. The increased levels of ATP in the cell prompt the potassium ion channels (ATP-sensitive K^+ channels) to close and calcium ion (Ca^{2+}) channels to open leading to Ca^{2+} influx and insulin secretion (Figure 1.1 B).

(A)



(B)

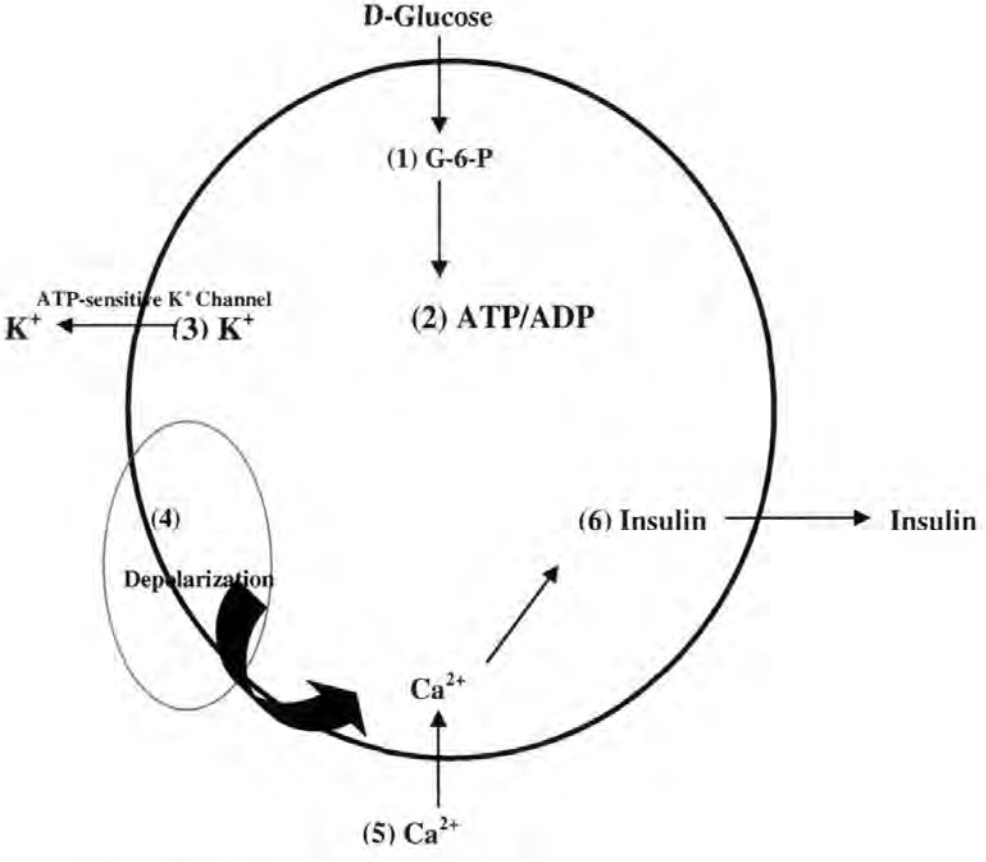


Figure 1.1: Glucose homeostasis

Figure (A) is a graph that shows blood glucose level rising from a fasting level of ~5 mM to a level of 8mM within 30-60 minutes after a meal and back to the fasting level within 2 hours. Figure (B) shows the insulin release from the β cell. Glucokinase (G-6-P) is the rate-limiting step in glycolysis (1), which leads to an increase in ATP/ADP ratio (2), closure of K^+ channel (3), depolarisation (4), and opening of the Ca^{2+} channel (5). Ca^{2+} influx initiates insulin release (6).

This mechanism is underpinned by cycles of changes across the cell membrane and is supported by a dense network of blood vessels, an array of neurons and signalling information. Subsequently, a cascade of protein kinases are recruited to amplify the effects of insulin to lower blood glucose levels, which is over a million fold higher than that of insulin. β cell-released insulin correlates with the glucose levels in the blood stream. β cell contains insulin granules and its precursor molecule (C-peptide) in a 1:1 molar ratio. The insulin dose of 40 IU (IU= $25x10^9$ insulin granules) is needed for physiological demand /day. The major actions of insulin are: 1) facilitation of glucose transport through certain membranes; 2) enhancement of the conversion of glucose to glycogen; 3) slowing down of gluconeogenesis in liver and muscle cells; 4) promotion of protein synthesis and growth (Marks et al., 1996; Kloppel et al., 1997).

1.2.4 Pathogenesis of β cell destruction

TIDM is an incurable progressive autoimmune disease due to immune-mediated pancreatic β cell destruction. There is no clear explanation for the onset of the disease. Glutamic acid decarboxylase (GAD) (Baekkeskov et al., 1990), insulin autoantibodies (IAA) (Serreze et al., 1988), and islet cell antibodies (ICA) (Bottazzo et al., 1974) serve as auto-antigens and direct the immune system to attack the β cell.

Macrophages, B cells, and T cells are all implicated in the pathogenesis of autoimmune TIDM (Van Noort et al., 1998). Macrophages are the first group of cells, which infiltrate the islets during the early stage of insulitis. They play an important role in β cell destruction through the generation of reactive oxygen species (ROS) and the release of cytokines including interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) (Yoon et al., 1998). Macrophage-derived cytokines recruit T

cells, and macrophages act as antigen presenting cell (APC) for β cell auto-antigens to T cells and initiate T cell- auto reactive response (Yoon et al., 2001). It is known that T cells are classified according to the glycoproteins co-receptors of two types: $CD4^+$ and CD8+. Antigen response occurs in two phases: 1) the activation phase in which the antigen is displayed by the APC with the help of class II MHC and binds to CD4 (incomplete immune response). 2) an elimination phase (immune response) in which co-stimulatory molecules activate transcription factors in T cells that bind to cytokines promoter region, initiate their transcription and contribute to T cell proliferation.

It has been suggested that apoptosis (programmed cell death) is the main mode of β cell damage leading to TIDM (Eizirik et al., 2001a and 2001b; Mandrup-Poulsen 2001). Apoptosis is a complicated cascade of pathological events that starts with an initial signal and ends in DNA fragmentation. Studies have shown that IL-1β, TNF-α and IFN-γ either alone or in combination, are able to induce β cells apoptosis (Green et al., 1999; Kukreja et al., 1999; Eizirik et al., 2001a and 2001b). IL-1β plays a crucial role in the pathogenesis of TIDM through three major pathways: the activation of the transcription factor, NFκB, mitogen-activated protein kinases (MAPKs), and PKC (Eizirik 1996; Eizirik et al., 2001; Cardozo et al., 2001). Specific inhibition of IL-1β could be a target for therapeutic programs (Cailleau et al., 1997). Studies have shown that TNF-α enhances the presentation of islet antigen to both effectors CD4+ and CD8+ T cells and it can participate in the autoimmune reaction through activation of NFκB (Green et al., 1999; Yoon et al., 1999).

Animal studies have shown that the process of β cell destruction is chronic and takes days to weeks (Kukreja et al., 1999), while this process could proceed for several months or years in the human (Eizirik et al., 2001). Human islet β cell has been shown to be more resistant than animal's suggesting an interspecies difference in the destruction and repair mechanisms (Eizirik et al., 1994). It is shown that cytokines in general and IL-1β in particular can induce HSPs and superoxide dismutase that may protect against damage

(Bellmann et al., 1995; Eizirik, 1996; Burkart et al., 2000).

1.2.5 Clinical diagnosis of TIDM

Clinical diagnosis of TIDM includes two main stages: 1) pre-clinical (insulitis); 2) overt diabetes, which often leads to the development of acute and chronic complications (Rewers et al., 1997).

1.2.5.1 Insulitis

Autoimmune destruction of β cells is thought to be completely asymptomatic until 80-90% of the cells are lost (Tisch et al., 1996). The mechanism of destruction is variable from patient to patient (Kukreja et al., 1999). However, animal studies have shown that insulitis does not progress to diabetes in all cases (Benoist et al., 1997; Noorchashm et al., 1997; Van Noort et al., 1998). Human insulitis starts several years before the clinical stage of the disease and can be diagnosed by antibodies. ICA, IAA and GAD are the prevalent immunonological factors in patients with TIDM (Bottazzo et al., 1974; Serreze et al., 1988; Baekkeskov et al., 1990; Leslie et al., 1999).

1.2.5.2 Overt TIDM

The ADA and the WHO state that FPG level of 7 mmol /L or above confirms a diagnosis of diabetes (Alberti et al., 1998; ADA, 2001). The diagnosis of TIDM is straightforward in almost all cases. In asymptomatic subjects, the diagnosis of diabetes is based on at least two abnormal FPG results. The detection of glucose in the urine is often the first indicator of the diagnosis of the disease. However, about 20-40% of patients present in diabetic ketoacidosis (DKA) (DCCT, 1993).

Symptoms of diabetes are clearly identified. A classical phenotype of the disease is weight loss, polyuria, polydipsia, polyphagia, blurred vision and fatigue (DCCT, 1993; The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998). There are no clear macroscopic pathological changes of the pancreas from patients with recent TIDM (<1 year), but the chronic disease shows a noticeable decrease in the weight and size of the pancreas due to cell atrophy. Those changes depend on the duration of the disease, age at onset, and the degree of the microangiopathy. Immunocytochemistry and electron microscopy reveal that the majority of islets are devoid of β cells in patients with TIDM (Kloppel et al., 1997).

1.2.6 Susceptibility factors to TIDM

TIDM is a complex trait; both genetic and environmental factors are involved in the aetiology of the disease. Family and twin studies have shown that genetic factors play an important role in the susceptibility to TIDM. The frequency of the disease in siblings of diabetics is 15 times more common than in the general population (Field, 2002). Twin studies have demonstrated a higher rate in monozygotic twins (100 % shared genes) than in dizygotic twins (average 50% shared genes) (Barnett et al., 1981; Kumar et al., 1993). However, concordance rates in monozygotic twins are not 100%, suggesting that non-genetic factors must also contribute to the development of TIDM. The genetic component to TIDM is polygenic and heterogeneous. Mapping the whole genome identified several combinations of susceptibility and protective genes. The power of genome wide screening studies depends on the number of families, the quality of clinical phenotyping and the selection criteria (reviewed in Cavan, 1992; Todd, 1995; Field, 2002).

1.2.6.1 Genetic factors

TIDM accounts for 10% of cases of diabetes in the UK, approximately 60-70 % of genetic susceptibility is associated with MHC class II markers (Cavan et al., 1992; Barnett et al., 1994). The identification of non-MHC loci was helped by the generation of polymorphic markers, which are spread throughout the human genome, PCR-based microsatellite markers and DNA from numerous multiplex families. The genetic mapping of susceptibility loci for TIDM has proposed more than 20 putative diabetes-predisposing genes. Most of the identified risk markers may modify a T cell-dependent progression from a benign to a destructive stage and contribute to the development of the disease. However, these genetic loci display a much weaker linkage to disease than the MHC region susceptibility genes and they are in strong linkage disequilibrium (Cavan et al., 1992; Field, 2002). All identified susceptibility loci are thought to be equally important and necessary for the disease development. Perhaps, if the predisposing genes act together, they could alter the immunological responsiveness and cause disease.

1.2.6.1.1 Major histocompatibility complex (MHC)

The human version of the MHC is the human leukocyte antigen (HLA) on chromosome 6. The MHC is critical for the function and regulation of the immune response. HLA molecules play a fundamental role in presenting self and foreign antigens to the T cells (Klein et al., 2000). It consists of several genes, which are highly polymorphic, closely linked and fall into three classes. Class I genes consist of HLA-A, B, and C. Class II genes are divided into the main sub-regions DP, DQ and DR. Each family contains two subunits α and β chain respectively (Owerbach et al., 1996; Klein et al., 2000). Class III genes encode complement components (Owerbach et al., 1996).

In diabetes, the early serological studies have shown that class I HLA antigen was associated with TIDM (Nerup et al., 1974). Further studies have shown that HLA class II on 6p21.3 contains one or more major genetic determinants of susceptibility to TIDM. The DQ and DR molecules have been suggested to influence the efficiency of autoantigen presentation and the degree of pancreatic β cell destruction during the course of the disease (Platz et al., 1981; Wolf et al., 1983; Todd, 1987; Becker, 1999).

In Caucasoid populations, family studies have shown that HLA DR3 and/or DR4 alleles are strongly associated with susceptibility to TIDM. The relative risk for TIDM in individuals who have both DR3 and DR4 susceptibility alleles (heterozygous) is greater than in those homozygous for either DR3 or DR4 (Platz et al., 1981; Wolf et al., 1983). Sequencing studies have shown that DQB1 is strongly associated with susceptibility to TIDM when it encodes a non-aspartic acid (Asp) at residue 57 of the β-chain. Studies have revealed that disease associated haplotypes containing HLA-DR3 and HLA-DR4 have alanine (Ala) or serine (Ser) at residue 57 of the β-chain, while protective haplotypes contain HLA-DR2 and Asp at position 57 (Todd et al., 1987; Khalil et al., 1990).

DR3, DR4 and DQ markers are associated with TIDM in populations with widely different ethnic origins. However, this association was not confirmed in a Chinese or Japanese populations suggesting that their presence is necessary but it is not the primary disease determinant (reviewed in Cavan et al., 1992; Todd, 1995; Field, 2002). The most potent susceptibility genes to TIDM in the *HLA region* are collectively referred to as IDDM1 (Davies et al., 1994; Hashimoto et al., 1994).

1.2.6.1.2 Non- MHC loci

1.2.6.1.2.1 Insulin-region

Any abnormality of insulin secretion or processing is a possible etiological factor for TIDM. The human insulin gene (INS) was cloned and mapped to chromosome 11 (Bell et al., 1980). A polymorphic region in the 5` untranslated region on the INS gene was first shown to be associated with TIDM in a case-control study in 1984. The marker consists of a variable number of tandem repeats (VNTR) 14 basepair (bp) oligonucleotides and revealed alleles of three sizes (Bell et al., 1984). In unrelated Caucasoid populations, RFLP analysis showed that the frequency of short-length alleles (class I) was increased in patients with TIDM as compared with control populations and the long-length allele (class III) decrease the risk of the disease (Bell et al., 1984; Hitman et al., 1985). General population association studies have also shown an increase in the frequency of the class I alleles in patients with TIDM. However, some family studies have been unable to confirm linkage of these alleles to the disease (reviewed in Todd, 1995; Field 2002). This marker is now referred to as IDDM2 (Davies et al., 1994; Hashimoto et al., 1994).

Genes for tyrosine hydroxylase (TH) and insulin-like growth factor II (IGF2) have been identified on the INS gene region (Bell et al., 1985). They may influence pro-insulin gene expression or may initiate the insulin resistance process. For more detailed analysis, they have been investigated as marker loci. They are in strong linkage disequilibrium with each other and have revealed in-conclusive results (Lucassen et al., 1993; Bennet et al., 1995).

1.2.6.1.2.2 Other putative susceptibility loci for TIDM

Previous studies have reported an association between TIDM and several candidate genes. Current diabetes susceptibility loci have their provisional IDDM gene symbol as assigned by the Human Gene Mapping Nomenclature Committee (Field, 2002) (Table 1.1).

1.2.6.2 Environmental factors

Studies have revealed that the lifestyle contributes to the development of TIDM as well as genetic components. Data from monozygotic twins have shown that 20-40% of identical twins may be susceptible for the disease because of environmental factors (Barnett et al., 1981; Kumar et al., 1993; Leslie et al., 1994). Environmental factors could have a precipitating effect for those biochemical changes in individuals genetically susceptible to TIDM and its complications. Geographical factors, nutritional habits, and infectious diseases are termed as environmental factors, which are believed to influence the onset of TIDM and alter the course of the disease.

1.2.6.2.1 Geographical factors

The incidence rate of TIDM varies between countries. When individuals migrate from a country with a low frequency to a country with a high frequency they become more susceptible to the disease (DERIG, 1988; Patrick et al., 1989). The great majority of patients with TIDM are seen in Europe and North America (Onkamo et al., 1999). Southern Europe regions had generally higher rates (Green et al., 1992). More information concerning the geographical variance has been mentioned in the epidemiology of TIDM (1.2.2).

Marker	Gene map	Reference
IDDM 1	6p21.3	Platz et al., 1981; Wolf et al., 1983
IDDM2	11p15.5	Bell et al., 1984; Hitman et al., 1985
IDDM3	15q26	Field et al., 1994
IDDM 4	11q13	Hashimoto et al., 1994
IDDM 5	6q25	Davies et al., 1994; Delépine et al., 1997
IDDM 6	18q21	Merriman et al., 1996
IDDM 7	2q31	Davies et al., 1994
IDDM 8	6q27	Delépine et al., 1997
IDDM 9	3q21-q25	Todd, 1995
IDDM 10	10p11-q11	Reed et al., 1997
IDDM 12	2q33	Kukreja et al., 1999
IDDM 13	2q35	Field, 2002
Unnamed	12q14-15	Jahromi et al., 2000 a
Unnamed	7p21	Jahromi et al., 2000 b

Table 1.1: Genetic susceptibility loci to TIDM

The table presents some genetic factors that contribute to the development of TIDM as identified by the Human Gene Mapping Nomenclature Committee. The table was modified from the original table (Field, 2002).

1.2.6.2.2 Dietary agents

Studies have shown that nutritional elements could affect the course of TIDM. Feeding babies with cows' milk and cessation of breast-feeding have been associated with TIDM. With the help of DERIG-supplied diabetes registries, Dahl-Jorgensen et al in 1991 found a significant positive correlation with TIDM incidence rate in children 0-14 years of age and fluid cow's milk consumption in 12 countries including UK, Denmark, Sweden, Finland and USA (Dahl-Jorgensen et al., 1991). There was an increased incidence of antibodies to several cows' milk proteins. Some of these proteins may have a similar molecular structure to ICA (Schrezenmeir et al., 2000). Interestingly, an unusually high incidence of TIDM in boys born in October in Iceland has been linked to the high nitrosamine content of a smoked mutton, which is traditionally consumed at Christmas (Helgason et al., 1981).

1.2.6.2.3 Infectious diseases

Viral infections may also act as initiators of the immune-mediated destruction of the pancreatic β cell in genetically susceptible individuals. Epidemiological reports have shown that there was a seasonal peak in the incidence of acute TIDM in autumn and viral infection preceding the onset of the disease (Von Herrath et al., 1998; Karvonen et al., 2000; Yoon et al., 2001). Mumps, rubella, measles, chickenpox and Coxsackie B viruses induce an immune-inflammatory response that may play a role in β cell destruction (Parkkonon et al., 1992; Von Herrath et al., 1998; Yoon et al., 1999, 2001). Animal studies have shown that antibodies to viral proteins might cross-react with islet cells (Hyöty et al., 1995, Yoon et al., 1999). Gestational infection (congenital rubella syndrome) is a major risk factor for the development of TIDM due to persistent virus infection (Noorchashm et al., 1997).

Currently, it is suggested that rubella vaccine could prevent a small proportion of TIDM, which is due to congenital rubella infection (Rewers et al., 1997).

1.2.6.2.4 Other factors

Several reports have shown that the incidence of TIDM varies according to weather and temperature with a peak incidence in autumn and winter (DERIG, 1990). There are many other environmental factors, which might play an important role in the pathogenesis of the disease such as hygiene and toxins. Further, reports from different countries have demonstrated that the incidence of TIDM is related to the economic status. The disease is positively associated in the richest countries such as USA, Germany, and among wealthy Jewish people suggesting that stressful life styles might contribute to the development of TIDM (DERIG, 1988; 1990).

1.3 Diabetic vascular complications

Diabetes is the leading cause of blindness, heart disease, end stage renal disease (ESRD) and debilitating neuropathies (DCCT, 1993; UKPDS, 2000). The endothelium is a major target of damage in susceptible tissues and leads to macro and micro vascular diabetic abnormalities (Calles-Escandon et al., 2001).

Macro-vascular diseases refer to atherosclerotic changes due to deposition of low-density lipoprotein (LDL) to vascular smooth muscle cells (VSMC). These types of complications are more common in TIIDM. The association of TIIDM with altered lipid metabolism, obesity and hypertension would accelerate the development of myocardial infarction and stroke (UKPDS 34, 1998; Donnelly, 2000; Calles-Escandon et al., 2001). Hyperglycaemia and insulin resistance are implicated in the pathogenesis of late diabetic vascular complications in TIDM and TIIDM (UKPDS 33, 1998).

The natural history of diabetic microvascular complications has a multistage course and has an inter-individual variation, which reflects the difference in the underlying pathogenic, genetic and environmental aetiologies. Diabetic microvascular diseases may require 10-20 years to develop in patients with TIDM (DCCT, 1993; NIH, 1994; 2002) while they may develop within 9 years of diagnosis in patients with TIIDM or be present at the time of diagnosis (UKPDS 35, 2000). In general, the intensity and the progression of diabetic vascular complications may depend on the duration of the disease, the degree of hyperglycaemia, age at onset of the disease, gender and family history (DCCT, 1993; UKPDS 33, 34, 1998).

1.3.1 Patho-physiology of late diabetic microvascular complications

Microcirculation processes are responsible for the exchange of metabolites between blood and tissues. The exchange processes act via fine capillaries of arterioles and venules that have less smooth muscle and they are extensively innervated by sympathetic nerve fibres. These critical processes are regulated by the local concentration of metabolites (Pocock and Richards, 1999).

Endothelium is the internal lumen of all vessels and the physical barrier between blood and VSMC. In normal conditions, Endothelial cells (EC) exert their action on both blood cells and VSMC to maintain the vascular homeostasis through the action of a complicated system of chemical mediators that include nitric oxide (NO) and endothelin (ET) (Anrather et al., 1997). The EC play a key role in the pathological mechanisms including inflammatory responses and controlling the trafficking of immuno-components.

The mechanisms of diabetes-induced endothelial abnormalities are not clear. During the course of diabetes, chronic hyperglycaemia and its metabolic consequences are highly expressed in endothelial and mesangial cells (Derubertis et al., 1994). These biochemical anomalies increase the vascular permeability and abnormal blood flow, which reflects vasoconstriction (Calles-Escandon et al., 2001). However, chronic diabetic microvascular complications are associated with alterations in the capillary basement membrane thickness, permeability, and variances in blood flow regulation within the retina, renal glomeruli, and nerves (Sheetz et al., 2002).

EC activation is associated with transcriptional induction of ET-1, E-selectin, vascular cell adhesion molecules (VCAM-1), intracellular adhesion molecules (ICAM-1), cytokines (Guha et al., 2000) and other proinflammatory genes. All of these factors are up regulated by NFκB, which plays a pivotal role in inflammation in different types of cells (Pieper et al., 1997; Quehenberger, 2000). Furthermore, endothelial dysfunction is associated with a variable degree of insulin resistance in patients with TIDM since hyperglycaemia can induce insulin resistance (Vuorinen-Markkola et al., 1992; Yib et al., 1993).

Polyol pathway hyperactivity, oxidative stress, increased advanced glycated end-proteins (AGEs), and activation of PKC isoforms are implicated in the pathogenesis of diabetic-induced endothelial dysfunction and the development of diabetic complications (King et al., 1996; Nishikawa et al., 2000a; Brownlee 2001; Raptis et al., 2001; Way et al 2001).

1.3.1.1 Polyol pathway

Under normal conditions, the polyol pathway plays a minor role in glucose metabolism (Marks et al., 1996; Yabe-Nishimura, 1998). However, in diabetes, hyperglcemia elevates polyol pathway activity (Nishikawa et al., 2000a; Brownlee 2001). The rate-limiting step of the polyol pathway is the reduction of glucose to sorbitol by aldose reductase (ALR2) with the reduced form nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Sorbitol is one of the organic osmolytes that balance the osmotic pressure in the body. Sorbitol accumulation may play a key role in the pathogenesis of diabetic complications (Yabe-Nishimura, 1998).

In the polyol pathway, sorbitol is subsequently converted to fructose by sorbitol dehydrogenase (SORD), which is the second regulating enzyme (Figure 1.2). This reaction is catalysed by nicotinamide adenine dinucleotide (NAD^+). The polyol pathway flux activates ALR2 with overflow of the sorbitol, redox imbalance, pseudohypoxia, ROS, and hyperosmolality (HO). Such metabolic imbalances provoke the early tissue damage in the target organs, such as lens, retina, peripheral nerves, and renal glomeulus (Williamson et al., 1993; Rossing 1998).

Recently it has become evident that polyol pathway hyperactivity is a major contributor to the pathogenesis of vascular diseases in diabetes through redox imbalance and oxidative stress (Williamson et al., 1993; Obrosova et al., 1999 Nishikawa et al., 2000a; 2000b; Brownlee 2001). Previous studies have yielded that the inhibition of polyol pathway may reduce the development of late diabetic microvascular complications (Obrosova et al., 2001; Okayama et al., 2002).

1.3.1.2 Oxidative stress

Oxidative stress occurs when redox homeostasis within the cell is altered. Normally, ROS are formed continuously in the body due to many sources of stress including metabolism, exercise, growth, aging and repair. ROS are in equilibrium with antioxidants and HSPs (Jacquier-Sarlin et al., 1996; Fehrenbach, 2001). Antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and methionine reductase. ROS includes free oxygen radical (O^-), superoxide (O_2), hydroxyl radical (-OH), NO, and hydrogen peroxide (H_2O_2). Oxidative destruction of tissues has been linked to many diseases such as arthritis, hypertension, cancer, tuberculosis and diabetes (Telci et al., 2000).

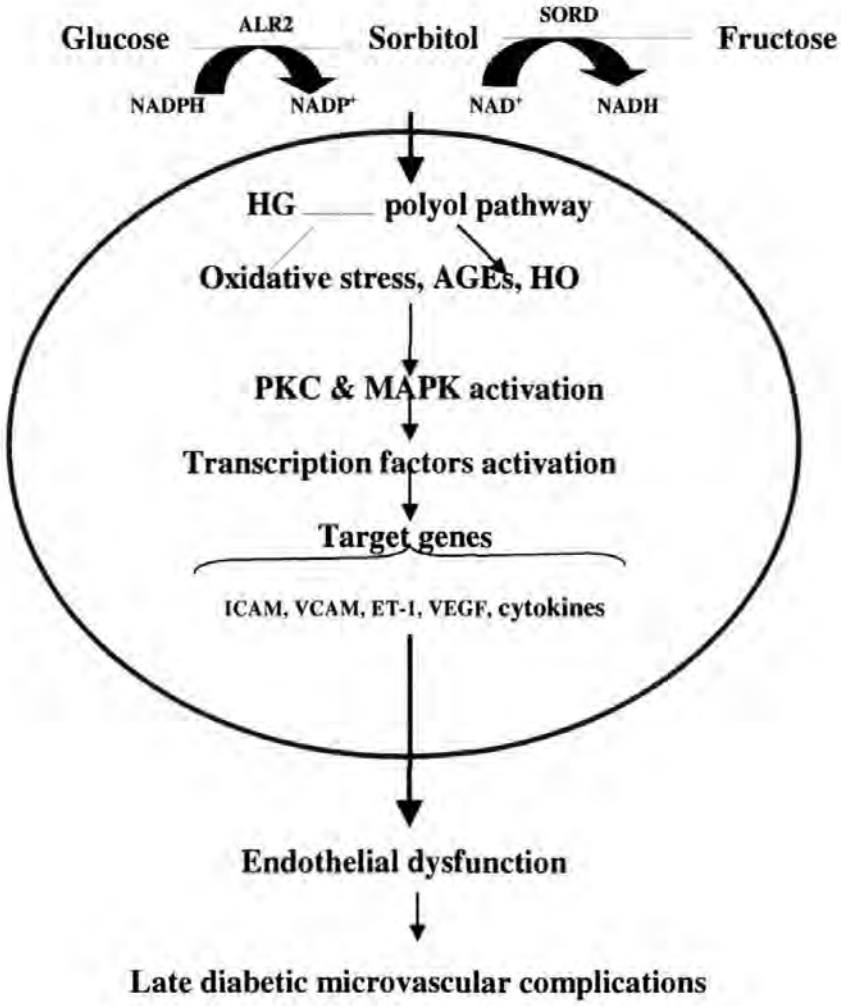


Figure 1.2: Patho-physiology of late diabetic microvascular complications

Hperglycemia–activated polyol pathway and its metabolic consequences. AGEs and redox-imbalance activate signal transduction pathways and transcription factors. The subsequent activation of cytokines and adhesion molecules contribute to the development of the late diabetic microvascular complications. HG=hyperglycaemia, ALR2= aldose reductase, SORD= Sorbitol dehyrogenase, NADPH= reduced form nicotinamide adenine dinucleotide phosphate, $NADP^+$=nicotinamide adenine dinucleotide phosphate, NAD^+= nicotinamide adenine dinucleotide, NADH= reduced form nicotinamide adenine dinucleotide. PKC= protein kinase C, MAPK= mitogen activated protein kinases. ICAM= intracellular adhesion molecules. VCAM= vascular cell adhesion molecules. ET-1= endothelin-1, VEGF= vascular endothelial growth factor.

The chronic hyperglycaemia activated polyol pathway is the main source of ROS in addition to glucose autoxidation, AGEs, and decreased antioxidants (Ceriello et al, 2000; Nishikawa et al., 2000a; b). Generally, ROS may lead to apoptosis and necrosis through DNA damage (Soulis-Liporota et al., 1995; Mohamed et al., 1999; Fehrenbach et al., 2001). Oxidative stress may be responsible for vascular abnormalities and hypertension through the deformation of red blood cells, osmotic fragility, coagulation and delayed replication of the endothelium cells (Nishikawa et al., 2000a). Additionally, ROS leads to endothelial damage and impaired production of endothelium derived relaxation factor, which is commonly known as NO and required to relax arterial smooth muscle. Decreased NO level has been linked to hypertension. ROS are implicated in several metabolic pathways and may function as intracellular messengers that modulate signalling pathways leading to the activation of PKC, MAPK and a number of transcription factors, including the NFκB and activator protein-1 (AP-1) (Jacquier-Sarlin et al., 1996; Mohamed et al., 1999). ROS control the inducible expression of a number of genes involved in cell growth, differentiation, inflammation and immune responses.

Studies have suggested that oxidative stress contributes to the development of the diabetic complications through the regulation of the redox-sensetive transcription factor NFκB (Mohamed et al. 1999) and heat shock proteins (Yabunaka et al., 1995; Johannesen et al., 2001).

1.3.1.3 Advanced glycated end products (AGEs)

The reaction between glucose and primary amino groups of proteins produces AGEs. Hyperglycaemia-induced intra and extra cellular non-enzymatic AGEs are chemically irreversible and recognised by specific cell surface receptors (RAGE) (Hudson et al., 2001). AGEs damage the target cells through different mechanisms: 1) they may alter the

structure and functions of intracellular and extracellular proteins (Brownlee, 2001); 2) AGEs are angiogenic factors and may contribute to the development of diabetic angiopathy by promoting monocyte migration, production of cytokines and growth factors (Soulis-Liparota et al., 1995; Morigi 1998; Friedman 1999; Yamagishi et al., 2002); 3) AGEs can be formed on DNA and may cause mutations and alter the genetic functions (Nishikawa et al., 2000a); 4) AGEs formation is accompanied by oxidative stress and represents a major source for ROS and subsequent activation of NFκB (Bierhaus et al., 1997; Mohamed et al., 1999) and MAPK p38 (Hattori et al., 2002). Studies have detected the role of AGEs in the pathogenesis of diabetic complication and shown that AGEs level correlates with duration of diabetes and severity of complications, especially nephropathy (Doria, 1998; Ceriello et al., 1999). Current data have shown that AGEs inhibitors inhibited a broad range of diabetic complications (Polak et al., 1997; Brownlee 2001).

1.3.1.4 Signal transduction pathways

Extra-cellular signal stimulates cell receptors. The signal transduction pathways transfer signals across the cytoplasm to the nucleus. This mechanism is regulated by a group of protein kinases and phosphatases. Serine/threonine and tyrosine are specific kinases, which initiate the appropriate transcriptional response (Considine et al., 1993; Keenan et al., 1997; Greenham et al., 1998; Murphy et al., 1998; Ceolotto et al., 1999).

1.3.1.4.1 Protein kinase C

PKC is regulated by calcium, phospholipase C, and diacylglycerol (DAG) (Derubertis et al., 1994) and is classified into three main types according to its structures and physiological role. Conventional PKC (cPKC) are Ca^{2+} and phospholipase-dependent protein kinases. Novel PKC (nPKC) and atypical PKC (aPKC) are Ca^{2+} and phospholipid-independent protein kinases (Ceolotto et al., 1999). The PKC system consists of 9 isoenzymes, whose activity varies in different tissues. PKC-β isoform is present in pancreatic islet cells, monocytes, and many vascular tissues including retina, renal (Derubertis et al., 1994) and neural tissues (Way et al., 2001).

In diabetes, hyperglycaemia directly induces PKC activation (DAG-PKC pathway) through the glycolytic pathway and increased levels of intracellular glyceraldhyde-3-phosphate. Also, PKC can indirectly be activated by ROS and AGE (Derubertis et al., 1994; Haneda et al., 1997; Koya et al., 1998, Ceolotto et al., 1999; Tomlinson, 1999; Igarashi, et al., 1999). Once PKC is activated, it induces a number of biochemical events that have a devastating effect on EC and VSMC (Aiello et al., 1997; Koya et al., 1998; Igarashi et al., 1999). In addition, PKC activation can also impact other signalling pathways such as those using MAPKs and nuclear transcription factors including the proinflammatory NFκB (Pieper et al., 1997; Tomlinson, 1999). Reduced PKC activity has been linked to reduced nerve conduction and Na^{+}-K^{+}-ATPase activity in neural tissues (Derubertis et al., 1994) while increased PKC activity have been observed in glomeruli, retinae, aorta and heart (Derubertis et al., 1994; King et al., 1996; Ishii et al., 1998). In vitro and in vivo studies have shown that specific PKC-β isoform inhibitors can prevent the vascular dysfunction (Ishii et al., 1998; Murphy et al., 1998; Ceolotto et al., 1999; Park et al., 2000; Way et al., 2001; Frank, 2002). Inhibition of PKC-β can block hyperglycaemia -induced NFκB

activity, suggesting that PKC induces its devastating effects in diabetes through NFκB activation (Yerneni et al., 1999).

1.3.1.4.2 Mitogen activated protein kinases

The MAPKs consist of three main groups: extra-cellular signal regulated kinases (ERK), C-Junction N-terminal kinases (JNK) and p38. Each of these has many groups. ERKs are growth factor signalling inducers through tyrosine-kinase receptors leading to cell proliferation and differentiation. JNK are stress-activated protein kinases (SAPK), which are triggered by cytokines and environmental stimuli and implicated in cellular responses (Tomlinson, 1999). Studies have suggested that MAPKs have an important role in the pathogenesis of diabetic complications (Haneda et al., 1997; Tomlinson 1999; Igarashi et al., 1999). MAPKs have direct downstream effect on HSPs (Haire et al., 1988) and NFκB (Schulze- Osthoff et al., 1997).

1.3.2 Types of microvascular diabetic complications

1.3.2.1 Diabetic nephropathy

Diabetic nephropathy (DN) is a microvascular complication of diabetes marked by albuminuria and a deteriorating course from normal renal function to ESRD (Figure 1.3). More than 30-40 % of patients with TIDM develop DN after 25 years of the diagnosis of the disease, the peak incidence of DN is at 15-16 years of diabetes duration then it declines rapidly (Kofoed-Enevoldsen et al., 1987). DN is the most common cause of ESRD in the Western world (Rossing 1998).

1- Normal Kidney

2- Diabetic Kidney

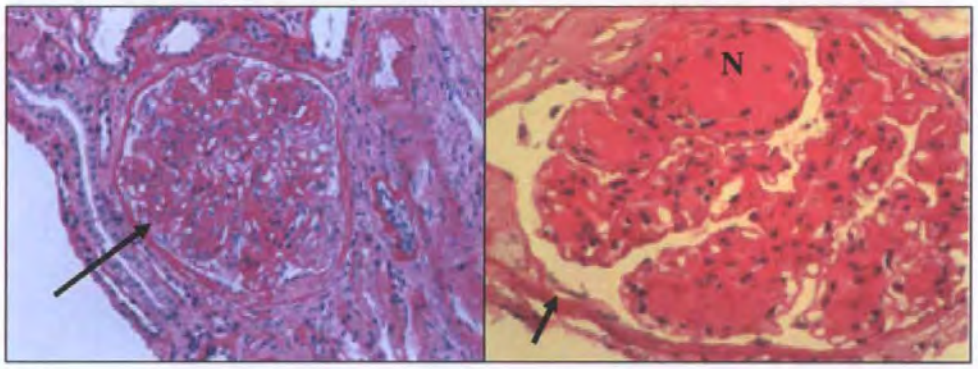
3- ESRD

Figure 1.3: Gross anatomy of kidney during the course of diabetes.

The pictures show the deteriorating course from normal renal function to end stage renal disease (ESRD). Picture 1 shows a normal healthy kidney, diabetic kidneys are shown in picture 2. Small sized atrophied kidneys in the ESRD as shown in picture 3(www.nephron.com).

DN develops in 5 stages (Ibrahim et al.; 1999): 1) Elevated glomerular filtration rate (GFR) by 40% and begins within 0-2 years of disease onset. 2) Increased extra cellular matrix (ECM) and thickening of the basement membrane starts from 2-15 years of disease onset and is clinically silent. This stage is the major histological characteristic of DN (Figure 1.4). The severity of mesangial expansion correlates with the loss of the renal function (Schwartz et al., 1998; Osterby et al., 2002). 3) Microalbuminuria (MA), an established risk factor for renal disease progression in TIDM and its presence is the earliest clinical sign of DN. MA occurs after 10-15 years of disease. DN is suspected when the urinary albumin excretion rate (UAER) is 20-200μg/min. To determine microalbuminuria, 2 out of 3 samples collected over a 6-month period should show abnormal UAER, which is <20 μg/min in normal. 4) Overt nephropathy (onset 15-20 years), the UAER is more than 200mg /24h for three frequent samples. 5) ESRD develops after 20-25 years of disease with a loss of kidney function of up to 3% per month (Bilous, 1997b; Ibrahim et al.; 1999).

Patients with DN are at high risk for macrovascular diseases and always accompanied with diabetic retinopathy (DR) (Bilous, 1997a and 1997b; Schwartz et al., 1998). In patients with TIDM, there is a familial clustering of diabetic renal disease and development of cardiovascular diseases at younger ages (Raptis et al., 2001). Studies have shown that the progression of DN correlates with arterial hypertension, smoking, the family history of nephropathy, the duration, the control of the disease and age at onset (Rossing, 1998; Ibrahim et al.; 1999).



(A) (B)

Figure 1.4: Histo-pathology of diabetic nephropathy

The figure shows pictures by light microscopy of renal tissues with acid-Schiff stain. (A): mesangial hypercellularity with a diffuse expansion, small nodule formation, and thickened glomerular basement membrane (original magnification X200).

(B): established nephropathy, illustrated with large nodules of matrix within mesangial areas with lesser cellularity and apparent thickness in the glomerular basement membrane, original magnification X400). The arrows point the glomerular basement membrane thickening. N = nodule. Pictures were adopted from atlas of renal pathology No.5 (Fogo, 1999).

1.3.2.2 Diabetic retinopathy (DR)

DR is the progressive damage to the retina and its blood supply in diabetes. The abnormalities of the capillary bed of the retina in diabetes include microaneurysms, exudates, and proliferative (angiogenesis) changes. DR is the most common cause of impaired vision, cataract and blindness in people aged 30-69 years (Hamman, 1997; DCCT, 2000). The incidence of DR correlates with duration of diabetes. DR develops in 17% of patients with TIDM of less than 5 years duration and 97.5% in those who had diabetes for 15 or more years (DCCT, 2000). At the beginning, most patients do not notice any changes in their vision, but as DR progresses, macular oedema and a few microaneurysms will develop and lead to blurring vision (Figure1.5) (Freund, 1997). In the advanced (proliferative) stage, retina stimulates the growth of new blood vessels (Figure 1.6). The new vessels can cause vitreous hemorrhage, retinal detachment, and uncontrolled glaucoma (Freund, 1997). The pathological changes are clinically diagnosed by ophthalmoscope, wide angle camera (45°) and stereofundus camera (30°) (Hamman, 1997).

1.3.2.3 Diabetic neuropathy

Diabetic neuropathies (DNU) are a family of nerve disorders caused by diabetes. The damage may involve small fibres, the large fibres or both. People with diabetes can develop nerve problems at any time. The prevalence rate of DNU is 7.5% to 50% in patients who had diabetes for 25 years (Hamman, 1997; NIH, 2002).

(A) (B)

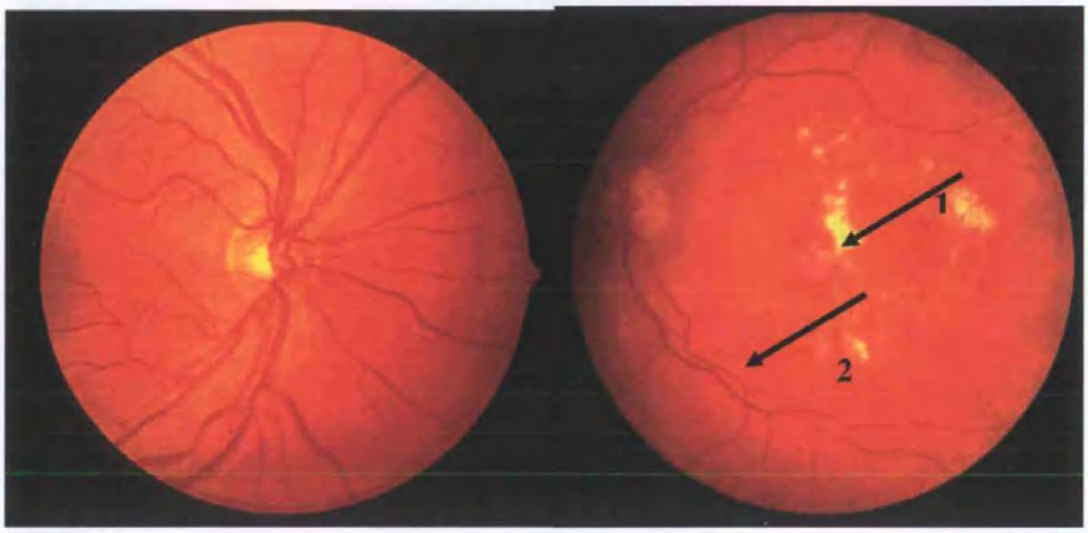


Figure 1.5: Diabetic retinopathy

Ophthalmoscopic examination. Figure A is a photograph of normal retina. Figure B shows a macular oedema (1) and angiogenesis (2) (Freund, 1997).

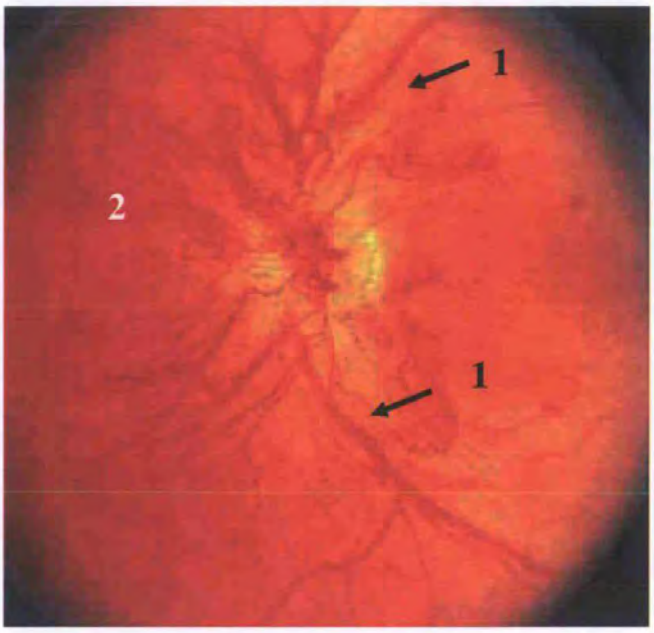


Figure 1.6: Proliferative diabetic retinopathy

Ophthalmoscopic examination. The picture demonstrates proliferative DR (neovascularization) (1) and vitreous hemorrhage (2) (Freund, 1997).

Chronic sensory DNU is the most common type in diabetes and occurs in up to 66% of all diabetics. It is symmetrical and affects the distal sensory nerves and may involve small and large nerve fibres of the arms and legs. It causes either pain or loss of feeling in the toes, feet, legs, hands, and arms (Figure 1.7).

Motor DNU is symmetrical or asymmetrical and causes muscle weakness in hips or thighs with or without pain (Sima et al., 1997). Autonomic DNU causes changes in digestion, bowel and bladder function in addition to sexual dysfunction. It can also affect the nerves that serve the heart and control blood pressure. The symptoms include diarrhoea or constipation, dizziness or faintness due to a drop in postural blood pressure. Focal neuropathy results in the sudden weakness of one nerve, or a group of nerves, causing muscle weakness or pain (Edmonds et al., 1997).

Diabetic foot can be classified into sensory and motor neuropathy (Huntley, 1995). In sensory DNU, the patient is unable to feel the trauma due to loss pain sensation that initiates the tissue damage leading to callus and ulceration (Figure 1.8). Repetitive trauma, persistent hyperemia and chronic inflammation lead to progressive loss of bone strength. X-rays can show the bone and joint destruction. Consequently, many patients eventually progress to amputation following infection or severe deformity (Huntley, 1995; Sima et al., 1997). In motor neuropathy, there is a weakness of foot muscles results in dorsiflexion and the toes drown upwards.

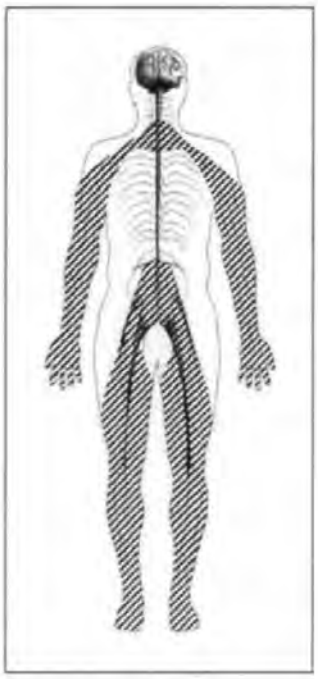


Figure 1.7: Peripheral neuropathy

The shadow parts present the distribution of chronic sensory neuropathy in a symmetrical pattern. The picture is adopted from NIH Publication No. 02-3185, May 2002 (NIH, 2002).

Figure 1.8: Charcot foot

The left side is a picture for the diabetic sensory neuropathy of the foot, which is oedematous and swollen (1). The right side picture shows callus (2) and ulcer (3) in the affected foot (Huntley 1995).

The DNU is diagnosed on the basis of signs and symptoms. A comprehensive foot examination assesses skin, circulation, and sensation. Physical examination for muscle strength, reflexes, and sensitivity to position, vibration and temperature gives a clear neurologic assessment. Electromyography and quantitative sensory testing show the response activity of the muscles to stimuli and ultrasound produces an image of internal organs (Edmond et al., 1997, Sima et al., 1997).

1.3.3 Susceptibility factors to diabetic microvascular complications

1.3.3.1 Genetics susceptibility

Microvascular diseases in TIDM have different patho-physiological background. They are multistage diseases in which several genetic and environmental factors promote the phenotype of each stage. The fact that some diabetic subjects suffer from or are protected against diabetic complications suggests a strong role of genetic factors. Family studies can confirm the role of genetic variation of the candidate genes that influence the risk of micro-vascular diseases in TIDM. Microvascular complications more frequently occur in first-degree relatives (parents, siblings, or offspring) of patients with diabetes with a history of the disease (DCCT, 1997). Studies have shown that a parental history of diabetes or cardiovascular disease is significantly associated with the presence of microvascular complications (Earle et al., 1992). Siblings of patients with TIDM with DN have shown a higher risk to develop DN (Seaquist et al., 1989). A family study on Pima Indian families with TIIDM has determined that proteinuria developed in 45.9% of the offspring if both parents had diabetes while 23% if only one parent was with DN and 14% if both parents had no renal disease (Pettitt et al., 1990).

Genomic screening of the factors involved in glucose metabolism, vascular homeostasis and growth factors may facilitate the identification of the individuals at risk for developing microvascular complications in patients with TIDM. Many candidate genes conferring susceptibility to microvascular complications in diabetes, some of the putative susceptibility loci are listed in Table 1.2.

1.3.3.1.1 Aldose reductase

The action of this enzyme was described in the polyol pathway (1.3.1.1). Studies have suggested that ALR2 activation contributes to the development of chronic diabetic microvascular complications (Yabe-Nishimura, 1998; Ramana et al., 2002). Some studies have shown that ALR2 expression was significantly elevated in patients with diabetic microvascular complications (Shah et al., 1998; Hodgkinson et al., 2001b).

The ALR2 gene is localised to human chromosome 7q35 (Graham et al., 1991). The (CA) dinucleotide repeats (5`ALR2) located 2.1kb upstream of the transcription site of the ALR2 gene was first shown an association with DR and DN in Chinese patients with TIIDM (Ko et al., 1995). The risk allele (Z-2) of this polymorphism was found in association with DN and DNU in Caucasoid patients with TIDM (Heesom et al., 1997). Another bi-allelic polymorphism (C -106 T) at the promoter region of the ALR2 gene may have a functional role on the gene expression (Kao et al., 1999). The C-106 and the Z-2 alleles are strongly associated with DR in patients with TIDM (Kao et al., 1999; Demaine et al., 2000). However, the T -106 allele together with the Z-2 5` ALR2 were associated with DN in patients with TIDM (Moczulski et al., 2000). The association between diabetic complications and two polymorphisms in the promoter region of the ALR2 gene implicates the polyol pathway in the development of microvascular diseases in TIDM.

Map locus	Complication	Reference
7q35 (AKR1B1)	TIDM-DN, DNU and DR TIIDM-DN	Moczulski et al., 2000 Heesom et al., 1997 Kao et al., 1999; Demaine et al., 2000 Ko et al., 1995
1q42-43 (AI)	TIDM-DN	Marre et al., 1997
17q23 (ACE)	TIDM-DN TIIDM-DN	Rigat et al., 1990; Marre et al., 1994; Parving et al., 1996; Fujisawa et al., 1998; Solini et al., 2002
16p11.2 (PRKCB1)	TIDM-DN	Araki et al., 2000a
1p35 (GLUT-1)	TIIDM-DN TIDM-DN	Liu et al., 1999 Hodgkinson et al., 2001a
7q21.3 (PON 1)	TIDM-DR	Kao et al., 2002
7q21.3 (PON 2)	TIDM-DN	Kao et al., 2002
6p12 (VEGF)	TIIDM-DR TIDM-DN	Awata et al., 2002 Yang et al., 2003
19q13.2 (ApoE)	TIDM-DN	Araki et al., 2000b
6p21.3 (RAGE)	TIIDM-DR TIDM-DN	Hudson et al., 2001 Poirier et al., 2001

Table 1.2: Candidate genetic loci for susceptibility to diabetic microvascular complications

The table presents some genetic factors that contribute to susceptibility to microvascular complications in diabetes. AKR1B1 = aldo-keto reductase family 1, member B1. AI= angiotensin 1. ACE= angiotensin1-converting enzyme. PRKCB1= protein kinase C, beta-1. GLUT-1= glucose transport. PON1& 2= Paraoxonase 1& 2. VEGF= Vascular endothelial growth factor. ApoE= apolipoprotein E. RAGE= Receptor of the advanced glycated end products. q= long arm and p= short arm of the chromosomes.

However, other studies have looked at 5`ALR2 microsatellite in diabetic complications and found no association between any of the Z-2 allele and DN or DR and suggested that 5`-ALR2 microsatellite polymorphism is a weak marker for susceptibility to diabetic complications in TIDM (Dyer et al., 1999; Maeda et al., 1999; Ng et al., 2001; Fanelli et al., 2002).

1.3.3.1.2 Renin-angiotensin system

The renin-angiotensin system is important in the control of hemodynamic status. This system consists of four components: renin, angiotensinogen, angiotensin-converting enzyme (ACE), and the angiotensin receptor. Renin is a proteolytic enzyme that is secreted by the kidneys in response to a fall in the sodium concentration. Renin converts angiotensinogen (inactive peptide in the blood) to angiotensin I (AI), which in turn is converted to angiotensin II (A II) by ACE. The later stimulates the secretion of aldosterone from the adrenal cortex and causes vasoconstriction. The system contributes to the development of hypertension and DN (Pocock and Richards, 1999).

The ACE can modulate the risk of DN due to hyperglycaemia through its generating effect for A II, which increases the intraglomerular hydraulic pressure leading to glomerulosclerosis and nephropathy. ACE and angiotensinogen levels may be related to polymorphisms within the genes (Doria, 1998, Rossing 1998). The I/D polymorphism at intron 16 of the ACE gene (17q23), first described by Rigat et al. and was associated with differences in the serum levels of ACE (Rigat et al., 1990). This polymorphism has been extensively proposed as a genetic marker for DN. Several studies have proposed that the D allele is an independent risk factor for the progression of DN in TIDM (Marre et al., 1994; Parving et al., 1996) and TIIDM (Fujisawa et al., 1998; Solini et al., 2002). However,

several studies have found no association between the D allele and DN in either TIDM or TIIDM (Tarnow et al 2000; Fujisawa et al., 1995). GENEDIAB study group in France and Belgium have revealed that the T235 allele of the angiotensinogen gene has been associated with renal function impairment in patients with TIDM (Marre et al., 1997).

1.3.3.1.3 Glucose transporter

Glucose transporter-1 (GLUT1) facilitates cellular glucose uptake. It is an important modulator for cellular abnormalities (Marks et al., 1996). GLUT1 might contribute to the development of renal complications through hyperglycaemia -induced extra-cellular matrix production in glomerular mesangial cells (Wahab et al., 1996). The polymorphic site (Xba-I) has been identified in the second intron of the GLUT1 gene. The RFLP analysis revealed that the 1.1 allele is associated with DN in Chinese patients with TIIDM (Liu et al., 1999) and in Caucasoid TIDM patients with DN (Hodgkinson et al., 2001a).

1.3.3.1.4 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a major mediator of vascular permeability and angiogenesis. The VEGF is an important growth factor in the pathogenesis of DR and intra-ocular neovascularization (Aiello et al., 1995). Studies have shown that VEGF may contribute to the development of diabetic complications in either TIDM or TIIDM through activation of PKC (Aiello et al., 1997). Several polymorphisms have been identified in the promoter region of the VEGF gene. In a Japanese population, a significant association was found between the C allele of the C (-634) G polymorphism and DR in patients with TIIDM (Awata et al., 2002). The D allele of the (I/D) polymorphism at –2549 has been significantly associated with DN in Caucasoid patients with TIDM (Yang et al., 2003).

1.3.3.2 Environmental risk factors to diabetic microvascular complications

1.3.3.2.1 Hyperglycaemia

The risk of developing diabetic complications greatly increases with uncontrolled glucose levels either in TIDM or TIIDM (DCCT, 1993; 1995; UKPDS 33, 1998; Gabir et al., 2000). Intensive insulin therapy markedly reduces the risk of microvascular complications (DCCT, 1993; 2000). Hyperglycaemia induces endothelial dysfunction and activation of inflammatory cells adhesion resulting in impairment of cellular supply with oxygen and nutrients in microvessels of retina, kidney, and nerves (King et al., 1996; Donnelly et al., 2000; Calles-Escandon et al., 2001; Sheetz et al., 2002).

In many populations, uncontrolled hyperglycaemia has been implicated in the pathogenesis of microvascular complications. A prospective study in the Berlin has shown that the rate of DR was found to increase with deteriorating glycaemic control (Danne et al., 1994). In Denmark, another study has determined that the overall increase in AER and the development of persistent microalbuminuria was increased in patients with poor glycaemic control (Rossing et al., 2002). A study in Norway showed a decrease in motor and sensory nerve conduction with uncontrolled HbA1c (Amthor et al., 1994). However, it is shown that the intensive treatment of hyperglycaemia with insulin in patients with diabetes retards the development of these complications (DCCT, 1993).

1.3.3.2.2 Duration of diabetes

The duration of DM is an important contributor to the development of late microvascular complications (DCCT, 1993). Studies have shown that DR may develop shortly after 5 years of the diagnosis of the disease. Prevalence of DR increases with increasing duration of the disease, since one third of patients who have diabetes for 10 years develop DR while up to 75% of those with duration of 20 years or over have DR (Hamman, 1997). The duration of diabetes may have less effect on the development of DN than on DR. The DN develops after 25 years of the diagnosis of the disease; the peak incidence is at 15-16 years of diabetes duration (Kofoed-Enevoldsen et al., 1987). There is no doubt that the duration of diabetes has a negative influence on peripheral nerve function (DCCT, 1993).

1.3.3.2.3 Hypertension

Hypertension is diagnosed when the average of diastolic blood pressure is 90 mmHg or greater or systolic blood pressure is 140 mmHg or greater. National Heart, Lung, and Blood Institutes in the NIH have shown that hypertension with diabetes together strongly increase the rate of morbidity and mortality (NIH, 1994). Hypertension poses more risk of cardio-vascular diseases to patients with diabetes, more frequently in patients with TIDM that have nephropathy (Rossing et al., 1996; Weidmann et al., 1997). Patients who have both diabetes and hypertension have more atherogenic risk factors, including dyslipidemia, hyperuricemia, and elevated fibrinogen. These factors increase the incidence of renal diseases and also contribute to the development of DR and DNU (Perin et al., 2001; NIH, 2002). The progression of DN correlates with a rise in blood pressure that might be prevented by aggressive blood pressure reduction (DCCT, 1993; 2000; NIH, 1994). A strong family history of essential hypertension and diabetes appears to identify those

persons who are most likely to develop hypertension and diabetic complications.

1.3.3.2.4 Diet

Type of food, amount of proteins, alcohol intake, overweight, and no physical activity are the main factors, which contribute to the development of vascular diseases. Studies have shown that diet contributes to the development of diabetic complications. Protein content of the diet can affect glomerular filtration rate and the function of proximal tubular cells (Pedrini et al., 1996). Dietary intake of pre-formed AGEs reacts with proteins in the body to form effective AGEs, which promote the pathogenesis of vascular diseases (Brownlee, 2001). Excessive fat may increase cholesterol and LDL, which are associated with hypertension and DN (Groop et al., 1996). A vegetarian diet and vitamins may be beneficial to delay the development of vascular diseases (Bursell et al., 1999).

1.3.3.2.5 Smoking

Smoking is the most important modifiable cause of premature death in general and patients with diabetes in particular (Rossing et al., 1996). The combination of smoking and diabetes accelerates the development of macrovascular and microvascular disease (UKPDS, 1991; NIH, 1994; ADA, 2000). Smoking affects circulation, slows down healing and accelerates the cell damage (Haire- Joshue et al., 1999). Tobacco smoke contains glycotoxins, which can rapidly react with proteins and increase AGEs formation in blood vessels (Nicholl et al., 1986). Studies have shown that smoking associates with the progression of DN in patients with TIDM (Sawicki et al., 1994).

1.4 Prevention and treatment strategies of TIDM and its complications

1.4.1 Prevention of TIDM and its complications

To limit the progress of diabetic microvascular complications it is essential to keep blood glucose under control. However, DCCT have reported that good glycaemic control was insufficient to prevent the development of complications, strongly suggesting a role of genetic factors (DCCT, 1993). Individuals with a family history of TIDM should be screened for the disease. ICA, IAA and GAD-positive relatives of patients with TIDM are the first targets in clinical trials to prevent the disease (Rewers et al., 1997; Yoon et al., 1998; 1999; 2001). It has been shown that the risk for TIDM varies from 0-1% in individuals with only one positive antibody to 62-100% in subjects who were positive for three or more antibodies (Yoon et al., 2001).

Controlling hypertension and regular check of renal function are the preventive strategies to avoid vascular and renal diseases in TIDM. Blood pressure control can reduce microvascular disease by approximately 33% (NIH, 1994). The NIH urges all patients with diabetes to have a proper foot care and urine analysis for microalbuminuria and a dilated pupils eye examination at least once a year (McGill et al., 1997; NIH 2002; 1994).

Lifestyle modifications are particularly relevant for all patients with diabetes. Weight management, diet, moderation of alcohol intake, increased physical activity, and cessation of smoking are the cornerstones of metabolic control. Weight loss can improve both hypertension and diabetes; it may reduce cholesterol levels and triglycerides. It has been suggested that weight reduction may enhance tissue sensitivity to insulin. Reduction of total energy intake maintains the balance of metabolic rate and avoids the risk to cardiac

heart diseases (DCCT, 1993; NIH, 1994). Protein intake is recommended to be 15-20% of a diabetic diet, this can reduces albuminuria, improve renal haemodynamics, lower the incidence of nephropathy and other microvascular complications in TIDM (Mann et al., 1997).

1.4.2 Treatment of TIDM and its complications

1.4.2.1 Insulin therapy

Patients at risk for the development of late microvascular complications require aggressive control of blood glucose levels and regular clinical examination. Insulin therapy can modulate the vascular tone and improve endothelial cell functions through the activation of NO and inhibition of PKC (Kuboki et al., 2000). Some hypoglycaemic agents prevented the glomerular dysfunction through prevention of the glomerular hyperfiltration, albuminuria, decrease ECM proteins, and reduce glomerular hyperglycaemic-induced PKC (Isshiki et al., 2001).

Statistical studies have shown that the development of diabetic microvascular complications can be reduced by 50-75% by intensive insulin therapy (DCCT, 1993; 2000; NIH, 1994; UKPDS, 1998; 2000). Studies have reported a reduction in the incidence of DR by 76%, 54% lower for DN, and 60% lower in DNU compared with the conventional insulin therapy (NIH, 1994; 2002; UKPDS, 1998; 2000). The successful improvement in glycaemic control was proposed with psychological support, parents' education, and patients self-monitoring 3-times/ day (McGill et al., 1997). However, insulin overdose could induce hypoglycaemia, which may lead to death (DCCT, 2000).

1.4.2.2 Supportive therapy

Previous studies have shown that treatment of patients with TIDM with special classes of drugs may improve and limit the progress of microvascular complications. Such therapeutic agents include PKC-β inhibitors (Murphy et al., 1998; Meier et al., 2000; Ways et al., 2000), antioxidants, and vitamins (Bursell et al., 1999). It is suggested that these drugs could compensate the oxidant damage in the vascular system and reduce the devastating effects of hyperglycaemia and the other biochemical abnormalities.

ACE inhibitors have a special interest in the treatment of diabetic complications. It is shown that they reduce albuminuria and progression of renal complications to a greater extent than other agents that lower blood pressure. It is recommended that small doses of ACE inhibitors can be given to protect the kidneys in patients with TIDM without lowering the blood pressure (DCCT, 2000).

ARIs are a new class of drugs. Most of the putative therapeutic mechanisms are dependent on the first half of the polyol pathway and inhibition of ALR2. ARIs can attenuate the action of the ALR2 enzyme and reduce levels of sorbitol in several tissues including lens, retina, kidney, blood vessels, and peripheral nerves (Cameron et al., 1997; Mizuno, et al., 1999). On the other side, the inhibition of the SORD enzyme in the second half of the polyol pathway will reduce the production of fructose, which is a very effective glycating agent and could contribute to the pathogenesis of diabetic complications (Cameron et al., 1997).

Furthermore, a precise knowledge of the defects underlying TIDM is essential for designing appropriate therapeutic strategies. Gene therapy is approaching an improvement in the insulin expression and immuno-modulation to treat diabetes. A considerable amount of effort has yielded gene delivery vectors based on adenoviral and retroviral. Genetically engineered rodent models have created their impact on treatment of human diabetes. Despite the number of successes in vitro and in animal models, safety trials in humans have not yet been attempted (Olefsky, 2000). HSP 60 has been shown to act as auto-antigen and initiate the autoimmune β cell destruction (Elias et al., 1990; Birk et al., 1996). Studies have suggested that the DiaPep277, a laboratory-made peptide, which mimics the action of HSP 60 could prevent the autoimmune-destruction of β cells (Elias et al., 1995; Vastag, 2002).

1.5 Candidate genes and possible pathways in the pathogenesis of TIDM and its late microvascular complications

1.5.1 Transcription factor nuclear factor kappa B

NFκB is an eukaryotic transcription factor that is intricately involved in mediating the immune-inflammatory response through a wide array of pro-inflammatory molecules (Baeuerle 1991; Brand et al., 2001).

1.5.1.1 Molecular biology of NFκB

The NFκB is a multicomponent protein of the Rel-family (Baeuerle et al., 1991; 1996; Brand et al., 2001). The classical dimer is composed of p50 and p65 (Rel A) (Ghosh et al., 1998; Christman et al., 2000). The p50 unit is necessary for nuclear translocation and p65 contributes to the transcription mechanism, both subunits of the NFκB dimer bind DNA (Lin et al., 1995; Anrather et al., 1997). The inactive forms of the p50/p65 dimer are bound to the inhibitor kappa B (IκB) and sequestered in the cytoplasm. Phosphorylation of IκB leads to the breakdown of IκB/NFκB complexes. Degraded IκB unmasks a nuclear localisation sequence that allows translocation of the NFκB dimer into the nucleus. Activated NFκB binds to a cognate DNA sequence (5`- GGG GAC TTT CC) on the target genes and activates the transcription mechanism (Hunter et al., 1992; Baeuerle et al., 1998). Figure 1.9 illustrates the activation pathway of NFκB and transcription mechanism.

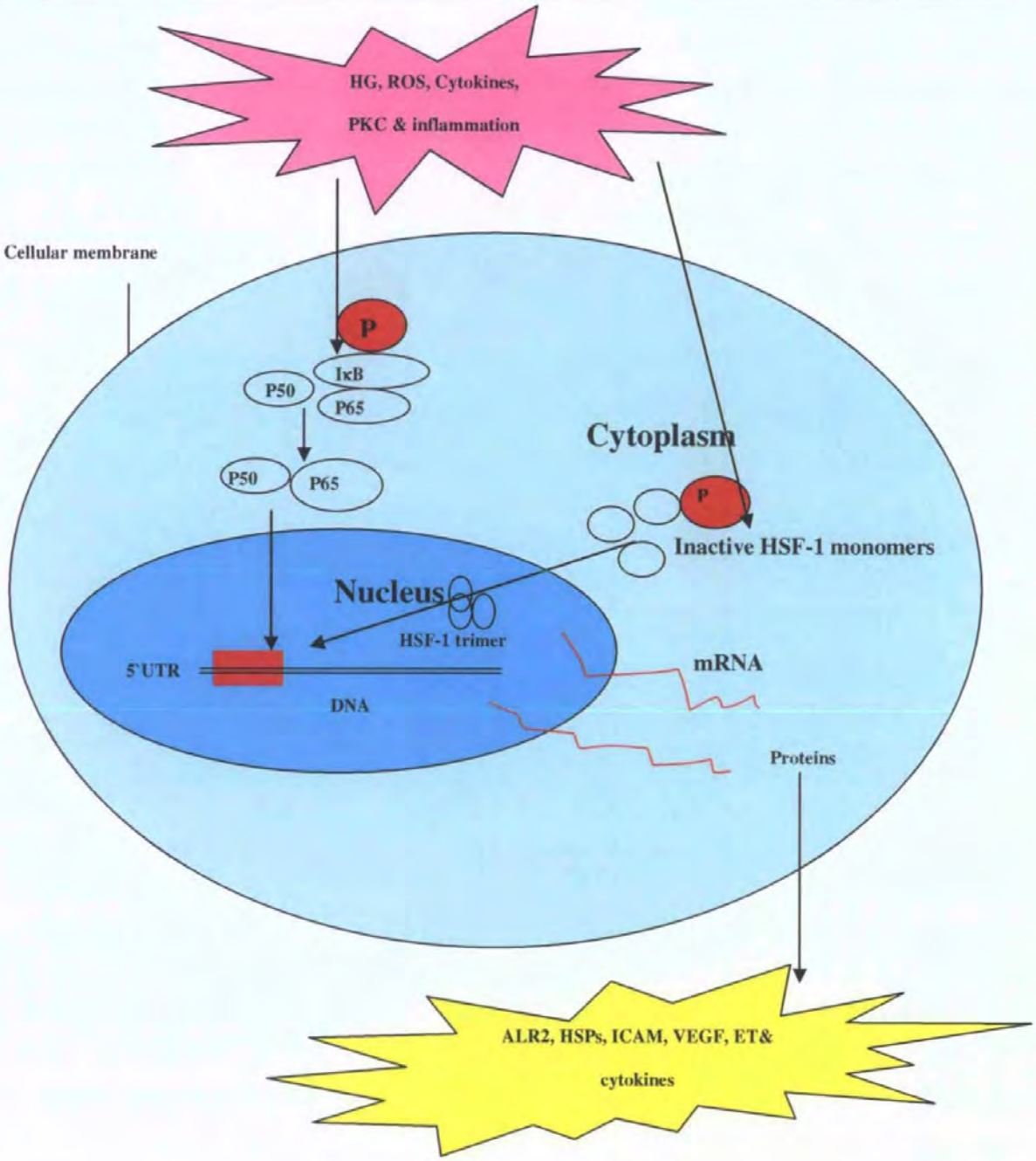


Figure 1.9: Illustration of the transcription factors activation

Inactive forms of the transcription factors NFκB and HSF-1 are in the cytoplasm. On phosphorylation (p), NFκB dimer (p50/p65) dissociates from the complex with IκB and HSF-1 monomers aggregate in a trimers form. Activated factors translocate to the nucleus and bind to the promoter region of the target genes to initiate the transcription mechanism. 5` UTR= un-translated region= promoter region.

1.5.1.2 Pathological importance of NFκB

NFκB activation is a redox-sensitive mechanism (Baeurele et al., 1996; Barnes et al., 1997). It is suggested that the role of NFκB in the pathogenesis of the inflammatory process is through the up-regulation of the synthesis of IκB-α (Du et al., 1999; Yamamoto et al., 2001). NFκB promotes the expression of over 150 target genes including those coding for cytokines such as TNF-α, IL-1β, chemokines, adhesion molecules as well as increased expression of HLA molecules (Taylor et al., 1998; Yamamoto et al., 2001). It has been shown that NFκB is involved in the induction of human inducible nitric oxide synthase (iNOS), which is over-expressed in a number of human inflammatory diseases (Taylor et al., 1998). NFκB is involved in the pathogenesis of many inflammatory diseases such as atherosclerotic diseases (Brand et al., 2001; Hamuro et al., 2002), MS and RA (Tak et al., 2001), β cell apoptosis (Eizirik et al., 1996, 2001a; 2001b; Cardozo et al., 2001), cancer (Yamamoto et al., 2001).

The molecular mechanisms that provoke the tissue damage in response to hyperglycaemia stress are still not clear. Until 1997, there had been no studies on the effect of hyperglycaemia on the expression of NFκB (Pieper et al., 1997). After then, several studies have shown that NFκB expression is increased in patients with diabetes and its microvascular complications (Hofmann et al., 1998; 1999, Bierhaus et al., 2001). In experimental models of diabetic complications, the increased expression of NFκB is found in retinal pericytes (Romeo et al., 2002) and VSMC (Yerneni et al., 1999) in response to high concentration of glucose.

There is increasing evidence that NFκB may play an important role in accelerating the tissue damage caused by hyperglycaemia (Yerneni et al., 1999; Marumo et al., 2000;

Tanaka et al., 2000). Studies have postulated that NFκB accelerates the development of diabetic vascular complications through inhibition of endothelial cell migration (Hamuro et al., 2002). It has been shown that NFκB activity correlates with monocyte chemoatractant protein-1 expression (MCP-1), which mediate monocytes infiltration (Rovin et al., 1995).

1.5.1.3 NFκB is a therapeutic target

Several studies have shown that inhibition of NFκB activity might control or cure the progress of inflammatory and immuno- destructive mechanisms such as cytokines-induced β-cell destruction (Giannoukakis et al., 2000), RA (Bondeson et al., 1999), cancer (Yamamoto et al., 2001). Blocking NFκB activity can also inhibit production of the proinflammatory molecules in intestinal epithelial cells (Jobin et al., 1998). Antioxidant enzymes such as Mn superoxide dismutase, catalase and glutathione peroxidase may attenuate NFκB-induced inflammatory effects (Christman et al., 2000).

1.5.2 Heat shock proteins

HSPs are a family of proteases, which are known as stress proteins, they mediate the degradation of damaged proteins and have apoptosis-regulatory function (Garrido et al., 2001). HSPs are intracellular molecules and encoded by several genes. They can be released in pathological situations and have a range of immunoregulatory activities. For many years, HSPs have been regarded as cytoprotective proteins. However, studies have shown that stress proteins have the capacity to induce pro-inflammatory responses (Pockley et al., 2000).

1.5.2.1 Molecular biology of HSPs

HSPs have been classified into four major families according to their molecular weights: HSP 90 KDa, 70 KDa, 60 KDa, and the small HSPs (Hendrick et al., 1993). They are constitutively expressed under normal conditions and can be markedly induced by a range of cellular stressors including oxidative stress (Kurucz et al., 2002), protein glycation (Ganea et al., 1995) and the pro-inflammatory NFκB (Feinstein et al., 1996; DeMeester et al., 1997 & 2001). Heat shock factors (HSF1 and 2) are specific transcription factors that control HSPs' expression (Goldenberg et al., 1988; Fawcett et al., 1994; Wu 1995). They are part of a transcriptional signalling cascade with both positive (protective HSPs) and negative (proinflammatory) properties. HSF1 has the main transcription function for HSPs in vertebrates (Fawcett et al., 1994; Wu 1995). It is present as inert monomers within the cytoplasm. Under stressful conditions it is phosphorylated by MAPKs subfamilies, JNK and p38 in particular (Kim et al., 1997). Active HSF-1 is oligomerized to a trimer, which translocates to the nucleus and binds to a heat shock element (HSE, nGAAn) in promoters of different genes, HSPs in particular (Goldenberg et al., 1988; Wu, 1995; Schafer et al., 2000; Christians et al., 2002) (Figure 1.9).

1.5.2.2 Pathological importance of heat shock proteins

Expression of the stress proteins has been observed as an adaptive response to oxidative stress in eukaryotes and prokaryotes (Jacquier-Sarlin et al., 1996). Studies have indicated that HSF-1 is a redox-sensitive transcription factor (Bijur et al., 1999) and it could be activated in response to environmental changes such as osmolality (Caruccio et al., 1997). HSF-DNA binding activity is regulated by HSP70 (Morimoto, 1993; Caruccio et al., 1997; Schett et al., 1998; Schafer et al., 2000). It has been supposed that the protective effect of

HSPs is through the inhibition of the proinflammatory gene expression (Feinstein et al., 1996; Wong et al., 1997b). In vitro studies have postulated that HSPs decrease NFκB nuclear translocation and activation in lung epithelium cells (Wong et al., 1997a) and endothelium cells (DeMeester et al., 1997).

HSPs, particularly those of HSP60 and HSP70 families are implicated in the pathogenesis of autoimmune diseases (Pockley et al., 2000). Studies have shown a link between HSPs and the pathogenesis of multiple sclerosis (Georgopoulos, et al., 1993), systemic lupus (Minota et al., 1988) and acute lung injuries (Wong et al., 1997a). Furthermore, it has been suggested that HSPs influence the pathogenic processes of organ allograft rejection and vascular diseases and mediate endothelial cytotoxicity (Pockley et al., 2000). Individuals with borderline hypertension have shown an increase in the levels of HSP60 and HSP70 (Pockley et al., 2000). Also, studies have shown an increase in HSP70 level in peripheral and renal vascular diseases (Wright et al., 2000).

With respect to diabetes, studies have demonstrated that antibodies to HSP-65 and HSP-60 contribute to the development of autoreactive T-cells and β cell destruction and may play a role in the aetiology of TIDM (Elias et al., 1990; 1995; Birk et al., 1996). In addition, there is evidence that HSP70 may play a role in autoimmune destruction of β cell in TIDM (Strandell et al., 1995; Figueredo, et al., 1996). Studies have shown that HSP70 is increased in the mononuclear cells of patients with TIIDM (Yabunaka et al., 1995) and in IL-1β and streptozotocin-induced diabetic rats (Strandell et al., 1995; Yamagishi et al., 2001). Previous studies have shown that a MHC-linked HSP70 gene is linked with susceptibility to TIDM (Caplan et al., 1990). The RFLP investigations have revealed contradictory results. Pociot et al., in Denmark at 1993 have reported an association of PstI-8.5 kb allele of with TIDM (Pociot et al., 1993) and that was supported by another

study in Taiwan (Chuang et al., 1996). In contrast, a Japanese study has demonstrated that the same allele was not associated with TIDM (Kawaguchi et al., 1993).

Conclusion

There is no doubt that hyperglycaemia contributes to the pathogenesis of late diabetic microvascular complications (King et al., 1996; Nishikawa et al., 2000a; Brownlee, 2001; Sheetz et al., 2002). The effect of hyperglycaemia may be mediated by activation of the polyol pathway and its subsequent metabolites, which are cofactors for many metabolic pathways. Other putative mechanisms such as PKC activation and AGEs will take part in the pathogenesis mechanism (Figure 1.2).

Hyperglycaemia-activated polyol pathway induces ALR2 activation. The reduction of glucose to sorbitol by ALR2 has been linked to the development of diabetic vascular complications (Yabe-Nishimura, 1998). Recent evidence shows that ALR2 enzyme is upregulated by oxidative stress and is implicated in apoptotic cell death (Ramana et al., 2003), mitogenic stimuli (Ramana et al., 2002) and vascular inflammatory diseases (Kasuya et al., 1999; Ruef et al., 2000). It is possible that ALR2 induce those effects through NFκB activation (Ramana et al., 2002 & 2003).

The DAG-PKC pathway is activated in diabetes as a result of hyperglycaemia (King et al., 1996). AGE and oxidative stress can enhance signalling pathways (PKC and MAPK) causing transcriptional changes as well as alterations in cellular survival. PKC and MAPK regulate many important factors such as cytokines, growth factors and NFκB, these factors affect vascular permeability, contractility and cellular proliferation (King et al., 1996; Koya and King, 1998; Ha et al., 2000).

Oxidative stress is the clearest example of interactions among these different pathways. The transcription factors NFκB and HSF-1 are upregulated by oxidative stress (Jacquier-Sarlin et al., 1996; Mohamed et al., 1999). These factors bind to cognate DNA sequences on the target genes including ALR2 gene (Iwata et al., 1999). It is possible that the increased flux of glucose through the polyol pathway provoke the tissue damage in the target organs through the alteration of the transcription factors-DNA binding activity, which may lead to an increase in the ALR2 transcription, more activation of polyol pathway and more production of oxidative stress.

Intensive insulin treatment can slow the progression of diabetic microvascular complications (DCCT, 1993). However, it is difficult to keep the blood glucose level normal at all times throughout the life of patients with diabetes. Accordingly, chemical agents that reduce hyperglycemic injury would assist the insulin therapy. Previous studies have shown that the specific inhibition of the polyol pathway might limit the progress of microvascular diseases in chronic diabetes (Obrosova et al., 2001; Okayama et al., 2002). ARIs (tolrestat, sorbinil, zopolrestat and ponelrestat) were studied to prevent diabetic complications and showed promising results. However, it is not clear how the ARIs work?

Hypothesis of this study

This study addressed the following points: (a) Polymorphisms through the genes coding for proteins involved in glucose metabolism and immuno-regulatory mechanism may contribute to the susceptibility of TIDM and/or its late microvascular diseases. (b) The transcription factor-DNA binding activity is an important step in a complex program of transcriptional events; its importance confers to the target genes. Uncontrolled blood glucose level may influence the transcriptional regulation. (c) The pharmacological modulation of transcription factor activity represents an attractive therapeutic approach. Therefore, the treatment with ARIs may influence the common signal transduction through the transcription factor-DNA binding activity.

Aim of this study

The aims of this study are outlined as follows:

1- To identify genetic markers for the susceptibility to TIDM and its late microvascular complications. The candidate genes include the transcription factor NFκB gene (4q24), the HSP70 gene (14q22), the SORD gene (15q21) and the PKC-β gene (16p12).

2- To investigate the transcription factors, NFκB and HSF-1-DNA binding activity in response to high glucose concentration using cell lines and human PBMCs from patients with TIDM with and without microvascular complications.

3- To assess the effect of aldose reductase inhibitors (ARIs) on NFκB and HSF-1-DNA binding activity in human PBMCs with and without microvascular complications.

4- To study the MAPK p38 expression in relation to the NFκB and HSF-1-DNA binding activity.

Chapter 2

MATERIALS& METHODS

2.1 Entry criteria and subjects

Eligible patients for this study were British Caucasoid patients. The study was restricted to patients with TIDM with and without microvascular complications. However, 24 patients with TIIDM were included at a later stage to support the studies on the role of NFκB. Classification was evaluated according to the clinical, physical and biochemical records. The subgroups of patients and normal controls (NC) were defined in previous studies (Heesom et al., 1997).

The patients were classified as follows:

-Uncomplicated diabetic controls (DC); patients who have had TIDM for at least 20 years but remain free of retinopathy, proteinuria, and neuropathy.

-Nephropaths (DN); have had TIDM for at least 10 years with persistent proteinuria (urine Albustix positive on at least three consecutive occasions over 12 months or three successive total urinary protein excretion rates more than 0.5 g/24h) in the absence of haematuria or infection on midstream urine samples. The presence of DN is commonly associated with diabetic retinopathy (DR) (Schwartz et al., 1998).

-Retinopaths (DR); patients had retinopathy defined as more than 5 dots or blots per eye, hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for pre-proliferative or proliferative retinopathy and maculopathy or vitrous haemorrhage. None of these patients had proteinuria.

-Neuropaths (DNU); this was defined by clinical and electro-physiology examination as damage to the nerve. The common symptoms and signs in this group are loss of sensation, hypotension (giddiness), sweating and joints problems.

- Patients with short duration diabetes; have had diabetes for less than 10 years with or without complications. Patients with TIDM of short duration were included in this study as patients with TIDM irrespective of microvascular diseases.

-Normal healthy controls (NC); are ethnically matched cord blood collected sequentially after normal obstetric delivery from the labour ward, Derriford Hospital, Plymouth, UK, schoolchildren aged between 5 and 16 years, and healthy adult donors with no family history of diabetes.

2.2 Data collection

Data were obtained from a diabetes register that includes patient number, sex, hospital number, date of birth, date of diagnosis, age at onset, diagnosis and classification. HbA_{1c}% data were re-called from the Derriford Hospital registers, Plymouth, UK.

2.3 Statistical analysis

Any differences in frequencies of alleles and genotypes were calculated by Statgraphics software (Statistical Graphics Corp. USA. 1996). The comparison between patient's subgroups and control subjects was made by 2x2 contingency tables using the χ^2 test. The p values were corrected for the number of comparisons made using the Bonferroni method (Tiwari 1985), and p values of <0.05 were considered to be significant.

The protein assay study was performed to investigate the proteins –DNA binding activity in patients with diabetes mellitus compared to non-diabetic (healthy) controls. When

patients were classified according to the presence or absence of microvascular complications, some groups have a limited sample size that might reduce the power of the study and lead to a statistical error. To indicate a sufficient statistical power, all parameters have been standardized to reduce the intra-assay variation. Further, the intensity of the bands was expressed as means ± SE with each sample run in triplicate. The difference between means was calculated by student's *t*- test. The p value of <0.05 with 95% CI was considered to be significant. Diabetic subgroups and control groups were compared by ANOVA to specify the difference between groups, p values of <0.05 were considered to be significant. Simple linear regression analysis was used to detect relationships among NFκB, HSF-1, age at onset and duration of the disease as the independent variables (SPSS version 9.0). Also, the expression of p38 was compared with the binding activity of NFκB and HSF-1 by using linear regression analysis.

2.4 Hardy-Weinberg distribution

Hardy-Weinberg equation confirms the relation between the expected alleles and genotypes frequencies with the observed frequencies. In case of random mating with two alleles, the expected frequencies of genotypes are (p^2, 2pq, q^2), where is the p and q are presenting alleles frequencies. Hardy-Weinberg equation can be extended to multiple alleles, frequency of any homozygote= square of allele frequency and frequency of any heterozygote= 2X product of allele frequencies (Hartl et al., 1999).

2.5 Genotyping study

2.5.1 Population

The total number of the subjects included in the genotyping study is shown in Table 2.1. Clinical characteristics of patients' subgroups and NC are included with the results of genotyping studies.

Subjects	NFκB 4q24	HSP70-A2 14q22	PKC-β 16p12
	(CA) n 3` UTR	(AAATA) n 5` UTR	T (+126) C
NC	111	94	88
DC	38	44	34
DR	76	55	60
DN	48	38	39
DNU	55	40	42
SD	0	16	0
TOTAL	328	287	263

Table 2.1: Total number of the subjects included in the genotyping study

Patients with TIDM were sub-grouped according to the presence or absence of microvascular complications. DC= those patients with no microvascular complications after 20 years duration of diabetes. DN= those patients with diabetic nephropathy. DNU = those patients with overt diabetic neuropathy. DR= those patients with diabetic retinopathy. SD= diabetes for less than 10 years with or without complications. NC= normal healthy controls.

2.5.2 Nucleon DNA Extraction

Peripheral blood was collected into NaEDTA containing tubes. DNA was extracted using nucleon II DNA extraction kit following the manufacturer's instructions (Tepnel Life Science PLC, Manchester, UK). 3-10 ml of sample was transferred to a 50 ml Falcon tube. 4x volume of nucleon A (which contains 10 mM Tris-Hcl; 320 mM sucrose; 5 mM $MgCl_2$; 1% Triton X-100; pH= 8.0) was added into the tube. The tube was shaken for 4 min and centrifuged at 1300 rpm for 4 min (MSE Mistral 1000- Sanyo, Park House Meridian East, Leicester, UK). The supernatant was discarded and a 2 ml of nucleon B were added to the pellet and the tube vortexed and incubated at 37°C for 10-15 min. The contents transferred to a 15 ml Falcon tube, and a 500µl volume of 5 M Na percholate was added to the tube and the tube was inverted 10-15 times. A 2ml of chloroform (-20°C) were added into the tube, which was inverted 10-15 times and centrifuged at 1300 rpm for 3 min. 200µl volume of silica suspension was added into the tube without distributing phases and the tube was centrifuged at 1300 rpm for 3 min. Upper aqueous phase was transferred to a fresh 15-ml Falcon and was spun for 1 min at 1300 rpm. The supernatant was transferred to a fresh tube. 2x volumes of ethanol (-20° C) were added into the tube and the tube was inverted a few times. Finally, the DNA was hooked with a glass rod and washed in 70% ethanol, then dissolved in 100µl double distilled water (ddH2O) in an Eppendorf tube (Fisher Scientific, Leicestershire, UK).

2.5.3 Optical density value of DNA

Concentrations of DNA were determined by using spectrophotometer (Elegant technology, Cambridge, UK) at wavelengths of 260 nm. An optical density (OD) of 1 corresponds to approximately 50 µg/ml for double stranded (ds) DNA.

The range of DNA in samples can be calculated with the following equation:

$$\frac{OD_{260} \times 100 \times 50}{1000} = ng/\mu l$$

N.B: 100 is the dilution factor and 50 is a fixed value for DNA quantitation.

2.5.4 Design of primers

Primers sets were designed as pairs of oligonucleotides to flank the interested areas (Figure 2.1). They were 18-24 base pair (bp) in length and had GC contents of ~50 %. All primers were supplied by MWG-BIOTECH UK LTD, Milton Keynes, UK.

2.5.5 Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows selective DNA sequences to be amplified using a set of two oligonucleotide primers (Figure 2.2). Extracted DNA (~100ng) was subjected to PCR in a total volume of 30 µl containing a 0.5 µl of a suitably heat-stable DNA polymerase (2.5U) with 3 µl of 10X $MgCl_2$ free PCR buffer (HT Biotechnology, Cambridge, UK), a 0.5 µl of DNA precursors 100mM (dNTPs) (Amersham Pharmacia Biotech, Bucks UK), and the appropriate titration of 50 mM $MgCl_2$. PTC-200 Thermocycler (MT-Research, Essex, UK) was used for all PCR.

(A)

5`-
CTGAGGCAGGAGAATGGCTTGAACCCGGGAGGCAGAGCTTGCAGGAGCCGGA
TCGCACACTGTACTCCAGCCTCGCGACAAACAAACAAACAAACAAAAAAACA
GCGCGCTGCAGATGGAGCCAGCACCCGGGCTTCTCGCTCCCAGCCCCCTCCCG
AAGCCCCGCCCCGCCTCCGGGTCTGGAGGAGGCTGGAAGCCCCGCCCCTCCAC
GTAGCGCCGCCCAGGCTGGCACAAAGGAGGAAGCCTAGTC **–3`**

(B)

5`-
CTTCAGTATCTAAGAGTATCCTCATTTTATATGTACACACACACACACACACAC
ACACACACACACACACACACACACACACAAAGCCTTATCAAGGGGATAAGGGGA
CTTAGGGCTGGGACTCCGTAGAGTCTTACTTG **–3`**

(C)

5`-
CCAAGATACATTCCTGATTCAGAAATCGATAAGTAATATTTATTTTATTTTATT
TTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTGAGGCAGAGTCTAGC
TCTGTCACCCAGGCAGAAGTGCAGTGGC**-3`**

(D)

5`-
GCACAGATGAGCTCTATGCTGTGAAGATCCTGAAGAAGGACGTTGTGATCCAA
GATGATGACGTGGAGTGCACTATGGTGGAGAAGCGGGTGTTGGCCCTG**CCT(C)**
GGGAAGCCGCCCTTCCTGACCCAGCTCCACTCCTGCTTCCAGACCATGGTAAC
TTGTCCCATGGCCCTGGGTATTCCACCTCCAGGGCTTCACCAGGCTTTGGCCAA
AGCTATCTTAGCAGCACCCAGCACAGAGATACCTGCACCCTTCCCTTTCTAGAC
ACAGAAAGATGTAAGGC**-3`**

Figure 2.1: Primers design

(A) (CAAA) 5 repeats on the 5` UTR of the SORD gene on chromosome 15q21 (Genebank- L29249). (B) CA repeats on the 3` UTR of the NFκB gene on 4q24 (HGP working draft at UCSC Blat search). (C) the reverse strand shows the compatible nucleotides to AAATA repeats, which are located at the 5` UTR of the HSP70 gene on 14q22 at position 43133854- 43133813 (HGP working draft at UCSC Blat search). (D) The single nucleotide polymorphism (SNP) T (+126) C on exon 10 of the PKC-β gene on 16p12 (Genebank- AC002299). The SNP (T) is in bald and (C) is the mutation nucleotide. CCCGGG sequence is recognised by SmaI restriction enzyme (underlined). Primers are highlighted and the polymorphisms are underlined.

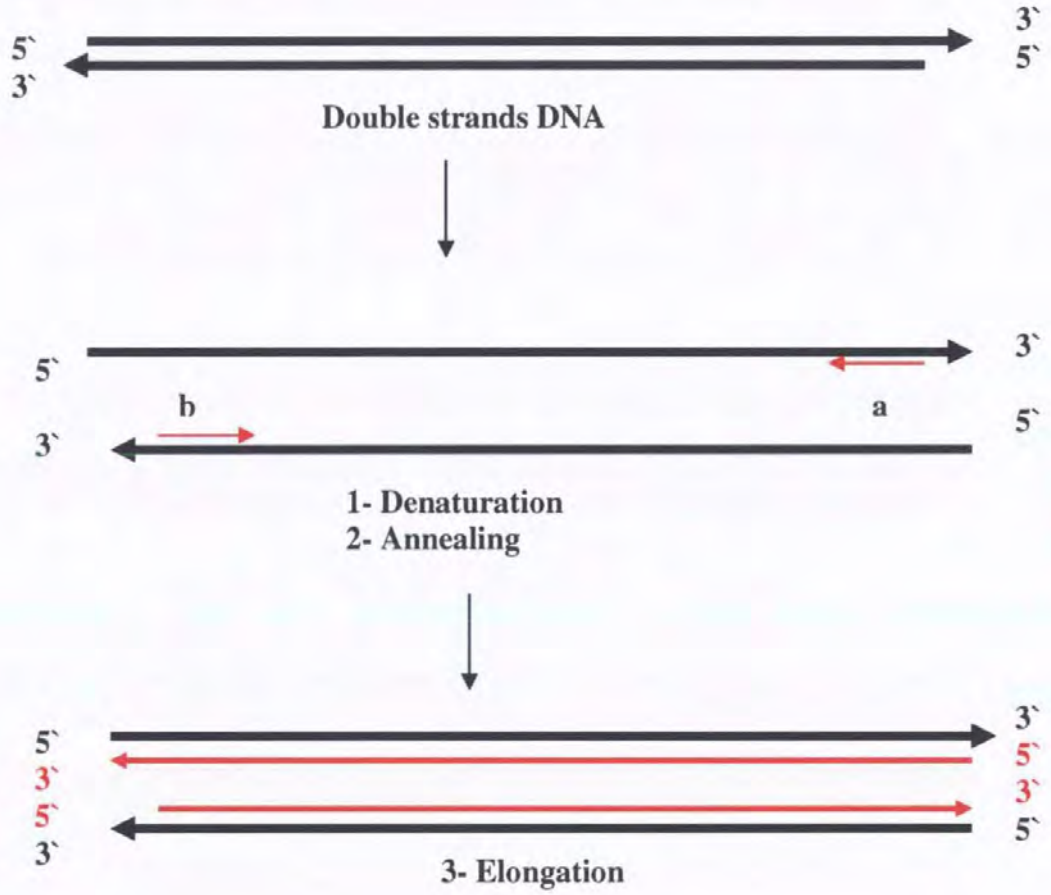


Figure 2.2: Polymerase Chain Reaction

PCR was performed with a starter step at high temperature of 94°C for 4 min followed by 30 cycles with the following cycle profile: 1) denaturation of the double strands at 94°C for 30 sec, 2) annealing stage at which the primers (a & b) join the main strand, the temperature varies according to the designed primers, 3) elongation at 72 °C for 30 sec.

Cycles basically involved a starter step at high temperature of 94°C for 4 min followed by 30 cycles with the following cycle profile: denaturation at 94°C for 30 sec, annealing temperature, which is calculated for each primer separately for 10-45 sec, and elongation at 72°C for 30 sec. Finally, a final elongation at 72° C for 10 min. PCR was performed with an annealing temperature of 70°C /45 sec, 53.5°C/30 sec, 54°C/10 sec, and 59.5°C /30 sec to amplify the wanted fragments of SORD, NFκB, HSP70-A2, and PKC-β respectively.

2.5.6 TBE buffer

The stock solution (10x) of tris/borate EDTA buffer (TBE) was prepared from 108gm of trisma base, 55 gm of boric acid, 8gm of ethylyne-diamine-tetra-acetic acid (EDTA) (Sigma chemicals, Poole, UK) and made up to 1000 ml using double distilled water (ddH2O).

2.5.7 Agarose gel preparation and visualization of the PCR products

The agarose gel was prepared (in different concentrations) by adding the appropriate amount of agarose (Invitrogen, Paisley, UK) to 100 ml volume of 0.5x TBE. It was melted in a microwave (Super shower wave, Sanyo, Park House Meridian East, Leicester, UK). Agarose was mixed with 10 µl of 10 % ethidium bromide (Sigma chemicals, Poole, UK) for visualising the DNA bands. The PCR products were mixed with 1.5 µl of tracking dye and loaded into the gel along side a molecular weight ladder (Invitrogen, Paisley, UK) and the gel was running in 0.5x TBE buffer at 150-200 v for 30 min. The gel was examined and visualised under the ultraviolet light (UV).

2.5.8 Restriction fragment length polymorphism (RFLP) genotyping

This method was used to genotype the PKC-β gene according to the T (+126) C on exon 10. This is a single nucleotide polymorphism (SNP), at which one nucleotide can be mutated to different one. C is the mutated form that makes a restriction site for Sma I endonucleases (Roche diagnostic limited, East Sussex, UK), which recognizes CCCGGG sequence. Sma I restriction enzyme (1U/µl) was used in digesting the amplified region (282 bp) at 25°C for 3 hrs; the digested PCR was checked for quantifying the bands on a 1.5 % agarose gel. The details are shown in Table 2.2.

2.5.9 The 5`end labelling of microsatellite primers

Forward primers for the microsatellites on the SORD, NFκB, and HSP70 A2 genes (Figure2.1) and 10 bp molecular weight marker were labelled with ATP [γ^{32}P] (20,000 cpm) using ready to go™ T4 polynucleotide kinase (T4PNK). Both chemicals were supplied by Amersham, Pharmacia, Biotech. Bucks, UK. 25 µl of ddH2O was added to one tube of ready to go™ T4PNK and the tube was incubated at room temperature for 2-5 min. 5 µl of the forward primer (10 pmol), 19 µl of ddH2O, and 1µl of the radioactive ATP [γ^{32}P] were added into the tube. Next, the tube was centrifuged at 13000 rpm for 30 sec and incubated at 37°C for 30 min. Then the tube was incubated on ice for 2 min to stop the reaction. The neat 5 µl of sodium chloride (5M), 2µl of Quick precip (Edge Biosystem, Gaithersburg, MD) and 160 µl of 100 % ethanol (-20°C) were added into the tube and the tube spun at 13000 rpm for 3 min. The tube was vortexed and spun for another 30 sec. The supernatant was discarded and the pellet washed with 70 % ethanol. The tube was air dried at room temperature for 5 min. The pellet was dissolved in 50 µl of ddH2O.

Contents	Measures	Conditions of Digestion
PCR product	20 µl	25 °C /16 hrs
H2O	6 µl	
Sma I buffer	3 µl	
Sma I enzyme	1 µl (1U)	
Total volume	30 µl	

Table 2.2: PCR-based genotyping for the T (+126) C on exon 10 of the PKC-β gene on 16p12 using SmaI restriction enzyme

The amplified fragment (282 bp) flanking the T (+126) C on exon 10 of the PKC-β gene at 16p12 was digested with the endonuclease restriction enzyme (Sma I). The total volume of digestion was incubated at 25° C for 16 hrs. The digestion products were checked on 1.5 % agarose gel and visualised under UV light.

2.5.10 Polyacrylamide gel electrophoresis and microsatellite analysis

PCR was performed with the radiolabelled forward primers to investigate the polymorphic dinucleotide $(CA)_n$ repeat at the regulatory region of the NFκB1 gene (4q24) (Ota et al., 1999), the pentanucleotid $(AAATA)_n$ repeat at the promoter region of the HSP70-A2 gene (14q22) (Roux et al., 1994) and $(CAAA)_5$ repeat on the promoter region of the SORD gene (15q21) (Iwata et al., 1995). The protocol of this method was illustrated in Figure 2.3.

BIORAD sequi-gen GT electrophoresis system (BIORAD Laboratories, Hempstead UK) was used for this technique (Figure 2.3). The system contains 30x50 cm glass plates with 0.25-cm spacers and a 40-space shark toothcomb. Before using, the glass plates and spacers were washed in detergent solution, rinsed thoroughly in tap water, followed with ddH2O, and finally rinsed with 70 % ethanol. The inner surface of the back-glass plate was siliconised with a small amount of a repel saline for 10 min (Amersham, Pharmacia, Biotech. Bucks, UK). The comb was inserted and the set was connected to the base. A polyacrylamide denaturing gel (6 %) was prepared by mixing 36 ml of concentrated Sequa-gel, 99 ml of diluted Sequa-gel, 15ml of Sequa-gel buffer, 10 ml of formamide, 1.2 ml of 10 % ammonium persulphate (A.P.S) and 70 µl of tetramethyl-ethylene-diamine (TEMED) (Sigma chemicals, Poole, UK). The mixture was cast to the set and left for 2 hrs to overnight at room temperature to allow complete polymerisation. The system was connected to a power pack (Biorad, Hempstead, UK), and the gel was running in 1x TBE at 1800-2000 v for 1-2 hrs to allow the temprature to reach 50°C.

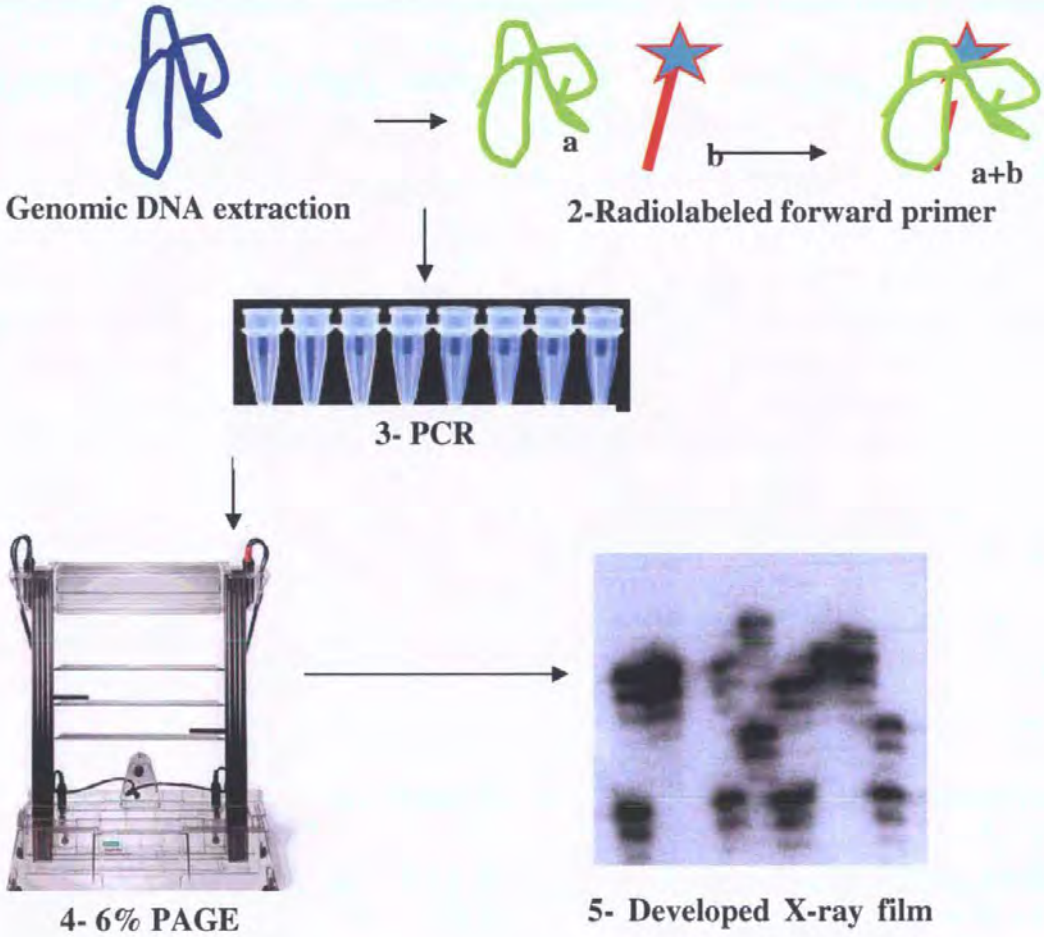


Figure 2.3: Protocol of polyacrylamide gel electrophoresis

1-Genomic DNA was extracted. 2- Forward primer (a) was radiolabeled with $\gamma^{32}P$ (b). 3-PCR was performed. 4- 6µl of the PCR product was mixed with 3µl of stop solution and loaded to the polymerised 6% polyacrylamide denaturing gel (PAGE), the picture shows BIORAD sequi-gen GT electrophoresis system. The system contains 30x50 cm glass plates with 0.25-cm spacers, a 40-space shark toothcomb and sandwich clamps. The electrophoresis time was ~2-3 hrs. 5- Gel was dried, exposed to X-ray film for 18 hrs at –80°C and developed. The picture was adopted from BIORAD website.

Afterwards, the system was disconnected from the power; 6µl of the processed PCR with radiolabeled forward primer were loaded with 3µl of stop solution (Amersham International plc, Buckinghamshire, England) and the gel electrophoresed for another 2-3 hrs.

2.5.11 Development of auto-radiographs

The gel was placed on Whitman filter paper, dried and exposed to X-ray film (Kodak xls5) in a firmly closed cassette for 18hrs at -80°C. The film was developed using X-ray developer, stop- solution and liquid fixer (Kodak Ltd, Scientific imaging system UK).

2.6 Cell culture and protein assay

2.6.1 Isolation of peripheral blood mononuclear cells

Patients with diabetes were recruited from the diabetic clinic, Derriford hospital, Plymouth, UK. They were classified according to the type of diabetes and type of microvascular complications as shown in Table 2.3. Clinical characteristics of patients' subgroups and NC are included with the results of the protein assay studies.

The fresh whole blood (20 ml) was collected into 5 % EDTA vacutainers (Becton Dickinson, Oxford, UK). PBMCs were isolated by using histopaque following the manufacturer's instructions (Sigma chemical, Poole, UK). Every 5 ml blood was transferred into a fresh 14 ml polystyrene round-bottom tube, which contains equal volume of histopaque without disturbing the surface. The tubes were centrifuged at 2000 rpm for 30 min (MSE Mistral 1000).

Study	NFκB-DNA		HSF-1-DNA	
Subjects	**HG**	**HGZ**	**HG**	**HGZ**
NC	10	5	12	0
TIDM-DC	7	3	6	1
TIDM-DR	12	0	12	0
TIDM-DN	5	0	6	0
TIDM-DNU	11	1	11	0
TIDM-SD	8	4	8	4
TIIDM-DR	7	7	7	7
TIIDM-DN	7	7	7	7
TIIDM-SD	10	10	10	10
TOTAL	77	37	79	29

Table 2.3: Total number of the subjects included in the protein study

Patients with diabetes were sub-grouped according to the presence or absence of microvascular complications and type of diabetes. DC = those patients with no microvascular complications after 20 years duration of diabetes. DN = those patients with diabetic nephropathy. DNU = those patients with overt diabetic neuropathy. DR = those patients with diabetic retinopathy. SD = patients with diabetes for less than 10 years **without** complications in both TIDM and TIIDM. NC = normal healthy controls.

HG= hypeglycaemia stressed cells with 31mM D-glucose. HGZ= hypeglycaemia stressed cells with 31mM D-glucose and treated with 10μM zopolrestat (ARI).

The white cell interface (PBMCs) was collected into a new polystyrene round-bottom tube with equal amount of phosphate buffers saline (PBS = 150 mM of NaCl, 10 mM of sodium phosphate, pH7.4) (Invitrogen, Paisley, UK) and the tube was centrifuged at 1400 rpm for 10 min. The cells were washed in 5 ml of RPMI-1640 medium with no supplement and were spun at 1400 rpm for 10 min. The pellet was resuspended in 2ml of RPMI-1640 medium and counted by haemocytometer slide. A concentration of 0.5x 10^6 was grown in culture flasks (200 ml) containing 20 ml of RPMI-1640 with 20 µl of phytohemagglutinin (PHA) (5µg/ µl). PHA was supplied by Sigma chemicals, Poole, UK. The cells were incubated at 37°C and 5% CO2 for 5 days in the incubator (Model GA 2SN- LEEC limited, Nottingham, UK). All Falcon equipments were supplied by Fahrenheit, Milton Keynes, UK.

2.6.2 Cell lines

2.6.2.1 Human Caucasoid hepatocyte carcinoma

Hepatocyte carcinoma cells have epithelial morphology (Hep G2). Cells were obtained from European Collection of Cell Cultures (ECACC No: 85011430).

2.6.2.2 Human Leukaemic T cell lymphoblast

This cell line is IL-2 producing lymphocytes (Jurkat E6.1). Cells were obtained from ECACC No: 88042803.

Both Hep G2 and E6.1 cell lines were supplied by centre for applied microbiology & research, Salisbury, Wiltshire.

2.6.3 Preparation of medium and culture

Roswell Park Memorial Institute 1640 medium (RPMI-1640) was used for growing PBMCs and E6.1 cell line. Minimum Eagle medium (MEM) with earl's salts was used for growing the Hep G2 cell line. Both types of medium were supplied by Invitrogen, Paisley, UK. A volume of 500 ml of RPMI medium was supplemented·with 50 ml of foetal calf serum (FCS) (10 %), 5 ml of L-glutamine (200 mM) and 5ml of penicillin/streptomycin (10 units/ml and 10 µg/ml respectively). MEM medium was supplemented with 5ml non-essential amino acids (100X) (Invitrogen, Paisley, UK).

2.6.4 Preparation of the cell lines for culture

Cell lines arrived from the company in a frozen tube. The tube was left for 1min at the room temperature and thawed in a water bath (37°C) as fast as possible. Cryopreserved cells were transferred to a culture flask (200 ml) containing 20ml of the recommended medium with supplements for growing. The flasks were placed in the incubator at 37° C and 5% CO2 for 2-3 days.

2.6.5 Cryopreservation

A concentration of 500,000/ ml has been preserved in a plastic cryotubes containing a total volume of 1 ml of 10% of Dimethyl Sulfoxide (DMSO), 30 % FCS and completed with the recommended medium and frozen gradually at –20°C for 2hrs and at –80° C overnight. The cryotubes were stored at the vapour phase of a liquid nitrogen freezer until required.

2.6.6 Hep G2 cells subculture

Hep G2 cells were detached by using 5 ml of trypsine EDTA (1X) for 20-30 min. The cells were scraped with a plastic scraper and neutralised with 5 ml of FCS. The cells were harvested into a 50 ml Falcon tube and centrifuged at1000 rpm for 4 min. The pellet was resuspended with 10 ml of PBS for washing and the tube was spun for another 4 min at 1000 rpm. The pellet was resuspended with 2 ml of medium with supplements, the cells were counted, divided, and placed into 2 culture flasks (200ml) and then were incubated at 37 ^{o}C.

2.6.7 E6.1 cells subculture

E6.1 cells are suspended. The cells were harvested into a 50 ml Falcon tube and the tube was spun at 1400 rpm for 4 min. The pellet was re-suspended with 10 ml of BPS for washing and the tube was centrifuged for another 4 min at 1400 rpm. The pellet was re-suspended with a 2 ml of supplemented medium and divided into 2 culture flasks (200 ml) containing 19 ml of the medium with supplements then incubated at 37 ^{o}C.

2.6.8 Cell count and viability

The heamocytometer slide was used for cell counting. Equal volume of cell suspension and trypan blue dye was mixed gently. Trypan blue stains the dead cells. The counting chamber was filled by the capillary action with the cells / trypan blue solution. Cells were allowed to settle briefly. Viable cells (unstained) were counted in the four large corner squares (each large corner square contains 16 smaller squares). The average of the four large squares was used to determine the concentrations of cells.

The following equation was used to calculate the viable cells:

$$n \times 2 \times 10^4 \text{ / ml of cell suspension.}$$

N.B. n = number of cells, 2 is a dilution factor and 10^4 is a constant factor.

2.6.9 Incubation of cells with stress factors

Cells were stressed, grown in the recommended medium and incubated at 37°C for 5 days as shown in Table 2.4 for the following investigations: 1) NF-κB-DNA binding activity in response to high glucose concentration in PBMCs isolated from patients with TIDM and TIIDM, 2) HSF-1-DNA binding activity in response to high glucose concentration in PBMCs isolated from patients with diabetes, 3) the effect of ARI on hypeglycaemia-induced NF-κB and HSF-1-DNA binding activity in PBMCs isolated from patients with diabetes, 4) NF-κB and HSF-1-DNA binding activity in response to hypeglycaemia and HO in cell lines for optimisation and supportive investigation, 5) the expression of the MAPK p38 in response to hypeglycaemia and HO in cell lines.

Experiments were set as following: 1) Normal condition, medium without stress factors, 2) hypeglycaemia, medium with high glucose concentration. A final concentration of 21 mM and 31 mM of D-glucose were studied on the protein activity for optimisation. Afterwards, the concentration of 31mM D-glucose was fixed in all experiments, 3) HO stress of 110mM of NaCl for 16 hrs.

Stress	Concentration	Time	Cells
N	Medium without stress factors	5 days	PBMCs Hep G2 E6.1
D-glucose	31mM	5 days	PBMCs Hep G2 E6.1
NaCl	110mM	16 hrs	Hep G2 E6.1
Zopolrestat	10 μM	5 days	PBMCs
Sorbinil	5 μM	5 days	PBMCs

Table 2.4: Stress conditions and incubation time

Cells were stressed according to the type of experiments, grown in the recommended medium and incubated for 5 days at 37°C and 5% CO2. Hypeglycaemia-stressed cells were stimulated with a final concentration of 31mM D-glucose. Hyperosmolar-stressed cells were exposed to 110 mM NaCl for 16 hrs then washed and grown in the recommended medium to complete the incubation time of 5 days at 37°C. N = unstressed -control cells. Cells were treated with zopolrestat and sorbinil for a complete time of 5 days.

For ARI study, at the beginning, the dose effect of zopolrestat (Pfizer, Groton, USA) on the protein-DNA binding activity was adjusted by using different doses of 0, 5, 10, 15 μM/ml zopolrestat. According to EMSA results, a final concentration of 10 μM/ml of zopolrestat was used in this study. PBMCs were isolated from patients with diabetes and NC and divided into 3 groups as following: NG, hypeglycaemia-stressed PBMCs with and without zopolrestat. A different ARI, sorbinil (Pfizer, Groton, USA) was used to confirm whether zopolrestat has a specific inhibitory effect on hypeglycaemia-increased protein activity. The dose of sorbinil was adjusted to a final concentration of 5 μM/ml.

2.6.10 Protein extraction

2.6.10.1 Buffers for protein extraction

Buffer A (cell membrane lysis buffer) contains 10mM of hydroxy-ethyl-piprazine-ethane-sulfonic acid (HEPES, pH 7.9), 10 mM of KCl, 1.5 mM of $MgCl_2$, 0.5 mM of di-thio-threiotol (DTT), 0.2 % of NP40 (Tergitol).

Buffer C (nuclear membrane lysis buffer) contains 20mM of HEPES (pH 7.9), 25% of glycerol, 0.42 M of NaCl, 1.5 mM of $MgCl_2$, 0.5 mM of DTT, 0.2 mM of EDTA.
To 1 ml of either buffer we added a 5 μl of 100 mM of amino ethyl benzo-sulfonyl fluoride (AEBSF), 10 μl of 18.4 mg/ml sodium orthovanadate, 10 μl of 42 mg/ml sodium fluoride, and 1 μl of 2.2 mg/ml aprotinin.

Sodium dodecyle sulphate (SDS) gel-loading buffer contains 3 ml of ddH2O, 1 ml of 0.5 M of tris-Hcl pH6.8, 1.6 ml of glycerol, 1.6ml of 10% SDS, 0.4 ml of β-mercapten and 0.4 ml of 0.5% bromophenol blue.

2.6.10.2 Nuclear protein extract

Cells were harvested into a 50 ml Falcon tube. The tube was spun at 1400 rpm for 4 min, the pellet was resuspended with 1 ml of PBS buffer and transferred into a 1.5 microfuge tube and spun for 30 sec at 7000 rpm. The cells were lysed in 300μl of buffer A, and incubated on ice for 10 min. Then the tube was centrifuged at 12000 rpm for 10 min. The supernatant (cytoplasmic protein) was kept at –80° C. The pellet was resuspended in 50μl of buffer C and incubated on ice for 10 min. The tube was spun at 13000 rpm for 3 min. The supernatant (nuclear protein) was kept at–80° until required for EMSA.

2.6.10.3 Whole cellular protein extract

E6.1 cells were harvested into a 50 ml Falcon tube, which spun for 4 min at 1400 rpm. The pellet was resuspended with 10 ml of PBS and the tube spun for a further 4 min at 1400 rpm. The pellet (cells) was lysed in 500 μl of SDS loading buffer and transferred into a microfuge tube. The cells were sonicated at 20,000 cpm for 15 sec by using an ultrasonic processor (Vibra cell T.M- VC 70, Newtown CT. U.S.A). The lid of the tube was pierced and the tube was incubated in a water bath at 95°C for 5 min and cooled on ice. The protein was kept at –80°C.

Hep G2 are adhesive cells. The medium in the flask was discarded and the cells were washed with PBS in the flask. The buffer was discarded, cells were lysed in 500 μl of SDS buffer in the flask and then the lysate was transferred into a microfuge tube following the same protocol as mentioned for E6.1cells.

2.6.10.4 Determination of Protein concentration

Concentration of protein extracts were determined by using a coomassie plus protein assay reagent kit (Pierce, Rockford, UK) following the manufacturers instructions. Bovine serum albumin (BSA) solution used as a standard (2mg/ml). BSA was mixed with ddH2O up to 50 µl in a set of test tubes with different concentrations. The dilutions of BSA (µl): H2O (µl) started with a blank (0: 50) and increased gradually as following: (5: 45), (10: 40), (15: 35), (20: 30), and (25: 25). The amount of protein in a tube with 5µl of BSA equals 10µg and so on. Then 1.5 ml of Coomassie dye was added to each tube and the tubes were vortexed carefully. The changes in absorbance were measured at 595 nm with a spectrophotometer. Data was plotted on the X coordinate with the corresponding OD values on the Y-axis. Standard curve was established (Figure 2.4). The curve was used to determine sample's protein concentrations. To determine concentration of protein extracts in the study's samples, a dilution of 1:10 of the protein (5 µl of protein + 45 µl ddH2O) was mixed with 5 ml of Coomassie dye and the tubes were vortexed carefully. The absorbance was measured at 595 nm with a spectrophotometer and the protein concentrations were determined by using the standard curve shown on Figure 2.4.

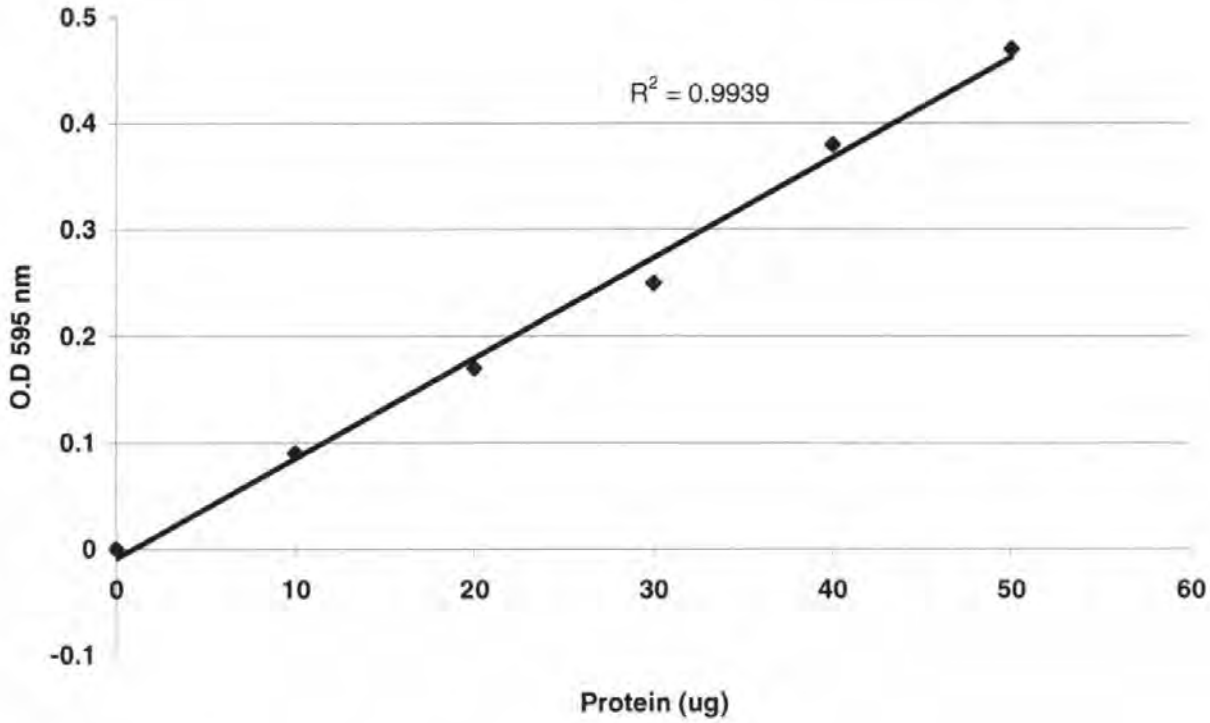


Figure 2.4: Protein standard curve

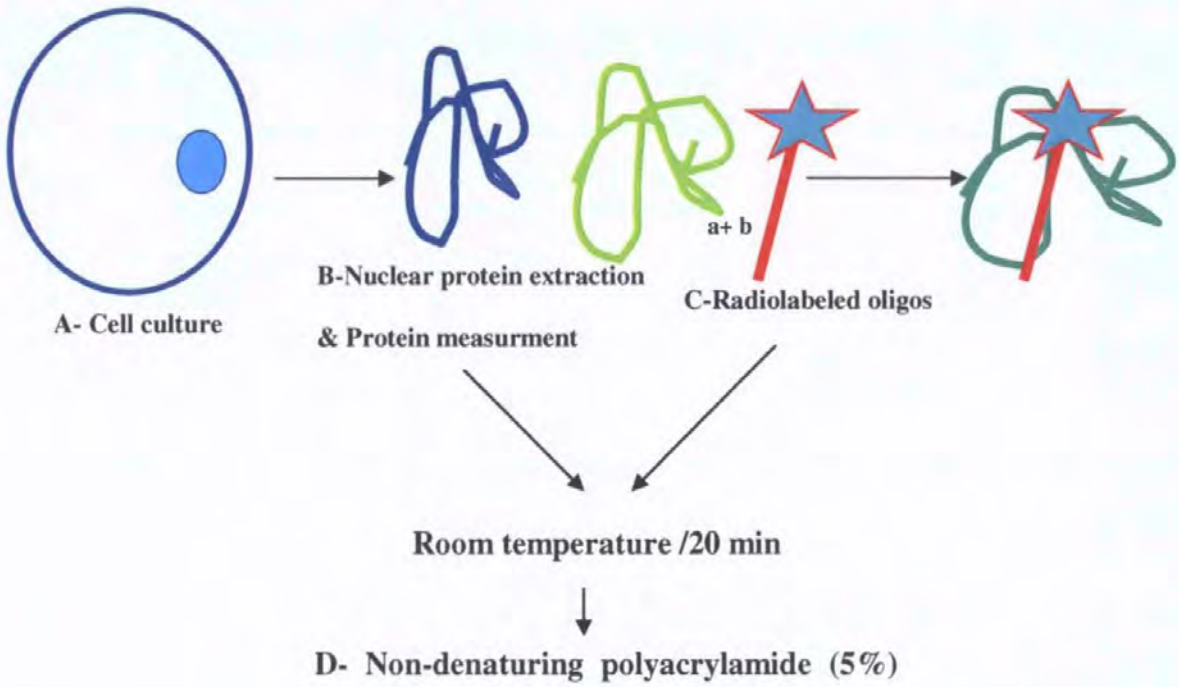
The concentration of BSA used as a standard is 2mg/ml (2µg/ µl). BSA was homogenized with ddH2O in a total volume of 50µl. The amount of protein was increased gradually from 0 (no protein= blank) to 10, 20, 30, 40, and 50µg. O.D. was measured with spectrophotometer at wavelength of 595nm. Data was scattered on X and Y-axis. The line was determined via linear regression.

2.6.11 Labelling of the oligonucleotide probes for EMSA

The reaction mixture was prepared from 2µl of T4 polynucleotide kinase (T4 PNK) 10x buffer, 2µl of T4 PNK (10 U/ µl) (Promega, Delta House, Southampton, UK), 4µl of ddH2O and 1µl of ATP [γ^{32}P] (20,000 cpm). The tube was incubated at 37°C for 20 min. 2µl of consensus oligonucleotides [NFκB: 5` TGA GGG GAC TTT CCC AGG C 3` (Promega, Delta House, Southhapton, UK) and HSF1: 5` GCC TCG AAT GTT CGC GAA GTT T 3` (Goldenberg et al., 1988) (MWG-BIOTECH UK LTD, Milton Keynes, UK)] were added into the tubes and incubated at room temperature for 10 min. The reaction was stopped with 1µl of 0.5M EDTA, diluted in 89 µl of TE buffer and was kept at -20°C.

2.6.12 Electrophoresis Mobility Shift Assay (EMSA)

This method was used to assess the translocation and activation of the transcription factors that bind to specific consensus DNA sequences (Mercié et al., 2000). In this study EMSA was performed to give us an estimate on NFκB and HSF-1 activation in PBMCs of patients with diabetes in response to stimulation with high D-glucose level. The protocol for this method was illustrated in Figure 2.5. BIORAD Protean^{R} II Xi cell (BIORAD Laboratories, Hempstead UK) was used for assessing the protein binding activity (Figure 2.5). The system contains glass plates, spacers, and a toothcomb. Before using, the glass plates and spacers were washed in detergent solution, followed by 70 % ethanol, rinsed thoroughly in tap water, and finally rinsed with ddH2O. The comb was inserted and the set was connected to the base.



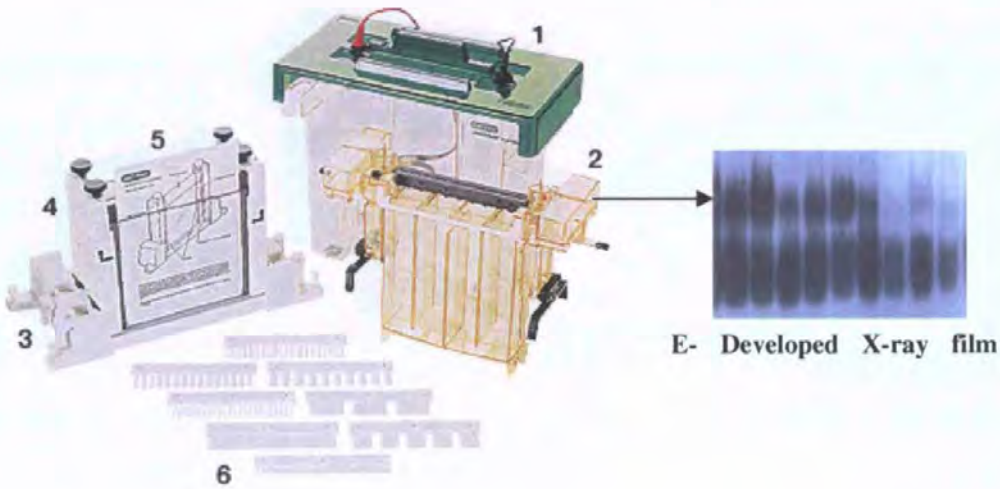


Figure 2.5: Protocol of electrophoresis mobility shift assay

A- Cells were grown in the recommended medium with and without stress factors.

B- Nuclear proteins were extracted and measured. C- DNA probes (a) were radiolabeled with ATP $\gamma^{32}P$ (b). An adequate volume of 10 μg of nuclear protein was processed with 2μl of gel shift binding buffer (5x), 1μl of radiolabelled oligonucleotide probe and loaded with 1μl of the loading dye to a 5% non-denaturing polyacrylamide gel. D- Gel was electrophoresed for 2-3 hr in BIORAD ProteanR II Xi cell. The system contains tank and lid (1), central cooling core (2), casting stand (3), sandwich clamps (4), alignment card (5), and combs (6). E- Gel was transformed to Whatman filter paper and exposed to X-ray film for 18 hrs at –80°C and developed. ProteanR II Xi cell picture was adopted from BIORAD website.

5 % nondenaturing polyacrylamide gel was prepared by mixing 5 ml of TBE 10x, 2.5 ml of 2 % bisacrylamide, 10ml of 40% acrylamide, 3.1 ml of 80% glycerol, 79.5 ml of ddH2O, 750 µl of 10 % of APS and 50 µl of TEMED. The mixture was cast to the set and left overnight at room temperature to allow complete polymerisation. The gel was run in 0.5x TBE at 100v for 1 hrs for warming up. An adequate volume of 10 µg of nuclear protein was measured and mixed with 2 µl of gel shift binding buffer (5x) and incubated at room temperature for 10 min, then mixed with 1 µl of radio-labelled oligonucleotides probes and incubated for another 20 min at room temperature. The samples were loaded with 1 µl of the loading dye and the gel was electrophoresed for another 2-3 hr. Gels were transferred to Whatman filter paper (Amersham, Pharmacia, Biotech. Bucks, UK), dried and exposed to X-ray film overnight at –80°C.

2.6.13 Super-shift assay for NFκB

The identity of the subunit proteins bound to the NFκB probe was determined by super-shifting with antibodies to the rel-family members p50 and p65, which have recently been shown to be the most common NFκB heterodimmer (Ghosh et al., 1998). EMSA was performed with four tubes as shown in Table 2.5. HelaScribe^{TM} nuclear extract was supplied with EMSA kit as a positive control protein. Antibodies, p50 and p65 were used as specific antibodies for p50/p65 NFκB heterdimer. Antibodies were supplied by New England Bio labs, Herts, UK.

Mixutre	Reaction 1 (-ve control)	Reaction 2 (+ve control)	Reaction 3 p50 antibody	Reaction 4 p65 antibody
dd H2O2	7 µl	5µl	4 µl	4 µl
GS-binding buffer	2 µl	2 µl	2 µl	2 µl
Hela extract	0 µl	2 µl	2 µl	2 µl
p50 antibody	0 µl	0 µl	1 µl	0 µl
p65 antibody	0 µl	0 µl	0 µl	1 µl
^{32}P labelled NFκB oligo	1 µl	1 µl	1 µl	1 µl
Total volume	**10 µl**	**10 µl**	**10 µl**	**10 µl**

Table 2.5: Super-shift assay for NFκB

The table shows the measurements of the reagents, which were used in the super-shift mobility electrophoresis for the NFκB heterodimer (p50/p65). ddH2O2 + GS-buffer + Hela + antibody were mixed and incubated at room temperature for 10 min. Then 1 µl of ^{32}P labelled NFκB oligo was added and incubated at room temperature / 20 min. GS- = gel shift binding buffer 5 X. 1 µl of the loading dye buffer was added into the reaction tubes and the mixture was loaded onto 5% nondenaturing gel. The gel was electrophoresed for 2-3 hr.

2.6.14 Competition shift assay

To confirm the specificity of the protein DNA complex, competition experiments were performed by a set of 4 tubes as shown in Table 2.6. The unlabeled AP1 oligonucleotide was used as a non-specific competitor that was supplied with EMSA kit. Unlabeled NFκB and HSF-1 oligonucleotides (50-fold excesses) were used as specific competitors.

2.6.15 Western Blot

BIORAD Protean^{R} II Xi cell was used in this method to assess the expression of p38 in hypeglycaemia and HO-stressed Hep G2 and E6.1 cells following the protocol outlines in 2.6.12. The SDS-PAGE gel was used for separating the proteins. The stack gel (4 % PAGE) and the resolving gel (12 % PAGE) were performed as shown in Table 2.7. The whole protein extracts (20 μg) were run through 12% polyacrylamide gels for 3 hrs and transferred to enhanced chemilumine scene (ECL) membrane (Amersham, Pharmacia, Biotech. Bucks, UK) by running the system at 1 volt overnight in the transfer buffer. The membrane was incubated with 5% milk buffer for 1hr to block non-specific bands. Afterwards, phospho-active antibody of p38 (rabbit IgG anti-human) in a dilution of 1:1000 was incubated with the blot for 2 hrs on the shaker and then washed. The membrane was incubated with conjugated secondary antibody (anti rabbit-IGg) in a dilution of 1/5000 for 1hr. Antibodies were supplied by New England Bio labs, Herts, UK. Super signal detection system was used to visualise the reactions (Pierce, Chester, UK).

Mixutre	Reaction 1 (-ve control)	Reaction 2 (+ve control)	Reaction 3 Specific	Reaction 4 Nonspecific
dd H2O2	**7 µl**	**5 µl**	**4 µl**	**4 µl**
GS-binding buffer	2 µl	2 µl	2 µl	2 µl
Hela extract	0 µl	2 µl	2 µl	2 µl
Unlabeled oligo	0 µl	0 µl	1 µl	0 µl
Unlabeled AP1 oligo	0 µl	0 µl	0 µl	1 µl
^{32}P labelled oligo	1 µl	1 µl	1 µl	1 µl
Total volume	**10 µl**	**10 µl**	**10 µl**	**10 µl**

Table 2.6: Competition shift assay

The table shows the measurements of the reagents, which were used in the competition experiments. ddH2O2 + GS-buffer + Hela + unlabeled oligos (50 fold) were mixed and incubated at room temperature for 10 min. Then 1 µl of ^{32}P labelled NFκB oligo was added and incubated at room temperature for 20 min. GS-binding buffer = gel shift binding 5 X buffer.

Reagent	Resolving 12% PAGE	Stack 4% PAGE
30% acrylamide / Bis	40 ml	3.9 ml
1.5 M Tris-HCl pH 8.8	25 ml	_
0.5 M Tris-HCl pH 6.8	_	7.5 ml
10%SDS	1.0 ml	300 µl
H2O	33.5 ml	18.3 ml
TEMED	50 µl	30 µl
10%A.P.S	500 µl	150 µl
Total volume	100 ml	30 ml

Table 2.7: SDS-Polyacrylamide gel for Western Blot

SDS-PAGE gel for Western Blot was performed from two layers, the resolving (running gel) is 12% PAGE to separate the p38 (MW=38KD) and the stack (high porosity gel) is 4% PAGE. The mixture was cast to the set and left overnight at room temperature to allow complete polymerisation.

2.6.15.1 Buffers for Western blot

Tris-Glycine electrophoresis buffer is the running buffer. The stock (5 x) buffer contains 45.0 g of Tris-Base, 216.0 g of glycine, and 15.0 g of SDS in a total volume of 3 litre were completed with ddH2O. For using, dilute 300 ml of 5x with 1.2 litre of ddH2O.

Transfer buffer contains 15.1g of Tris-base and 72g of glycine in 5 litres of ddH2O.

10% SDS buffer was made by dissolving 10g of SDS were dissolved in 60 ml dH2O and completing with ddH2O to 100 ml.

Washing buffer (TBS-Tween) contains 1900 ml of ddH2O, 100 mM of NaCl (80 ml of 2.5 M), 20 mM of Tris (20 ml of 1 M) and 0.1% of Tween 20 (2 ml).

Blocking buffer was made by mixing 5 gm of non-fat milk in 100 ml of Tween-20 buffer.

2.6.16 Quantification of bands

Phosphor-imager soft ware program was used for quantifying the bands in autoradiographs (Bio-Rad Multi-Analyst ™/ PC version 1.1).

2.7 Autoclaving

All solutions, glassware, and plastic ware used in the techniques of DNA analysis were autoclaved at a temperature of 121°C, and pressure of 15-pressure specific index (p.s.i) for 30 min in a steam autoclave (Prior Clave Limited, London, UK).

Chapter 3

RESULTS

3.1 Results of genotyping

3.1.1 Result of the (CA)n micosatellite genotyping of the NFκB gene on 4q24

Microsatellite analysis for the NFκB gene was performed in 217 patients with TIDM and 111 NC. Clinical characteristics of these groups are shown in Table 3.1. In our population, 18 alleles (A1-A18) of the (CA) repeat microsatellite in the regulatory region of the NFκB gene were detected. The size of the alleles ranged from 120 to 154 bp (A1-A18) (Figure 3.1). The frequencies of the alleles in the patients and controls are shown in Table 3.2. The A8 and A14 alleles were the most prevalent in the NC (19.8% and 28.4% respectively). In contrast, in those patients with TIDM there was a highly significant decrease in the frequency of both the A8 as well as the A14 allele compared to the NC (6.2% vs 19.8%, χ^2= 26.75, p=0.000004, OR= 3.7 and 3.9 % vs 28.4 %, χ^2= 79.80, p=0.000017, OR= 9.7 respectively). In those patients with TIDM, there was a highly significant increase in the frequency of the A10 (138 bp) allele compared with the NC (17.5% vs 2.7% respectively, χ^2= 32.8, p = 0.00002, OR= 9.4) (Table 3.2).

More than 70 genotypes were identified in our population. Table 3.3 shows the frequency of the common genotypes. In NC population, the most common genotype was A8/A14 but this was only found in 1 of the 217 patients with TIDM (12.6 % vs 0.5%, χ^2= 22.1, p= 0.00014). The second most prevalent genotype in NC group was A14/A14 and this was completely absent from the patient group (9.9% vs 0.0%, χ^2= 19.3, p=0.0007). Similarly, the A8/A11 genotype was found in 6.3% of the NC but was absent from the patient population (6.3% vs 0.0%, χ^2= 11.1). However, this difference was not significant after correction of the p-value (p=0.06).

In the patient group, the A9/A10 genotype was the most common genotype and was present in 4 of the 111 NC (11.9 % vs 3.6%, χ^2= 5.2, OR= 3.3, p=ns). The A10/A11 genotype was found in 9.2% of the patients but was absent from the NC group (χ^2= 9.3, p= ns). No difference was found between observed and expected frequency of the alleles in both the patient and the normal populations (Table 3.4).

Table 3.5 shows the frequency of the NFκB genotypes with respect to age at onset of TIDM and gender. The patients were categorised into <10, 10-20 or >20 years age at onset of diabetes. There were no significant differences in the frequency of the genotypes with age at onset although the A9/A10 genotype was found in only 4.8% of those diagnosed before the age of 10 years compared to 13.2% and 14.0% in the 10-20 years and >20 years age at onset respectively. There were no significant differences in the frequency of the NFκB genotypes with gender. Table 3.6 shows the frequency of the common NFκB alleles with respect to the presence or absence of diabetic microvascular disease after 20 years duration of diabetes. Whilst A9 was more common in those with diabetic microvascular disease compared to those with no complications after 20 years of diabetes, these differences were not significant. All the other frequencies of the alleles were similar between the patient sub-groups.

Patient subgroup	DC (n=38)	DN (n=48)	DNU (n=55)	DR (n=76)	NC (n=111)
Male: Female	14:24	22:26	26:29	37:39	58:53
AAO (years)	17.8 ±10.7	16.4 ±10.4	19.1 ±11.3	18.1 ±10.5	
Duration of diabetes (years)	29.2 ±10.5	34.5 ± 11.5	31.5 ± 10.8	32.1 ±11.7	

Table 3.1: Clinical characteristics of the patient sub-groups in the microsatellite analysis of NFκB

DC, those patients with no microvascular complications after 20 years duration of diabetes; DN, those patients with diabetic nephropathy; DNU, those patients with overt diabetic neuropathy; DR, those patients with diabetic retinopathy and NC, normal healthy controls. Data is shown as mean of years ± s.d. AAO= age at onset of diabetes.

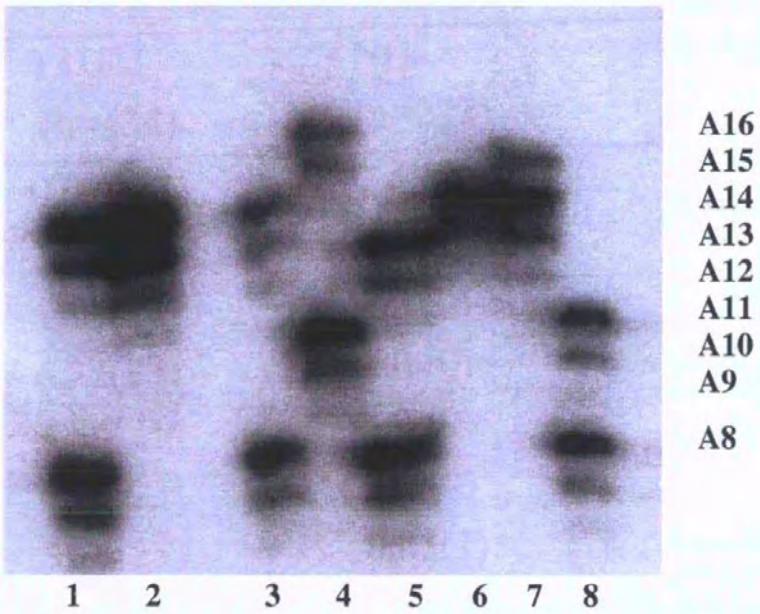


Figure 3.1: NF*k*B microsatellite analysis

Autoradiograph is showing some of the CA dinucleotide repeats polymorphisms in the human NF*k*B gene. Lane 1: A8/A14, Lane 2: A13/A14, Lane 3: A8/A14, Lane 4: A11/A16, Lane 5: A8/A13, Lane 6: A14/A14, Lane 7: A13/A14, Lane 8: A8/A11.

Allele	Patients with TIDM % (n=434)	Normal healthy controls % (n=222)
A1	0.0 (0)	0.5 (1)
A2	1.6(7)	0.5 (1)
A3	1.2 (5)	0.0 (0)
A4	6.2 (27)	0.0 (0)
A5	4.8 (21)	0.0 (0)
A6	5.8 (25)	0.0 (0)
A7	5.8 (25)	0.5 (1)
A8	6.2 (27)	19.8 (44) [a]
A9	14.9 (65)	9.9 (22)
A10	17.5(76) [b]	2.7 (6)
A11	10.6 (46)	9.9 (22)
A12	7.6(33)	2.3 (5)
A13	5.3 (23)	11.3 (25)
A14	3.9 (17)	28.4 (63) [c]
A15	6.7 (29)	7.2 (16)
A16	1.6(7)	5.4 (12)
A17	0.2 (1)	0.9 (2)
A18	0.0 (0)	0.9 (2)

Table 3.2: Frequency (%) of NFκB microsatellite alleles in patients with TIDM and normal healthy controls

Allele frequencies are expressed as percentage of the total number of chromosomes. The numbers in the top parentheses is the number of chromosomes.

[a] vs patients with TIDM, $p = 0.000004$

[b] vs normal healthy controls, $p = 0.00002$

[c] vs patients with TIDM, $p = 0.000017$

Genotypes	Patients with TIDM % (n=217)	Normal healthy controls % (n= 111)
A8/A9	3.2 (7)	4.5 (5)
A8/A11	0.00 (0)	6.3 (7)
A8/A14	0.5 (1)	12.6 [a] (14)
A9/A10	11.9 (26)	3.6 (4)
A9/A15	4.2 (9)	0.9 (1)
A10/A11	9.2 (20)	0.0 (0)
A14/A14	0.00 (0)	9.9 [b] (11)
X/X	70.9 (154)	62.2 (69)

Table 3.3: Frequency (%) of NFκB genotypes in patients with TIDM and normal healthy controls

The table shows the frequency of genotypes with prevalence >3%. X = all other genotypes. [a] and [b] vs patients with TIDM, p = 0.00014 and p = 0.0007 respectively. X/X = other genotypes.

	Patients with TIDM (n=217)				Normal healthy controls (n=111)			
Genotype	**Expected frequency**	**Expected number**	**Observed number**		**Expected frequency**	**Expected number**	**Observed number**	
A8/X	0.07	17	21	$\chi^2 = 13.02$	0.3	38	40	$\chi^2 = 3.64$
A9/X	0.18	38	42	**p = 0.07**	0.2	21	16	p = 0.8
A10/X	0.1	24	30		0.02	2	2	
A11/X	0.04	8	8		0.11	13	12	
A12/X	0.03	6	14		0.01	1	2	
A13/X	0.03	6	12		0.11	11	12	
A14/X	0.005	1	4		0.22	22	19	
Others	0.5	117	86		0.03	3	8	

Table 3.4: Hardy-Weinberg equation and NFκB genotypes in patients with TIDM and normal healthy controls

Hardy-Weinberg principle was compared with the observed results of the most common genotypes in patients with TIDM and normal healthy controls. Frequency of any heterozygote= 2X product of allele frequencies (Hartl et al., 1999). The observed numbers fit the predicted numbers in patients with TIDM and normal healthy controls.

	Age at Onset (years)		
	<10	**10-20**	**>20**
Genotype	**(n=42)**	**(n=68)**	**(n=107)**
	%	**%**	**%**
	Male: Female	**Male: Female**	**Male: Female**
A9/A10	4.8	13.2	14.0
	0:2	4:5	8:7
A10/A11	16.7	8.8	6.5
	1:6	2:4	4:3
A10/X	14.3	16.2	12.2
	2:4	7:4	5:8
X/X	64.2	61.8	67.3
	15:12	20:22	34:38

Table 3.5: Frequency (%) of the most common NFκB genotypes with respect to age at onset of TIDM and gender

Patients were classified into three groups according to the age at onset of TIDM: <10, 10-20, >20 years. No difference was found in the frequency of the genotypes with either age at onset or gender. X/X = other genotypes

Allele	DC % (n=76)	DN % (n=96)	DNU % (n=110)	DR % (n=152)
A10	19.8 (15)	17.7 (17)	16.4 (18)	17.1 (26)
A9	9.2 (7)	16.7 (16)	16.4 (18)	15.8 (24)
A11	11.8 (9)	10.4 (10)	9.1 (10)	11.2 (17)
X	59.2 (45)	55.2 (53)	58.1 (64)	55.9 (85)

Table 3.6: Frequency (%) of the most common NFκB alleles with respect to presence or absence of diabetic microvascular complications

There were no significant differences in the frequency of the NFκB alleles between any of the patient subgroups. DC, those patients with no nephropathy, retinopathy or overt neuropathy after 20 years duration of diabetes. DN, those patients with nephropathy. DNU, those patients with overt neuropathy. DR, those patients with retinopathy. X= other alleles.

3.1.2 Results of the (AAATA) microsatellite analysis of the HSP70-A2 (14q22)

Microsatellite analysis of the HSP70-A2 gene was performed in 193 patients with TIDM and 94 NC. Clinical characteristics of these groups are shown in Table 3.7. In our population, 11 alleles (H1-H11) were detected, their size ranged from 124 to 174 bp with a difference of 5 bp (Figure 3.2). The frequency of the alleles in the patients and controls are shown in Table 3.8. The H1 and H5 alleles (124 and 144bp) were the most prevalent in NC (19.7% and 39.9% respectively). In contrast, in those patients with TIDM there was a highly significant decrease in the frequency of both the H1 as well as the H5 allele compared to NC group (2.6 % vs 19.7%, χ^2= 46.9, p < 0.01, OR= 9.21 and 9.8% vs 39.9%, χ^2= 70.3, p < 0.01, OR= 6.08 respectively). In those patients with TIDM, there was a highly significant increase in the frequency of the H3 and H7 (134 and 154 bp) alleles compared with the NC (17.6% vs 8.0%, χ^2= 8.7, p= 0.03, OR= 2.5 and 29.5 vs 3.7, χ^2 = 49.1, p <0.01, OR= 9.8 respectively) (Table 3.8).

The results revealed 28 genotypes in this population. Table 3.9 shows the frequency of the common genotypes. In the NC group, the most common genotype was H5/H5 but this was found in only 11 of the 193 patients with TIDM (21.3% vs 5.7%, χ^2= 14.3, p= 0.006, OR= 4.47). The second most prevalent genotype in NC group was H1/H5 and this was completely absent from the patient group (11.7% vs 0.0% χ^2= 20.4, p = 0.00008). In the patient group, the H7/H7 genotype was the most common genotype and was present in 2 of the 94 NC (14.5% vs 2.1%, χ^2= 9.1, p = ns). The H3/H7 genotype was found in 10.4% of the patients but was 3.2% in NC group (χ^2= 4.4, p= 0.06). The observed and predicted frequency of the alleles was similar in patients with TIDM and NC population (Table 3.10).

Table 3.11 shows the frequency of the common HSP70 alleles with respect to the presence or absence of diabetic microvascular complications after 20 years duration of diabetes. There was no difference was observed between any of the diabetic complications and the DC group. Patients with TIDM were divided into three groups according to the age at onset of the disease: <10 (n=57), 10-20 (n=63), and >20 years (n= 73). There were no significant differences in the frequency of the genotypes with either age at onset or gender (Table A.1, appendix).

	DC	DN	DNU	DR	SD	NC
Patient subgroup	(n=44)	(n=38)	(n=40)	(n=55)	(n= 16)	(n=94)
Female: Male	29:15	21: 17	18:22	30:25	9:7	48: 46
AAO (years)	15.7 ± 9.4	17.1 ±11.2	19.2 ± 10.04	18.6 ± 11	4.5±3.3	
Duration (years)	30.4 ±9.5	32 ±11.6	31.8 ± 9.9	31.1 ± 11	6.6 ± 3.8	

Table 3.7: Clinical characteristics of the patient sub-groups in the microsatellite analysis of HSP70-A2

DC, those patients with no microvascular complications after 20 years duration of diabetes; DN, those patients with diabetic nephropathy; DNU, those patients with overt diabetic neuropathy; DR, those patients with diabetic retinopathy and NC, normal healthy controls. Data is shown as mean of years ± s.d. AAO= age at onset.

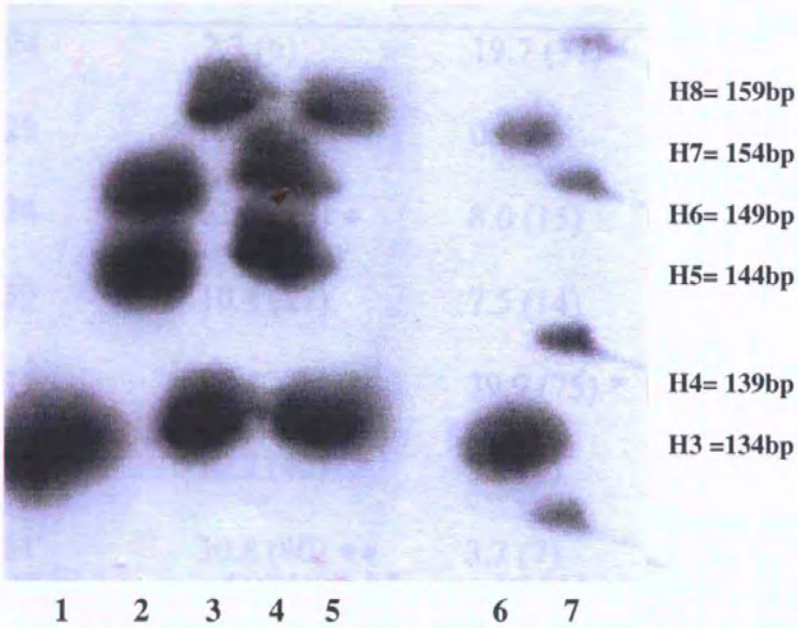


Figure 3.2: HSP 70-A2 microsatellite analysis

Autoradiograph is showing some of the 11 alleles (H1-H11) of the (AAATA) pentanucleotide repeat polymorphism in the human HSP70-A2 gene. Their size ranged from 124 to 174 bp with a difference of 5 bp. Lane 1: H3/H3, Lane 2: H5/H6, Lane 3: H3/H7, Lane 4: H5/H6, Lane 5: H3/H7, Lane 6: H3/H3, Lane 7: A molecular weight (10 bp).

Allele (H)	Size (bp)	Patients with TIDM % (n=386)	Normal healthy controls % (n =188)
H1	124	2.6 (10)	19.7 (37) [a]
H2	129	0.0 (0)	0.5 (1)
H3	134	17.6 (68) [b]	8.0 (15)
H4	139	9.8 (38)	7.5 (14)
H5	144	9.8 (38)	39.9 (75) [c]
H6	149	12.1 (47)	3.2 (6)
H7	154	29.5 (114) [d]	3.7 (7)
H8	159	6 (23)	0.5 (1)
H9	164	3.6 (14)	12.2 (23)
H10	169	1 (4)	4.8 (9)
H11	174	7.8 (30)	0.0 (0)

Table 3.8: The frequency (%) of HSP70-A2 microsatellite alleles in patients with TIDM and normal healthy controls

a and c vs patients with TIDM, $p < 0.01$ for both

b vs NC, $p = 0.03$

d vs NC, $p < 0.01$.

Genotypes	Patients with TIDM % (n=193)	Normal healthy controls % (n =94)
H1/H1	2.1 (4)	7.5 (7)
H1/H5	0.0 (0)	11.7 [a] (11)
H3/H3	9.3 (18)	3.2 (3)
H3/H7	10.4 (20)	3.2 (3)
H5/H5	5.7 (11)	21.3 [b] (20)
H7/H7	14.5 (28)	2.1 (2)
X/X	55.4 (112)	51.0 (48)

Table 3.9: The frequency (%) of the most common genotypes of HSP70 gene in patients with TIDM and normal healthy controls

X/X=all other genotypes.

[a] vs patients with TIDM, p= 0.00008

[b] vs patients with TIDM, p= 0.006

	Patients with TIDM (n= 193)				Normal healthy controls (n= 94)			
Genotype	**Expected frequency**	**Expected number**	**Observed number**		**Expected frequency**	**Expected number**	**Observed number**	
H1/ X	0.008	2	6	χ^2 =30.1	0.295	28	29	χ^2 =4.98
H3/X	0.26	50	50	$p < 0.05$	0.025	2	5	$p = 0.4$
H5/X	0.058	12	23		0.438	41	34	
H7/X	0.26	50	65		0.001	0	2	
H8/X	0.02	4	11					
H9/X	0.001	0	4		0.01	1	3	
Others	0.4	75	34		0.23	22	21	

Table 3.10: Hardy- Weinberg equilibrium and HSP70-A2 genotypes in patients with TIDM and normal healthy controls

Hardy-Weinberg principle was compared with the observed results of the most common genotypes in patients with TIDM and normal healthy controls. Frequency of any heterozygote= 2x product of allele frequencies (Hartl et al., 1999). The observed numbers fit the predicted numbers in normal healthy controls but not in patients with TIDM. X = other alleles.

Allele	DC	DN	DNU	DR
	%	%	%	%
(n=386)	(n=88)	(n=76)	(n=80)	(n=110)
H1	0.0	2.6	2.4	1.8
	(0)	(2)	(2)	(2)
H3	12.5	13.2	8.8	11.8
	(11)	(10)	(7)	(13)
H5	3.5	11.8	8.8	11.8
	(3)	(9)	(7)	(13)
H7	40.9	26.3	25	28.2
	(36)	(20)	(20)	(31)
X	43.1	46.1	55	46.4
	(38)	(35)	(44)	(51)

Table 3.11: Frequency (%) of the most common HSP70 alleles with respect to the presence or absence of diabetic microvascular complications

No difference was observed between any of the diabetic complications and diabetic controls. DC, those patients with no microvascular complications after 20 years duration of diabetes; DN, those patients with diabetic nephropathy; DNU, those patients with overt diabetic neuropathy and DR, those patients with diabetic retinopathy. X= other alleles

Results of the (CAAA) 5 microsatellite analysis of the SORD gene (15q21)

PCR-based microsatellite analysis was performed to detect the repeats in the promoter region of the SORD gene. A total number of 48 genomic DNA samples were randomly chosen. The PCR-amplified fragment size was 258 bp. PAGE results have revealed that the (CAAA) 5 microsatellite was not polymorphic. Figure 3.3 is an autoradiograph, which shows no difference in the fragment size of the genotyped group. Three PCR products have been sequenced by MWG-Biotech, UK LTD to confirm our results. The sequencing results showed that the (CAAA) microsatellite was five repeats in all samples (Figure 3.4 A, B, and C).

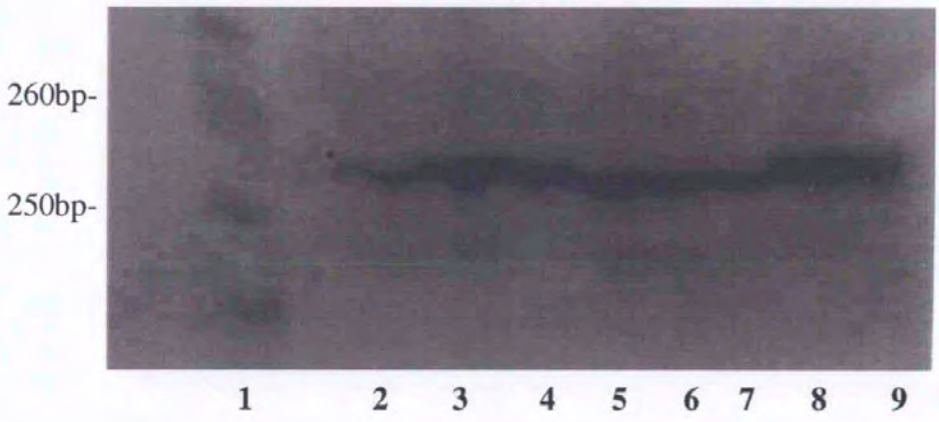


Figure 3.3: SORD microsatellite analysis

Autoradiograph shows the microsatellite (CAAA) 5 of the SORD gene. Lane 1 is a 10 bp molecular weight ladder. Lanes 2-9 demonstrate the fragment size of 258bp in 8 subjects.

A (MWG-order number VIII-73, template 1)

GAGCCGGGATCGCACCACTGTACTCCAGCCTCGGCGACAAACAAACAAACAA
ACAAAAAAAACAGCGCGCTGCAGATGGAGCCAGCACCCGGGCTTCTCGCTCCC
AGCCCCCTCCCGAAGCCCCGCCCCGCCTCCGGGTCTGGAGGAGGCTGGGAAGC
CCCGCCCCTCCACGCTAGCGCCGCCCAGGCTGGCACAAAGGAGGAAGCCTA

B (MWG-order number VIII-73 template 2)

CAGTGAGCCGGGATCGCACCACTGTACTCCAGCCTCGGCGACAAACAAACAAA
CAAACAAAAAAAACAGCGCGCTGCAGATGGAGCCAGCACCCGGGCTTCTCGC
TCCCAGCCCCCTCCCGAAGCCCCGCCCCGCCTCCGGGTCTGGAGGAGGCTGGG
AAGCCCCGCCCCTCCACGCTAGCGCCGCCCAGGCTGGCACAAAGGAGGAAGC
CTAGTCA

C (MWG-order number V-423 template SORD)

GCCGGGATCGCACCACTGTACTCCAGCCTCGGCGACAAACAAACAAACAA
ACAAAAAAAACAGCGCGCAGAGATGGAGCCAGCACCCGGGCT

Figure 3.4: Results of sequencing the amplified region of the (CAAA) 5 on the 5`UTR of the SORD gene (15q21)

The figure shows the 5` noncoding region of the SORD gene. The amplified fragment presents (CAAA) 5 repeats. A, B, and C are the sequences of three different samples. The underlined nucleotides are microsatellite's repeats, which are equal in all subjects.

3.1.3 Results of the T (+126) C polymorphism of the PKC-β gene (16p12)

PCR-based RFLP analysis was developed and applied to type PKC-β in 175 patients with TIDM and 88 NC. Clinical characteristics of subjects are shown in Table 3.12. The PCR product was a fragment size of 282 bp. Figure 3.5 demonstrates the results of RFLPs using SmaI endonuclease that revealed three genotypes, homozygous CC, TT and heterozygous CT. The frequency of the alleles and genotypes in patients with TIDM and NC groups are shown in Table 3.13. There was no significant difference in the frequency of either the T (wild) or the C alleles in patients with TIDM compared with NC (0.72 vs 0.68 and 0.27 vs 0.31 respectively). The frequency of genotypes TT, TC and CC in patients with TIDM was 54.3%, 36.6% and 9.1% respectively that were not significantly different from those of NC group (45.5%, 45.5% and 9.1% respectively). Hardy-Weinberg equation was applied, the predicted frequencies and numbers fit to the observed results (Table 3.14).

When patients were divided into subgroups according to the presence of complications (DC, DN, DNU, and DR). There was no significant difference in the frequency of either the alleles or genotypes in patients with complications compared with those in patients with DC (Table 3.15). Our results suggest that the T (+126) C polymorphism of the PKC-β gene is not associated with the susceptibility to TIDM or any of the late micro vascular complications.

	DC	DN	DNU	DR	NC
	n=34	n=39	n=42	n=60	n=88
Male: Female	13: 21	20: 19	26: 16	33: 27	44: 44
AAO (years)	15 ± 10.6	18.8 ± 12.4	21.5 ± 11.9	20.6 ± 12.1	
Duration (years)	30 ± 9.1	30.1 ± 12	29.3 ± 10	29.7 ± 10.9	

Table 3.12: Clinical characteristics of the patient sub-groups in RFLP analysis of PKC-β

DC, those patients with no microvascular complications after 20 years duration of diabetes. DN, those patients with diabetic nephropathy. DNU, those patients with overt diabetic neuropathy. DR, those patients with diabetic retinopathy. NC= normal healthy controls. Data is shown as mean of years ± s.d. AAO= age at onset.

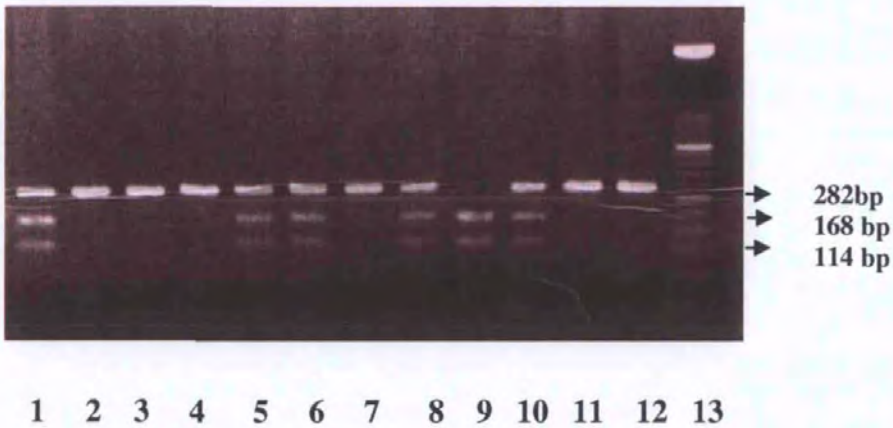


Figure 3.5: RFLP analysis of the C (+126) T on exon 10 of the PKC-β gene

A total volume of 20µl of the PCR product (282bp) was incubated with 1µl of Sma I restriction endonucleases for at least 3 hrs. A 2 % agarose gel electrophoresis was used for polymorphisms detection. Lane 13 is the molecular weight ladder. Lane 1, 5,6,8,10 are heterozygous (TC). Lane 2, 3, 4, 7, 11 and 12 are homozygous wild type (TT). Lane 9 is a homozygous mutant (CC).

Allele	Normal controls % (n=176)	Patients with TIDM % (n= 350)	Genotype	Normal controls % (n= 88)	Patients with TIDM % (n= 175)
T	68.2	72.6	TT	45.4	54.3
	(120)	(254)		(40)	(95)
C	31.8	27.4	TC	45.4	36.6
	(56)	(96)		(40)	(64)
			CC	9.2	9.1
				(8)	(16)

Table 3.13: Frequencies of alleles and genotypes of T (+126) C polymorphism in exon 10 of the PKC-β gene in TIDM and normal healthy controls

T allele (wild type) and C allele did not show a significant difference in either patients with TIDM or normal healthy controls. Genotypes did not show any difference between patients and normal healthy controls.

	Patients with TIDM				Normal healthy controls			
Genotype	**Expected frequency**	**Expected number**	**Observed number**		**Expected frequency**	**Expected number**	**Observed number**	
TT	0.52	91	95	$\chi^2 = 0.21$	0.47	41	40	$\chi^2 = 0.12$
TC	0.4	68	64	**p = 0.9**	0.43	38	40	**p = 0.9**
CC	0.08	16	16		0.1	9	8	

Table 3.14: Hardy-Weinberg equation and PKC-β genotypes in patients with TIDM and normal healthy controls

Hardy-Weinberg principle was compared with the identified genotypes in patients with TIDM and normal healthy controls. The observed numbers fit the predicted numbers in patients with TIDM and normal healthy controls.

Allele	DC % (n=68)	DN % (n=78)	DNU % (n=84)	DR % (n=120)	Genotype	DC % (n=34)	DN % (n=39)	DNU % (n=42)	DR % (n=60)
T	76.5	70.5	72.6	71.7	**TT**	55.9	51.3	57.1	53.3
	(52)	(55)	(61)	(86)		(19)	(20)	(24)	(32)
C	23.5	29.5	27.4	28.3	**TC**	41.2	38.4	31	36.7
	(16)	(23)	(23)	(34)		(14)	(15)	(13)	(22)
					CC	2.9	10.3	11.9	10
						(1)	(4)	(5)	(6)

Table 3.15: Frequencies of alleles and genotypes of T (+126) C polymorphism in exon 10 of the PKC-β gene in patients subgroups

No difference in the frequency of either alleles or genotypes was observed between diabetic subgroups. DC= uncomplicated diabetic patients, DN= diabetic pateints with nephropathy, DNU= diabetic pateints with neuropathy and DR= diabetic pateints with retinopathy. T is the wild allele and C is the modified nucleotide. TT is a homozygous (wild type) genotype, TC is heterozygous, and CC is the modified homozygous.

3.2 Results of cell culture and protein assay

3.2.1 NFκB-DNA binding activity

3.2.1.1 Super-shift experiment

The identity of the subunit proteins bound to the NFκB probe was demonstrated by super-shifting with antibodies to the rel-family members p50 and p65, which have been shown to be the most common NFκB heterodimmer (Ghosh et al., 1998). Figure 3.6 is an autoradiograph, which demonstrates the binding of the protein complex (p50/p65) with the radiolabelled DNA-oligonucleotide. Hela protein extract was used as a positive control as shown in lane 2 while no band was developed in lane 1 as this was the negative control with no protein. Lane 3 shows that an antibody to p50 did not affect the shift of the complex while the antibody to p65 in lane 4 delayed the migration of unit 65 as being the efficient part in the NFκB complex, leaving the lower band (p50) unaffected.

3.2.1.2 Competition experiment

To confirm the specificity of the protein DNA complex for NFκB sequence, we have performed competition experiments. Competition assays contained 50-fold excesses of unlabeled NFκB oligonucleotide as a specific competitor, which blocked all of DNA binding, while the non-specific competitor (AP1) failed to block the DNA binding in DNA-protein complex (Figure 3.7).

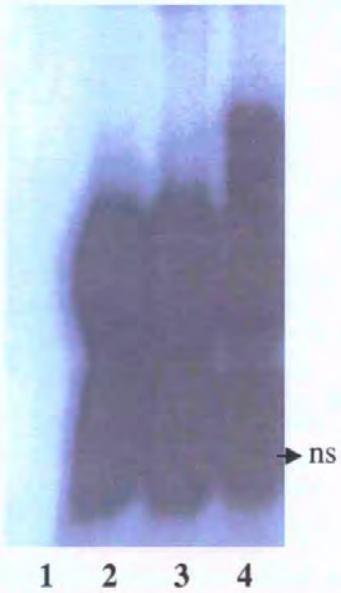


Figure 3.6: Super-shift assay for NFκB

Super-shift assay for the heterodimer NFκB (p65&p50) protein: Lane 1: Negative control, Lane 2: positive control, Lane3: p50 antibody in combination with NFκB hetrodimer, Lane4: p65 antibody in combination with NFκB hetrodimer. ns= non-specific band.

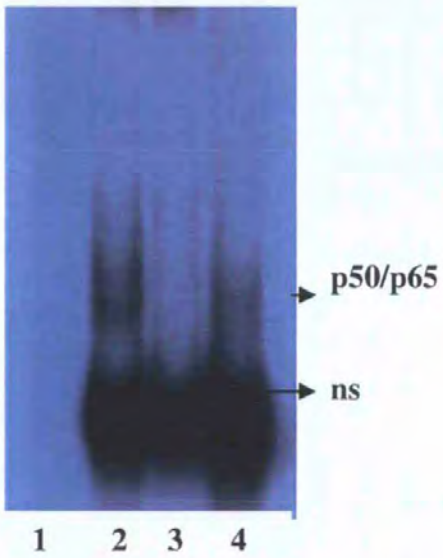


Figure 3.7: Competition shift assay for NFκB-DNA binding activity

Competition assays were performed with binding reaction mixtures containing 50-fold excesses of unlabeled NFκB oligonucleotide (lane 3). Lane 1: Negative control (no protein), Lane2: positive control (Hela extract). Lane 4: non-specific competitor AP1. ns=non-specific band.

3.2.1.3 NFκB-DNA binding activity in cell lines

3.2.1.3.1 Hyperglycaemia and NFκB -DNA binding activity in different cells

To simulate the diabetic state and the effect of uncontrolled chronic hyperglycaemia, cells were stimulated with increased D-glucose level and grown in RPMI-1640 medium for 5 days at 37^{0} C. EMSA results have shown that hyperglycaemia induced NFκB-DNA binding activity. Arbitrary units (AU) quantitation showed a significant increase in hyperglycaemia -stressed Hep G2 with 21 and 31mM D-glucose compared with unstressed (NG) (0.3 ± 0.01 and 0.5 ± 0.01 vs 0.2 ± 0.01, p= 0.002 and 0.001 respectively). The NFκB-DNA binding activity was correlated with glucose concentration since a concentration of 31mM D-glucose induced approximately a two-fold increase in NFκB-DNA binding activity compared with a concentration of 21 mM (0.3 ± 0.01 vs 0.5 ±0.01, p=0.006) (Figure 3.8).

The concentration of 31mM D-glucose was fixed in all experiments in this study. hyperglycaemia-induced NFκB-DNA binding activity in different cell types was assessed by phospho-imager quantitation (Figure 3.9). Results have revealed a 2.5 fold increase in basal activity in hyperglycaemia-stressed Hep G2 relative to NG (0.5 ± 0.1 vs 0.2 ± 0.04, p= 0.001) while no difference was observed in hyperglycaemia -stressed E6.1 compared with that in NG group (0.25 ± 0.05 vs 0.2 ± 0.02, p=ns). No significant increase in NFκB-DNA binding activity was observed in hyperglycaemia-stressed human PBMCs from NC individuals compared with unstressed cells (0.1 ± 0.09 vs 0.2 ± 0.1, p=ns) (Figure 3.10).

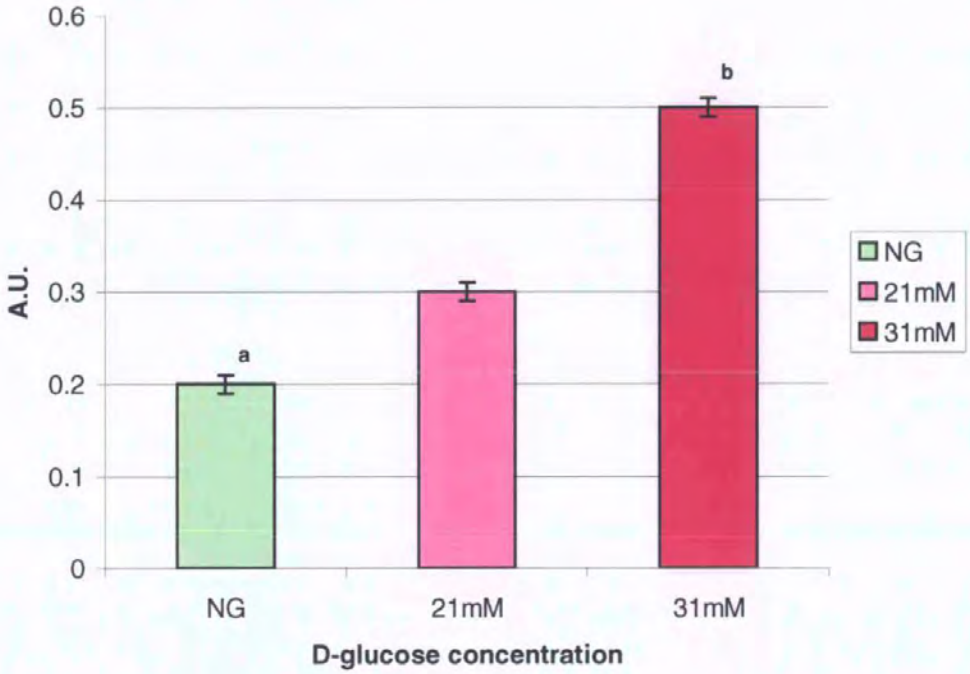


Figure 3.8: NFκB binding activity under different D-glucose concentration

A bar graph shows the results of three experiments. Arbitrary units quantitation (AU) revealed a significant increase in response to 21& 31 mM D-glucose. NG= normoglycemia.

[a] vs stressed Hep G2 with 21 and 31 mM, p= 0.002 and p= 0.001 respectively

[b] vs Hep G2 stressed with 21 mM, p= 0.006

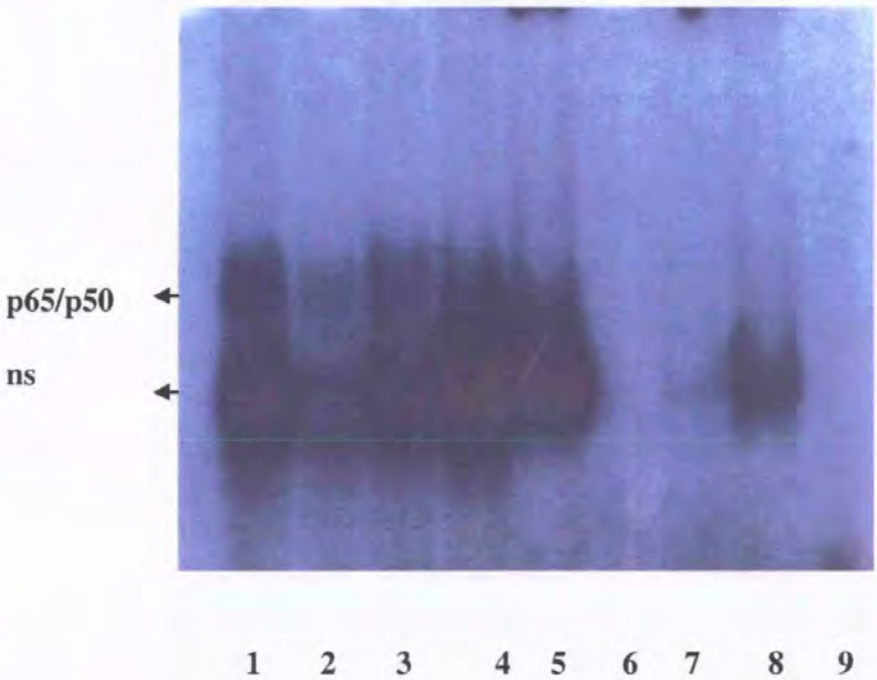


Figure 3.9: NFκB-DNA binding activity in stressed-cell lines

An autoradiograph, which depicts the binding of equal amounts of nuclear protein extracts from Hep G2 and E6.1 cells to an oligonucleotide containing NFκB motif. The heterodimer NFκB complex (p65/p50) is the upper band while the lower band is a non-specific (ns = free protein). Lane 1& 2:E6.1 cells contols, lane 3: hyperglycaemia -stressed E6.1 cells, lane 4: HO-stressed E6.1 cells, lane 5: hyperglycaemia -stressed HepG2 cells, Lane 6,7 HepG2 cells with HO stress, lane 8: unstressed-HepG2 cells, Lane 9: negative control.

Hyperglycaemia = a concentration of 31mM D-glucose

HO= hyperosmolar stress with a final concentration of 110 mM NaCl

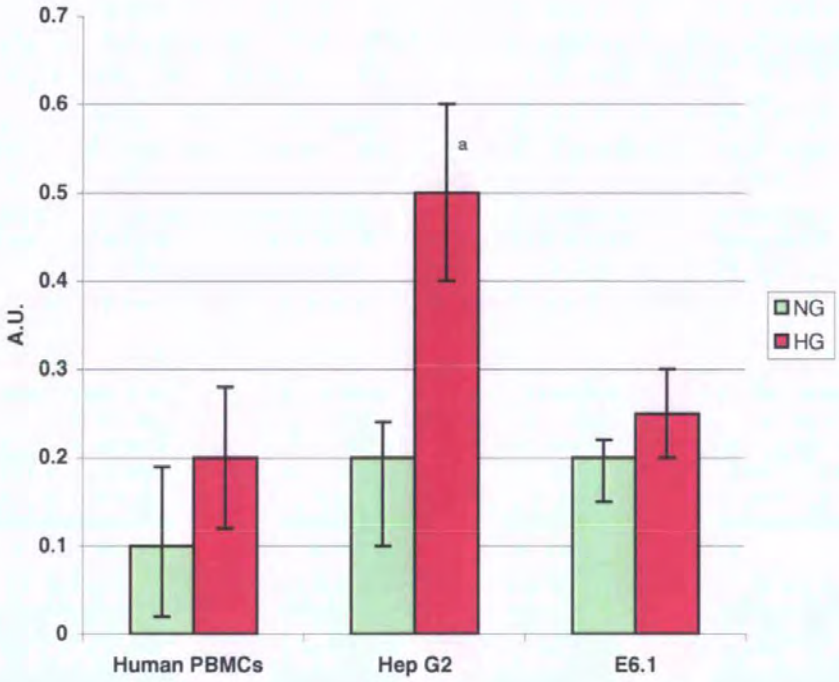


Figure 3.10: Hyperglycaemia -induced NFκB-DNA binding activity in different cell types

A bar graph shows the hyperglycaemia-induced NFκB-DNA binding activity in human PBMCs from normal healthy controls, Hep G2 and E6.1 cell lines. Cells were stressed with 31 mM D-glucose and grown in the recommended medium and incubated at 37 ^{o} C for 5 days. Experiments were repeated for 3 times. Results are the means of AU ± SE. NG= normoglycemia, HG= hyperglycaemia. AU= arbitrary units.

[a] vs Hep G2 controls, p= 0.001

3.2.1.3.2 HO and NFκB -DNA binding activity in cell lines

The EMSA results showed that HO stress of 110 mM NaCl did not induce NFκB-DNA binding activity either in Hep G2 or E6.1 cell lines. The autoradiograph (Figure 3.9) shows NFκB-DNA binding activity in HO-stressed E6.1 in lane 4 compared with that in E6.1 controls in lanes 1 and 2. The bands in lane 6 and 7 shown NFκB-DNA binding activity in HO-stressed Hep G2 while that in lane 8 for control cells. AU quantitation was illustrated in the bar graph (Figure 3.11). No change in the density of the bands was observed in HO-stressed Hep G2 compared with unstressed cells (0.2 ± 0.01 vs 0.2 ± 0.01) while HO-stressed E6.1 cells have shown a significant inhibition in the protein activity compared with controls (0.1 ± 0.02 vs 0.2 ± 0.01, p= 0.04).

3.2.1.4 NFκB-DNA binding activity in patients with diabetes

PBMCs were isolated from ten NC and sixty-seven patients with diabetes. Forty-three of the patients had TIDM, and 24 had TIIDM. The 43 patients with TIDM included 7 without complication (DC), 28 with microvascular complications (TIDM-Comp) and 8 with SD (TIDM-SD). Patients with TIIDM included 2 groups: 14 patients with microvascular complications (TIIDM-Comp) and SD (n=10). Herein, SD group in either TIDM or TIIDM are patients with less than 10 years without complications. The characteristics of the patients are shown in Table 3.16. Cells were stressed with 31mM D-glucose and incubated for 5 days at 37^{0} C then EMSA was performed to investigate NFκB-DNA binding activity.

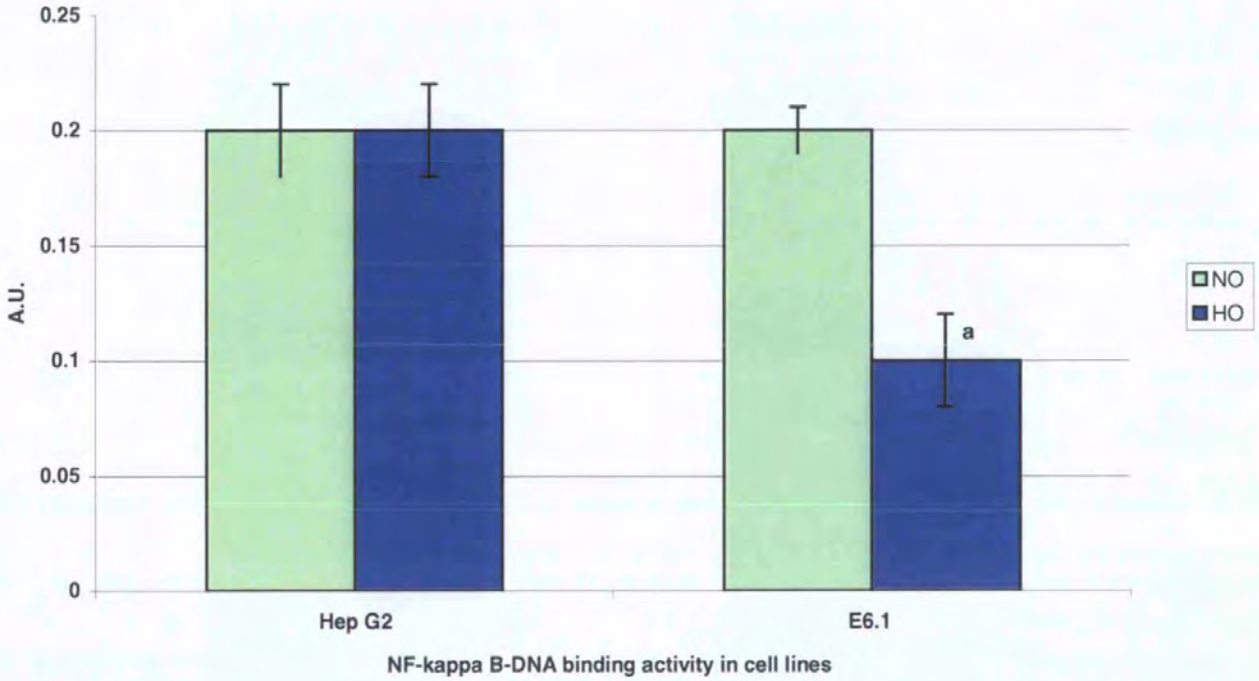


Figure 3.11: NFκB-DNA binding activity in HO-stressed cell lines

The bar graph shows arbitrary units (AU) quantitation in HO-stressed Hep G2 and E6.1 cell lines. Cells were stressed with 110 mM NaCl for 16 hrs then incubated at 37 ^{o} C to complete 5 days. Experiments were repeated for 3 times. NO= normal osmolarity and HO= hyperosmolarity. The results are the means of AU ± SE.

[a] vs E6.1 NO, p= 0.04.

Subjects	Male: Female	Age at onset	Duration
TIDM (n=43)			
TIDM-DC (n= 7)	2:5	8.25 ± 4.8	31.8 ± 6.8
TIDM-Comp (n= 28)	21:7	26.8± 15.5	29.8 ± 8.9
TIDM-SD (n=8)	2:6	26.3 ± 16.3	8.2 ± 4.2
TIIDM (n=24)			
TIIDM-Comp (n=14)	8:6	56 ± 4	5 ± 3
TIIDM-SD (n= 10)	8:2	66 ± 10	5 ± 2
NC (n=10)	4: 6		
Total	**45:32**		

Table 3.16: Subjects included in the study of hyperglycaemia and NFκB-DNA binding activity

A total number of 67 patients and 10 NC were included in this study. Patients were sub-grouped according to the presence or absence of microvascular complications and type of diabetes. DC = those patients with no microvascular complications after 20 years duration of diabetes. TIDM & TIIDM-Comp = those patients with microvascular complications including DN, DNU and DR. TIDM& TIIDM-SD = patients with short duration diabetes for less than 10 years without complications. NC = normal healthy controls. The table presents the classification according to sex, age at onset, and duration of the disease. Data are presented in means of years ± s.d.

AU quantitation showed a significant increase in the NFκB-DNA binding activity in hyperglycaemia -stressed PBMCs compared with the unstressed cells from patients with iabetes (0.5 ± 0.04 vs 0.3 ± 0.07, p= 0.003). hyperglycaemia induced a significant NFκB activation compared to NG in those patients with TIDM and those with TIIDM (0.3 ± 0.05 vs 0.2 ± 0.05, p= 0.009 and 0.7 ± 0.07 vs 0.5 ± 0.07, p= 0.03 respectively). Further, NFκB-DNA binding activity was significantly increased in hyperglycaemia-stressed cells compared with unstressed cells in patients with microvascular complications irrespective of type of diabetes (TIDM, 0.2 ± 0.09 vs 0.1 ± 0.09, p= 0.001 and TIIDM, 0.7 ± 0.2 vs 0.5 ± 0.2, p= 0.047). Hyperglycaemia did not induce NFκB activation in cells of NC, DC, and all patients with SD (n=18) (0.2 ± 0.1 vs 0.1 ± 0.09, 0.4 ± 0.1 vs 0.2 ± 0.1, and 0.6 ± 0.08 vs 0.5 ± 0.08 respectively) (Table 3.17).

Patients with diabetes (n=67) showed a significant increase in the hyperglycaemia-induced NFκB ratio (fold) compared with NC group (n=10) (2.1 ± 0.2 fold vs 1.65 ± 0.3 fold, p= 0.01) (Figure 3.12). **When all patients were divided into groups based on the type of diabetes,** a significant difference in the hyperglycaemia-induced NFκB ratio was observed between patients with TIDM (n=43) and TIIDM (n=24) (2.2 ± 0.12 vs 1.7 ± 0.17, p= 0.02) (Figure 3.12). **Further patients were divided into subgroups based on the presence of complications.** In patients with TIDM, those with complications (n=28) have shown a significant increase in the hyperglycaemia-induced NFκB ratio compared with the DC (n=7) (2.5 ± 0.15 vs 1.9 ± 0.3, p= 0.047) and those with SD (n=8) (2.5 ± 0.1 vs 1.6 ± 0.3, p= 0.003) (Figure 3.13). Patients with TIDM-comp (n=28) did not show a difference when compared with those with TIIDM-comp (n=14) (2.5 ± 0.15 vs 1.95 ± 0.24, p=ns) (Figure 3.13). Also, no difference was observed between TIDM-SD (n=8) and TIIDM-SD (n= 10) groups (1.6 ± 0.3 vs 1.3 ± 0.3, p=ns) (Figure 3.13).

M ± SE	NC (n=10)	DM (n= 67)	TIDM (n=43)	TIIDM (n=24)	DC (n=7)	All SD (n=18)	TIDM-Comp (n=28)	TIIDM-Comp (n= 14)
NG	0.1 ± 0.09	0.3 ± 0.07	0.2 ± 0.05	0.5± 0.07	0.2 ± 0.1	0.5± 0.08	0.1 ± 0.09	0.5 ± 0.2
HG	0.2 ± 0.1	0.5 ± 0.04	0.3 ± 0.05	0.7 ± 0.07	0.4 ± 0.1	0.6 ± 0.08	0.2 ± 0.09	0.7 ± 0.2
p	ns	0.003	0.009	0.03	ns	ns	0.001	0.047

Table 3.17: Results of hyperglycaemia and NFκB-DNA binding activity

Patients were sub-grouped according to types of diabetes and the presence or absence of microvascular complications. DM = all patients with diabetes, NC = non-diabetic controls, all SD= all patients with short duration diabetes without complications, TIDM & TIIDM= type 1& 2 diabetes mellitus. Comp= microvascular complications. ns= non-significant p value. NG= unstressed PBMCs, HG= stressed PBMCs with 31mM D-glucose. Results are in means of arbitrary units ± S.E and compared by student *t*-test.

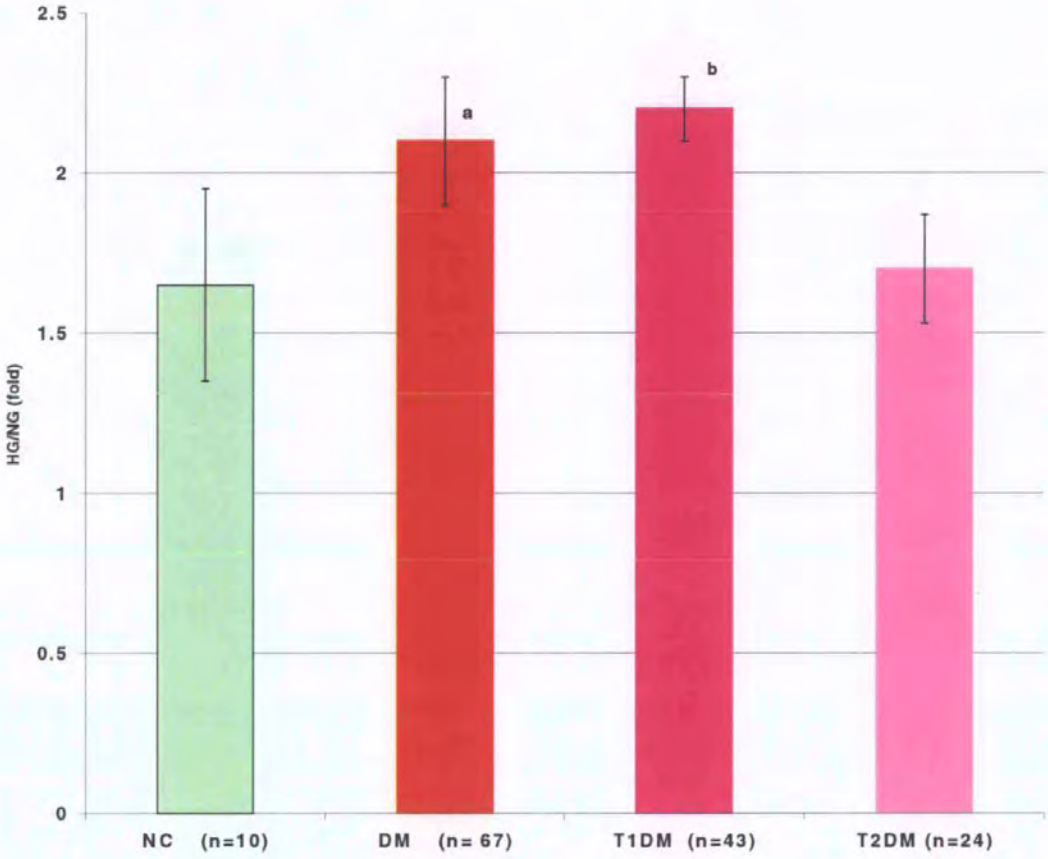


Figure 3.12: - Hyperglycaemia-induced NFκB in patients with diabetes compared with normal healthy controls

Hyperglycaemia-induced NFκB-DNA binding activity was calculated in folds in different groups. DM = all patients with diabetes, NC = non-diabetic controls, TIDM& TIIDM= type 1& 2 diabetes mellitus. Results are in means of folds ± S.E and compared by student *t*-test.

[a] vs NC, p= 0.01

[b] vs those patients with TIIDM, p= 0.02

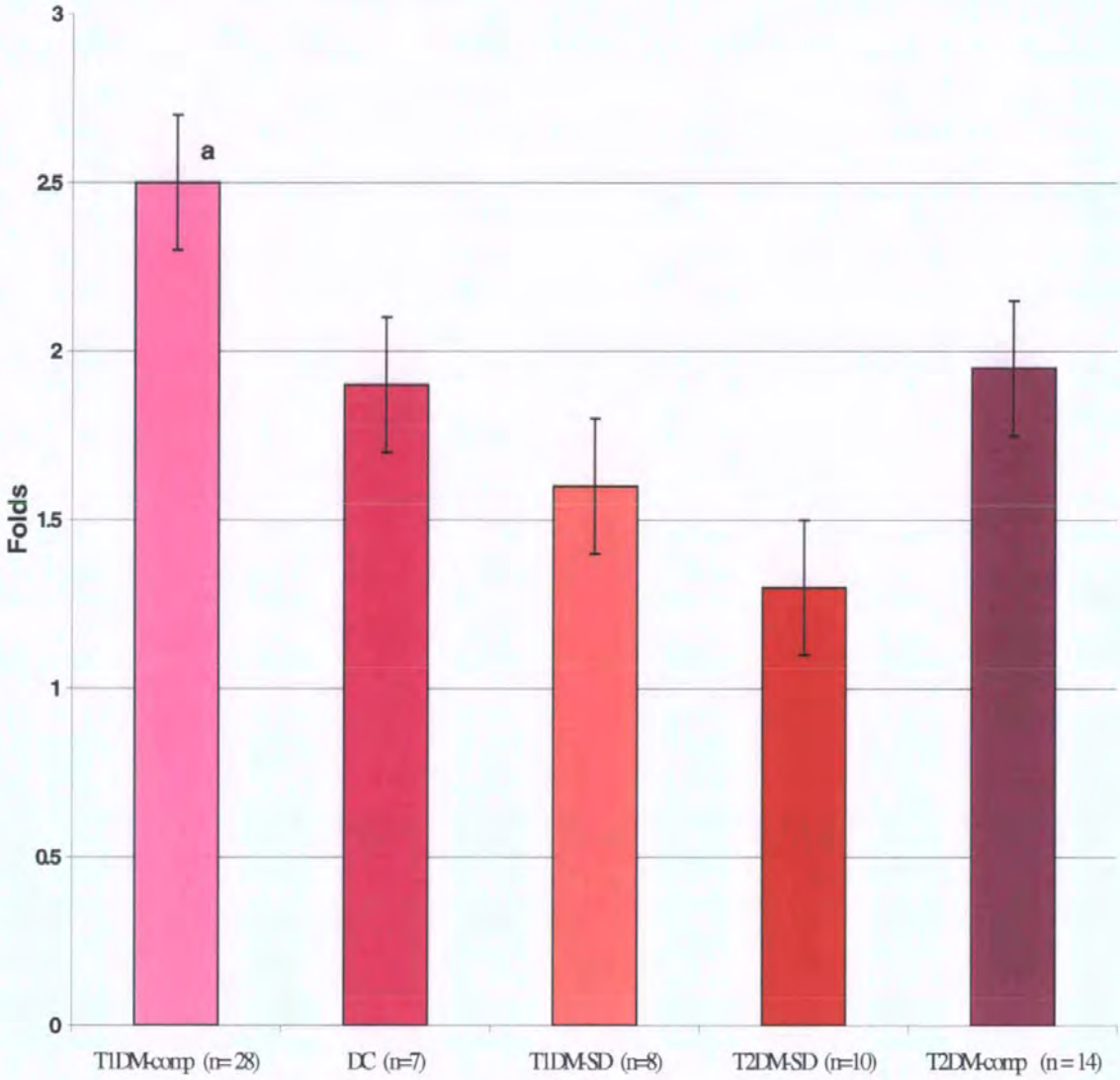


Figure 3.13: Hyperglycaemia-induced NFκB binding activity in diabetic subgroups

The bars represent the ratio of hyperglycaemia -induced NFκB binding activity in folds. The patients were classified to four groups according to the presence and absence of complications. TIDM-comp and TIIDM-comp are patients with both types of diabetes with microvascular complications. DC= patients with TIDM for at least 20 years with no complications. TIDM-SD and TIIDM-SD = patients with TIDM and TIIDM for less than 10 years without complications. Results are in means of the folds ± S.E and compared with student *t*-test.

[a] vs DC, p=0.047 and TIDM-SD, p=0.003

Patients with TIIDM with complications (n=14) have shown an increase in the hyperglycaemia-induced NFκB ratio compared with the TIIDM-SD (n=10), however it was not significant (1.95 ± 0.2 vs 1.3 ± 0.3, p = ns) (Figure 3.13).

When the patients in the TIDM-Comp group were divided into the following groups, DN (n=5), DR (n= 12) and DNU (n=11), there was no significant difference in hyperglycaemia -induced NFκB binding activity between complication subgroups (2.4 ± 0.3, 2.4 ± 0.2, and 2.5 ± 0.2 respectively, p=ns). **However, all patients with complications irrespective of type of diabetes** were combined and classified into three groups including all DN (n=12), all DR (n=19), and all DNU (n=11). These groups were compared with SD (n=18) in both TIDM and TIIDM. The results revealed a significant difference in the ratio (p= 0.009) (Table 3.18). Further analysis has shown that the fold was significantly higher in PBMCs of patients with both DR and DNU compared with those of SD group (p= 0.04 and 0.016 respectively).

The Pearson correlation was used to define the extent of the linear relationship between the ratios of hyperglycaemia -induced NFκB, duration of the disease, age at onset and HbA_{1c}%. Data analysis revealed a significant correlation with the duration of the disease (r = 0.291, p= 0.035) (Figure 3.14 A). A positive correlation was observed between hyperglycaemia-induced NFκB-DNA binding activity and HbA_{1c}% in patients with diabetes, however it was not significant (r= 0.255, p=ns) (Figure 3.14 B). No correlation was observed between age at onset and hyperglycaemia -induced NFκB-DNA binding activity (Figure 3.14 C).

	All DN (n=12)	All DR (n=19)	All DNU (n=11)	All SD (n=18)	ANOVA
Fold	2.2 ± 0.24	2.3 ± 0.19	2.5 ± 0.25	1.5 ± 0.19	0.009

Table 3.18: Hyperglycaemia -induced NFκB-DNA binding activity in complication subgroups

Hyperglycaemia induced approximately a two-fold increase in NFκB-DNA binding activity in patients with different microvascular complications compared with all patients with SD. All = the complication subgroup in both TIDM and TIIDM. Results are in means of folds ± S.E. DN, those patients with diabetic nephropathy; DNU, those patients with overt diabetic neuropathy and DR, those patients with diabetic retinopathy. SD = all patients with TIDM and TIIDM for less than 10 years without complications.

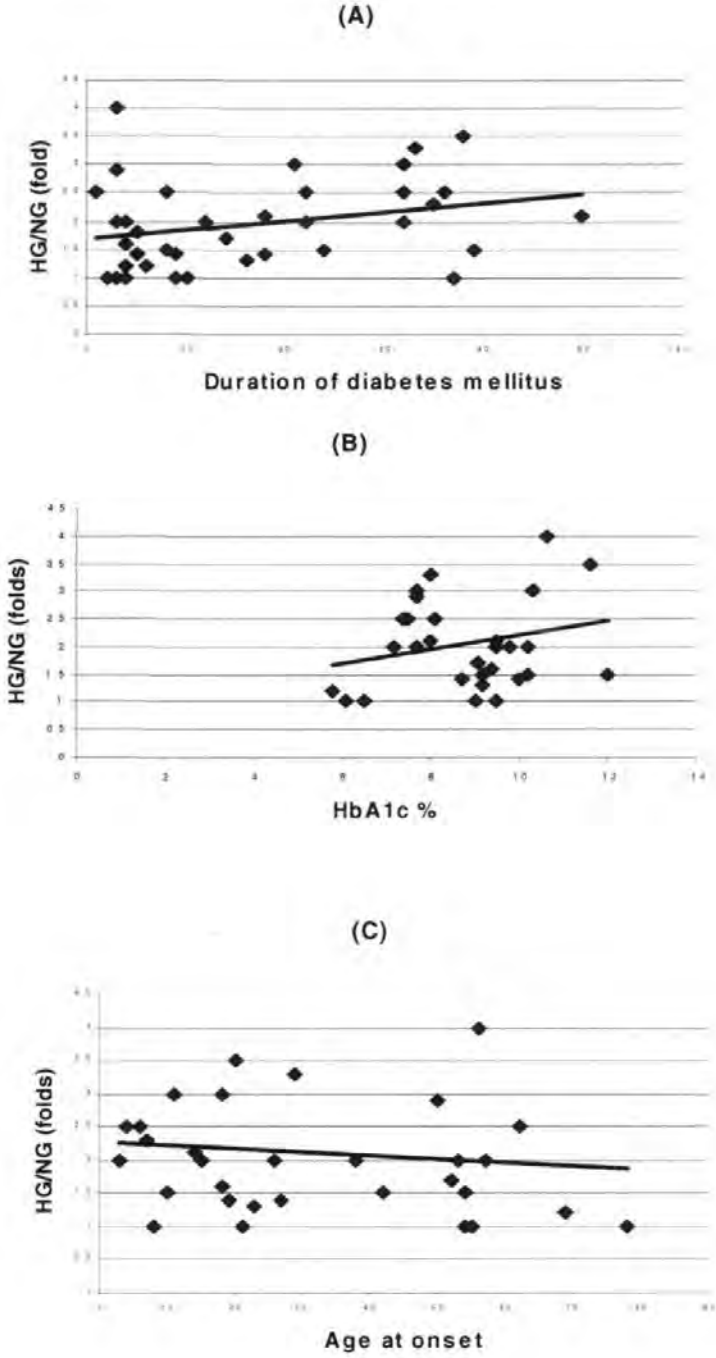


Figure 3.14: Hyperglycaemia-induced NFκB binding activity in PBMCs of patients with diabetes in relation to duration of diabetes, HbA_{1c}%, and age at onset

(A): Pearson correlation revealed a significant positive correlation between hyperglycaemia -induced NFκB-DNA binding activity and the duration of diabetes (r = 0.291, p= 0.035). (B) Shows a non-significant positive correlation with HbA_{1c}% (r= 0.255, p> 0.05). HbA_{1c}%= blood glucose level. Figure C shows no correlation with age at onset.

3.2.2 HSF-1-DNA binding activity

3.2.2.1 HSF-1-DNA binding activity in different cells

3.2.2.1.1 Hyperglycaemia and HSF-1-DNA binding activity in different cells

To confirm the specificity of the protein DNA complex for HSF-1 sequence, we have performed competition experiments. Competition assays contained 50-fold excesses of unlabeled HSF-1 oligonucleotide as a specific competitor, which blocked all of DNA binding, while the non-specific competitor (AP1) failed to block the DNA binding in DNA-protein complex. To assess HSF-1-DNA binding activity in response to high glucose concentration, cells were stressed with 31mM D-glucose and grown for 5 days at 37^{0} C. Cells were harvested, nuclear protein was extracted and EMSA was performed. A typical autoradiograph was shown in Figure 3.15, which shows two bands. The upper band is the complex of HSF-1 protein with the responsive element (GAA) (HSF-1 / HSE) while the lower band is non-specific band (free protein).

High concentration of D-glucose did not induce HSF-1-DNA binding activity in Hep G2. Phospho-imager quantitation revealed that HSF-1-DNA binding activity was 0.26 ± 0.03 in hyperglycaemia -stressed Hep G2 vs 0.15 ± 0.03 in unstressed cells, p=ns (Figure 3.16). While the protein-DNA binding activity was significantly increased in E6.1 cells compared with unstressed cells (1.09 ± 0.02 vs 0.65 ± 0.02, p= 0.004) (Figure 3.16). With respect to human PBMCs, there was a 1.2 fold increase in HSF-1-DNA binding activity in response to a 31mM D-glucose concentration compared to NG (0.5 ± 0.07 vs 0.6 ± 0.08, p> 0.05) (Figure 3.16).

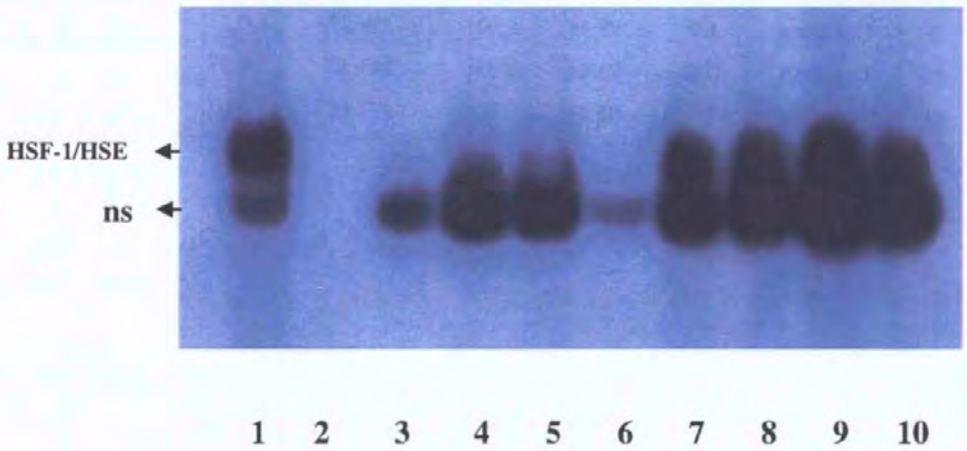


Figure 3.15: HSF1-DNA binding activity in HepG2 and E6.1 cell lines

An autoradiograph shows the binding of equal amounts of nuclear protein from Hep G2 and E6.1 cells to the HSF-1 probe. The upper band was the complex of HSF-1 protein with the responsive element (HSF-1/ HSE) while the lower band was non-specific (ns). Lane 1 is a positive control, lane 2 is a negative control, and lane 3 presents unstressed-HepG2. No bands were observed in hyperglycaemia -stressed HepG2 cells in lanes 4 and 5. Lane 7 shows HSF-1 in unstressed E6.1 cell while lane 8 and 9 are for hyperglycaemia -stressed cells. Lane 6 presents HO-stressed HepG2 cell while lane 10 presents HO-stressed E6.1 cells. Hyperglycaemia = a final concentration of 31mM D-glucose. HO= hyperosmolar stress

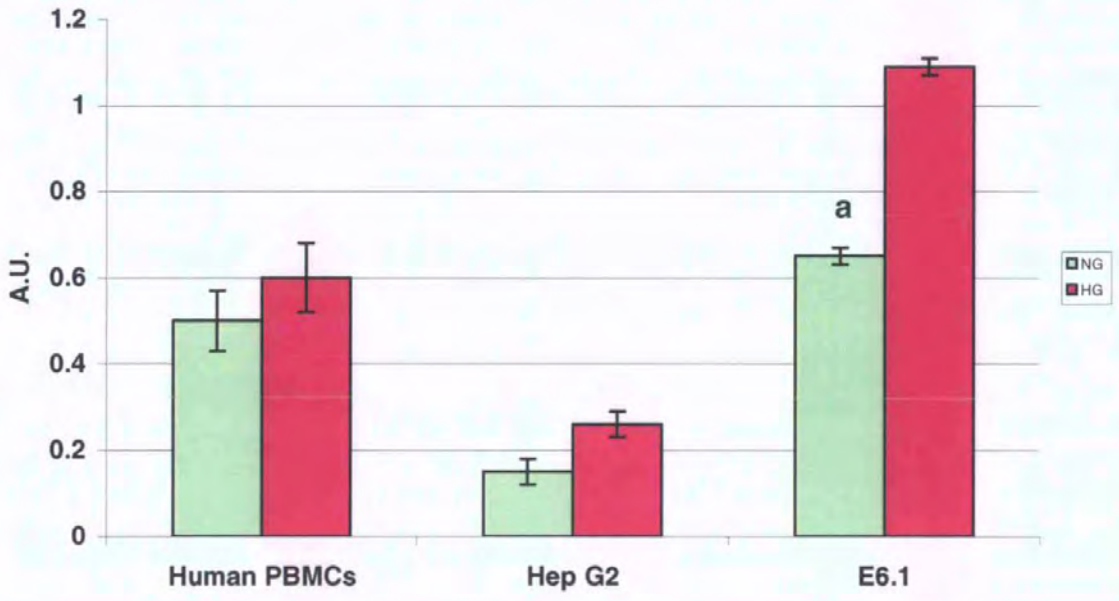


Figure 3.16: Hyperglycaemia-increased HSF-1-DNA binding activity in different cell types

A bar graph shows the hyperglycaemia -induced HSF-1-DNA binding activity in human PBMCs Hep G2 and E6.1 cell lines. Cells were incubated at 37°C for 5 days. The densitometric quantitation of the bands was obtained from 3 experiments. Results are in means ± SE.

[a] vs unstressed E6.1, $p = 0.004$

3.2.2.1.2 HO and HSF-1-DNA binding activity in cell lines

Figure 3.15 is an autoradiograph; the bands represent equal amounts of nuclear protein combined with the radiolabelled HSF-1 DNA probe. Lane 3 demonstrates the HSF-1-DNA binding activity in Hep G2 controls and lane 6 is HO-stressed Hep G2. Lane 7 is a band for E6.1 controls and lane 10 demonstrates the binding activity in HO-stressed E6.1. Densitometric quantitation of the bands obtained from 3 experiments in both cell types has shown that HO stress may induce HSF-1-DNA binding activity. There was a 1.3 fold increase in HO-stressed E6.1cells compared with unstressed cells; *t*-test has revealed a significant increase in the mean of AU quantitation (0.8 ± 0.02 vs 0.65 ± 0.02, p= 0.01). No difference was observed in HO stressed-Hep G2 compared with controls (Figure 3.17).

3.2.2.2 Hyperglycaemia and HSF-1-DNA binding activity in patients with diabetes

This study included 67 patients with diabetes mellitus (TIDM= 43 and TIIDM=24) and 12 NC. The clinical characteristics of the patients are shown in Table 3.19. The 43 patients with TIDM included 6 patients with DC, 29 patients with TIDM-comp and 8 patients with TIDM-SD. The ratio of men to women was 6.25 among patients with microvascular complications while it was 0.33 among patients with SD. Patients with TIIDM included 2 groups: TIIDM-comp (n=14), and TIIDM-SD (n=10). The ratios of men to women were 1.3 among patients with complications and 4 among SD. PBMCs were isolated from patients with TIDM and TIIDM, stressed with 31 mM D-glucose and grown for 5 days at 37^{0}C then EMSA was performed.

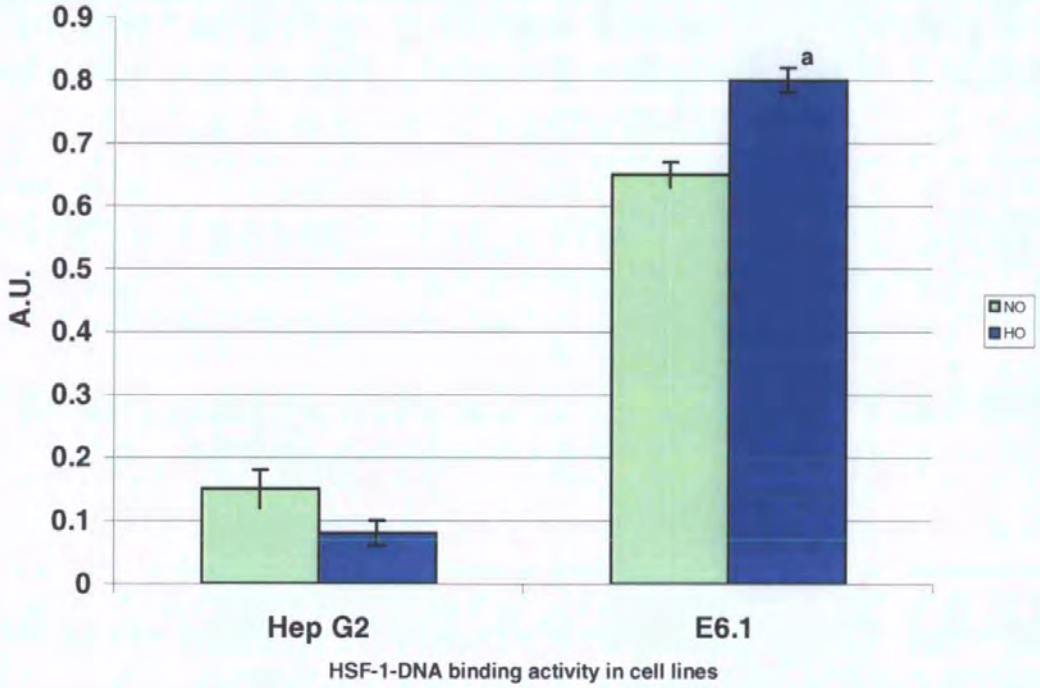


Figure 3.17: HSF-1-DNA binding activity in HO-stressed cell lines

The bar graph shows arbitrary units (AU) quantitation in HO-stressed Hep G2 and E6.1 cell lines. Cells were stressed with 110 mM Na Cl for 16 hrs then incubated at 37 ^{o} C to complete 5 days. Experiments were repeated for 3 times. NO= normal osmolarity and HO= Hyperosmolarity. The results are the means of AU ± SE. HSF-1-DNA binding activity was increased in response to HO in E6.1 but not in Hep G2.

[a] vs E6.1 NO, p= 0.01

Subjects	Male: Female	Age at onset	Duration
DC			
(n= 6)	2:4	8.25 ± 4.8	25 ± 5
TIDM-Comp			
(n= 29)	25: 4	23.2± 11.9	22.2 ±7.4
TIDM-SD			
(n= 8)	2:6	26.3 ± 16.3	8.2 ± 4.2
TIIDM-Comp			
(n= 14)	8:6	56 ± 4	5 ± 3
TIIDM-SD			
(n=10)	8:2	66 ± 10	5 ± 2
NC	5:7		
(n=12)			
Total	**50: 29**		

Table 3.19: Subjects included in the study of hyperglycaemia and HSF-1-DNA binding activity

The table presents the classification according to sex, age at onset, and duration of the disease. Data are presented in means of years ± SD. A total number of 67 patients and 12 NC were included in this study. Patients were sub-grouped according to the presence or absence of microvascular complications and types of diabetes. DC = those patients with no microvascular complications after 20 years duration of diabetes. TIDM-Comp & TIIDM-Comp = those patients with microvascular complications including DN, DNU and DR. TIDM-SD& TIIDM-SD = patients with short duration diabetes for less than 10 years without complications. NC = non-diabetic controls.

Quantitation of the bands showed a significant increase in HSF-1 activity in hyperglycaemia -stressed PBMCs relative to NG in patients with diabetes (0.5 ± 0.03 vs 0. 39 ± 0.03, p= 0.017) while no difference was observed in NC group (0.6 ± 0.08 vs 0.5 ± 0.07, p= ns) (Table 3.20). No difference in the ratio was observed between patients with diabetes (n=67) and the NC group (n=12) (1.3± 0.07 vs 1.2 ± 0.1, p= ns) (Figure 3.18).

With respect to type of diabetes, there was a significant increase in HSF-1 binding activity in hyperglycaemia -stressed PBMCs compared with unstressed cells in patients with TIDM (0.4 ± 0.04 vs 0.3 ± 0.04, p = 0.01) while no difference was observed in patients with TIIDM (0.6 ± 0.05 vs 0.5 ± 0.05, p=ns) (Table 3.20). Further, no difference in the ratio was observed between TIDM (n= 43) and TIIDM (n=24) (1.4 ± 0.09 vs 1.2 ± 0.1, p=ns) (Figure 3.18).

With respect to the presence of complications, HSF-1-DNA binding activity was significantly increased in response to hyperglycaemia-stressed PBMCs from patients with TIDM-comp compared with unstressed cells (0.44 ± 0.09 vs 0.3 ± 0.09, p= 0.008). No difference was observed in hyperglycaemia-stressed PBMCs from patients with DC compared with unstressed cells (0.26 ± 0.1 vs 0.24 ± 0.1, p=ns) (Table 3.20). Further, no difference in the ratio was observed between TIDM-complications (n= 29) and those patients with either DC (n = 6) or TIDM-SD (n = 8) (1.4 ± 0.1 vs 1.3 ± 0.1 and 1.2 ± 0.2 respectively, p=ns) (Figure 3.19).

No difference was observed in hyperglycaemia stressed cells of patients with TIIDM-comp compared with unstressed cells (0.7 ± 0.08 vs 0.5 ± 0.08, p= ns) (Table 3.20).

M ± SE	NC (n=12)	DM (n= 67)	TIDM (n=43)	TIIDM (n=24)	DC (n=6)	TIDM-Comp (n=29)	TIIDM-Comp (n= 14)
NG	0.5 ± 0.07	0.39 ± 0.03	0.3 ± 0.04	0.5 ± 0.05	0.24 ± 0.1	0.3 ± 0.09	0.5 ± 0.08
HG	0.6 ± 0.08	[a] 0.5 ± 0.03	[b] 0.4 ± 0.04	0.6 ± 0.05	0.26 ± 0.1	[c] 0.44 ± 0.09	0.7 ± 0.08

Table 3.20: Results of hyperglycaemia and HSF-1-DNA binding activity

The table shows the HSF-1-DNA binding activity in hyperglycaemia -stressed and unstressed PBMCs. Patients were divided according to the types of diabetes. Data presents the mean of the AU ± SE. Student *t*-test was used in the comparison of densito-metric quantitation. DC = those patients with no microvascular complications after 20 years duration of diabetes. TIDM-Comp & TIIDM-Comp = those patients with microvascular complications including DN, DNU and DR. NC = normal healthy controls. DM= all patients with diabetes. HG= = a final concentration of 31mM D-glucose. NG= unstressed cells

[a] vs NG of total diabetes, p= 0.017

[b] vs NG of TIDM, p= 0.01

[c] vs NG of patients with TIDM-Comp, p= 0.008.

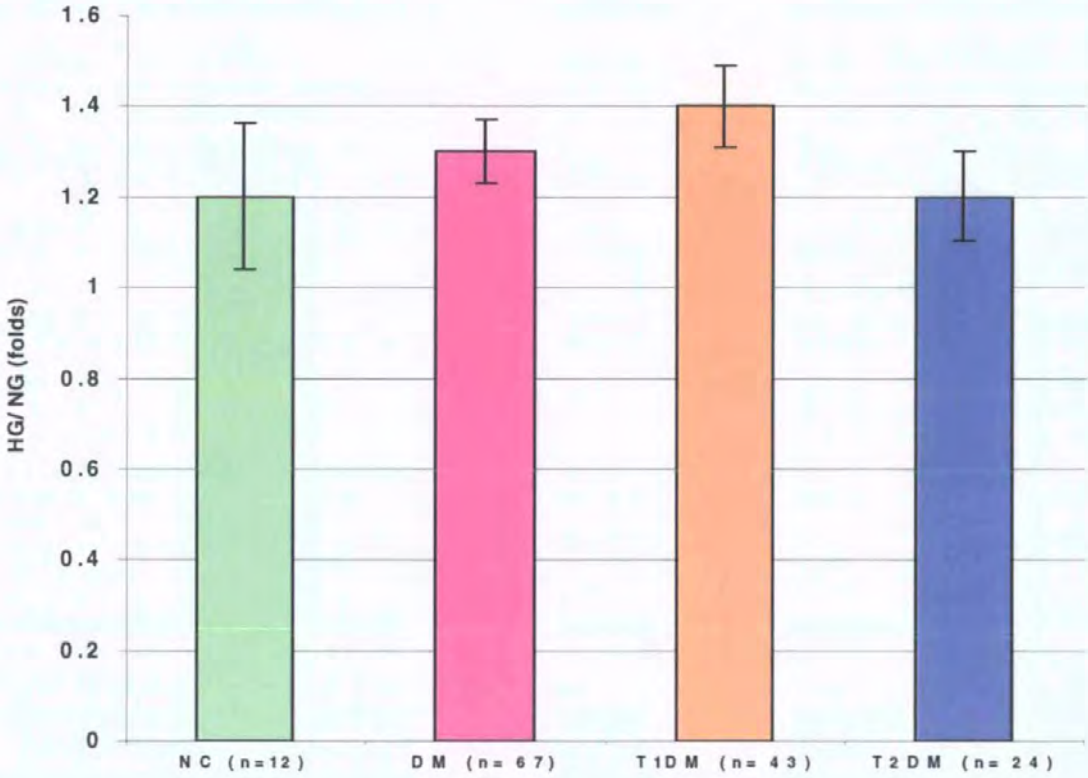


Figure 3.18: Hyperglycaemia -increased HSF-1-DNA binding activity in patients with diabetes compared with normal healthy controls

The graph shows the ratio of the HSF-1-DNA binding activity in patients and NC. Patients were divided according to the type of diabetes. Data presents the mean of the AU ± SE. DM=all patients with diabetes, NC= normal healthy controls, TIDM& TIIDM= type 1&2 diabetes mellitus. The values are the mean of the folds ± SE. One-way ANOVA did not show a significant difference in HG/NG between groups ($p > 0.05$). HG= = a final concentration of 31mM D-glucose. NG= unstressed cells.

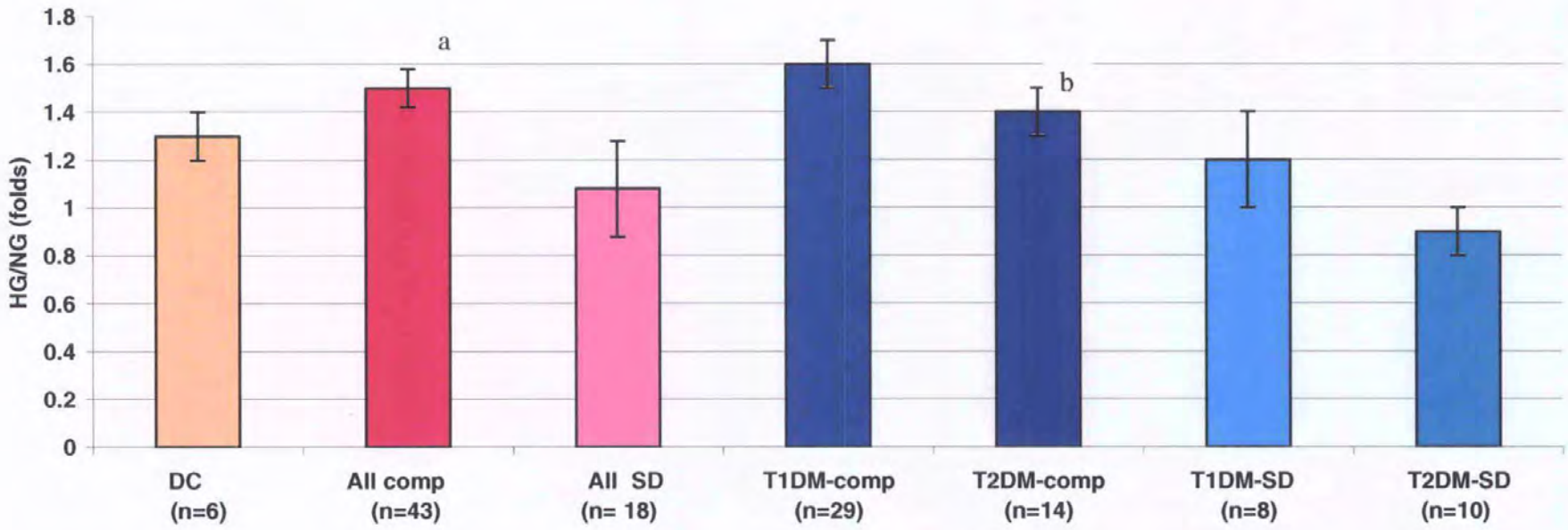


Figure 3.19: Hyperglycaemia -induced HSF-1-DNA binding activity in patients with diabetes mellitus

The graph shows the increased HSF-1-DNA binding activity in patients with diabetes mellitus with and without complications.

[a] vs all patients with all SD, p=0.001

[b] vs TIIDM-SD, p = 0.005

No difference was observed in the ratio between patients with TIDM-comp and those with TIIDM-comp (Figure 3.19). Patients with TIIDM-complications (n= 14) showed a significant increase in the ratio compared to the TIIDM-SD group (n= 10) (1.4 ± 0.1 vs 0.95 ± 0.1, p=0.005) (Figure 3.19). Further, no difference was observed between patients with TIDM-SD and those with TIIDM-SD (1.2 ± 0.2 vs 0.9 ± 0.1, p=ns) (Figure 3.19). Patients with complications (n=43) were compared to the SD patients (n= 18), a significant increase in ratio was observed in the former group (1.5 ± 0.08 vs 1.08 ± 0.2, p= 0.001) (Figure 3.19).

Patients were divided into the following groups DN (n=13), DR (n= 19), DNU (n= 11) and SD group (n=18). No difference in ratio was observed between any of these groups (Table 3.21). Further analysis revealed that there was no correlation between hyperglycaemia - induced HSF-1-DNA binding activity in patients with diabetes, age at onset, and duration of the disease or the level of HbA_{1c}%.

When we compared hyperglycaemia -induced NFκB to hyperglycaemia -induced HSF-1 DNA binding activity in response to 31mM D-glucose in PBMCs of patients with diabetes, a highly significant positive correlation between both proteins has been identified by the Pearson correlation test ($r = 0.915$, $p < 0.01$) (Figure 3.20).

	All DN (n=13)	All DR (n=19)	All DNU (n=11)	All SD (n=18)	ANOVA
NG	0.4 ± 0.1	0.35 ± 0.1	0.35 ± 0.1	0.46 ± 0.1	
HG	0.6 ± 0.1	0.53 ± 0.1	0.46 ± 0.1	0.49 ± 0.1	
HG/NG (Fold)	1.5± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.08 ± 0.1	ns

Table 3.21: Hyperglycaemia and HSF-1-DNA binding activity in diabetic microvascular complications

ANOVA test was used to compare (fold) between complication groups. All= patients with either TIDM or TIIDM with the complication. DN, those patients with diabetic nephropathy. DNU, those patients with overt diabetic neuropathy. DR, those patients with diabetic retinopathy. All SD = all patients with short duration diabetes without complications Data is shown as means ± s.d.

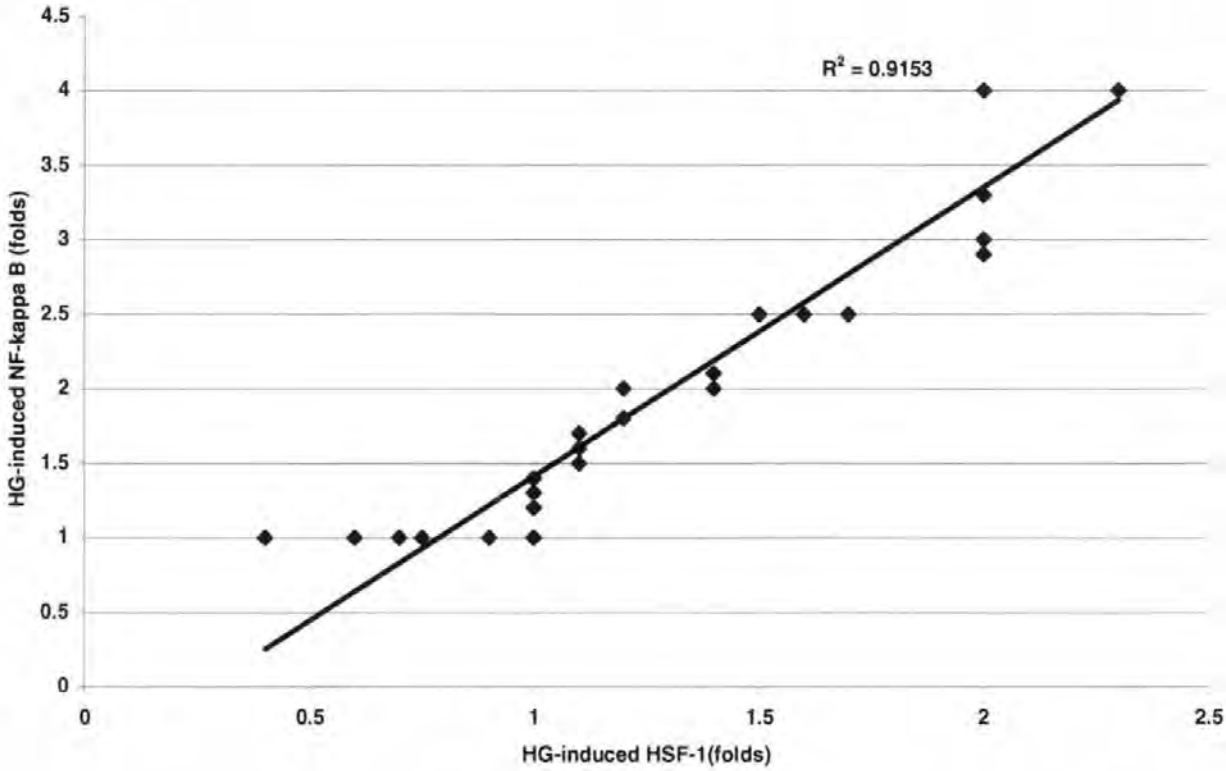


Figure 3.20: NFκB-DNA binding activity in correlation with that of HSF-1

NFκB and HSF-1 DNA binding activity were induced in response to a high concentration of 31mM D-glucose in patients with both TIDM and TIIDM with and without microvascular. Pearson correlation has revealed a highly significant positive correlation between proteins activities ($r = 0.915$, $p < 0.01$).

3.2.3 Aldose reductase inhibitor and transcription factors binding activity

3.2.3.1 ARI and hyperglycaemia -increased NFκB-DNA binding activity

Samples from 5 NC and 32 patients with diabetes were investigated for the effect of ARI on hyperglycaemia -induced NFκB-DNA binding activity. Eight of the patients had TIDM and 24 had TIIDM. Patients with TIDM included 3 with DC, 1 with DNU and 4 with SD. There are 7 patients with TIIDM with DN, 7 with DR and 10 with SD. The characteristics of the patients are shown in Table 3.22.

In order to optimize the dose of zopolrestat on the protein activity, NG and hyperglycaemia -stressed cells were treated with different doses of zopolrestat (0, 5, 10, and 15 μM/ml). EMSA results showed that zopolrestat significantly inhibited the hyperglycaemia -increased NFκB activity in a dose-dependent fashion. Densito-metric quantitation of the bands showed that hyperglycaemia stress without ARI treatment induced NFκB-DNA binding activity in human PBMCs (1.6 ± 0.2). When PBMCs were stimulated with hyperglycaemia and treated with doses of 5 μM, 10 μM and 15 μM of zopolrestat, AU quantitation of the protein activity was reduced to 1.06 ± 0.15, 0.8 ± 0.1, and 0.4 ± 0.05 respectively (p= 0.01, 0.04, and 0.004 respectively vs hyperglycaemia stressed cells without ARI) (Figure 3.21 A). NG-PBMCs did not show any difference in the protein activity when compared to treatment with ARI (0.3 ± 0.1 vs 0.3 ± 0.2) (Figure 3.21 B).

	DC	TIDM-Comp	TIDM-SD	TIIDM-Comp	TIIDM-SD	NC
	(n= 3)	(n=1)	(n=4)	(n=14)	(n= 10)	(n= 5)
Male: Female	1:2	1: 0	0:4	8:6	8:2	4:1
Age at onset	26.5 ± 0.7	7	22 ± 9.5	56 ± 4	66± 10	
Duration	20.5 ± 4.9	35	9 ± 5.8	5 ± 3	5 ± 2	

Table 3.22: characteristics of patients included in the study of zopolrestat and hyperglycaemia -induced NFκB-DNA binding activity

A total number of 32 patients included in the study of hyperglycaemia -induced NFκB–DNA binding activity in response to the treatment with zopolrestat. DC = those patients with TIDM with no microvascular complications after 20 years duration of diabetes. TIDM-Comp& TIIDM-Comp those patients with TIDM or TIIDM with microvascular complications. SD = those patients with diabetes for 10 years or less without complications. NC = non-diabetic controls. Data is shown as means of years ± s.d.

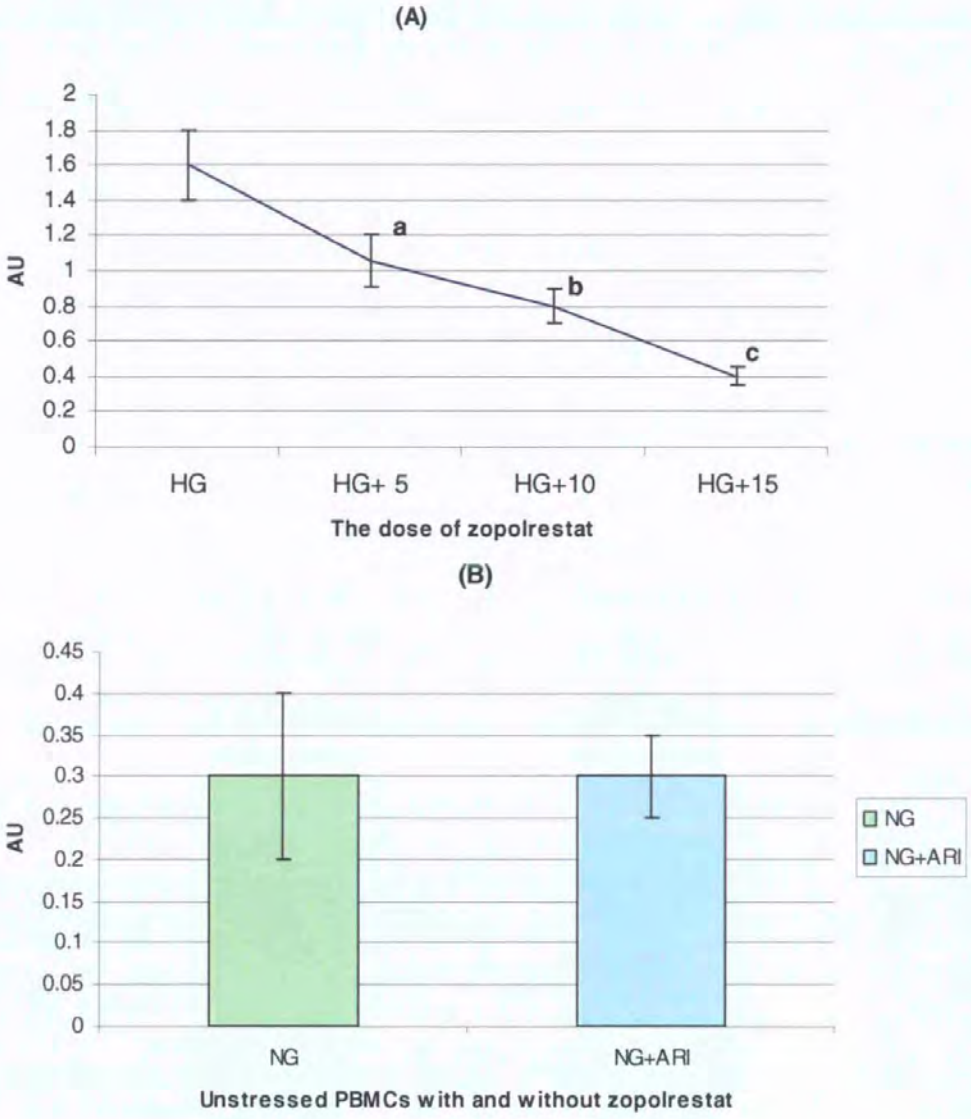


Figure 3.21: Zopolrestat and hyperglycaemia -increased NFκB-DNA binding activity

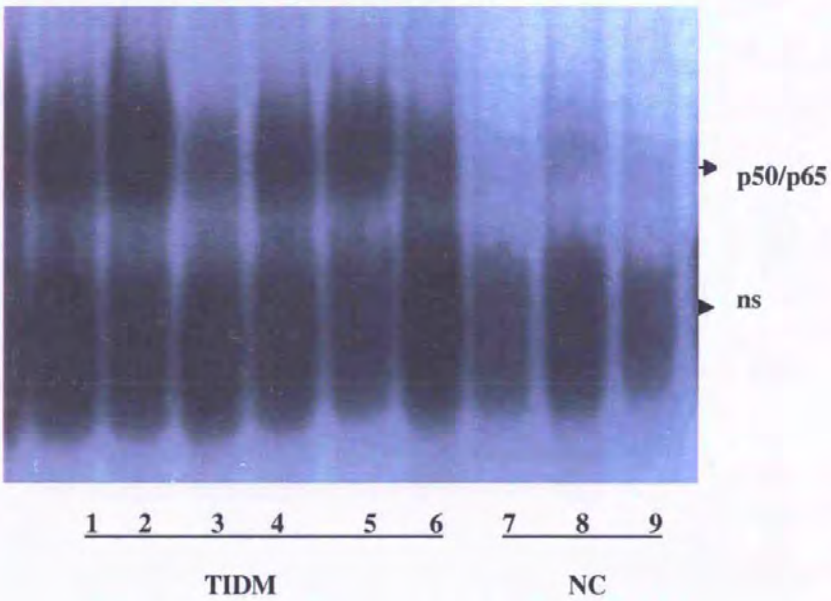
(A): The graph shows the NFκB-DNA binding activity in PBMCs in response to a high concentration of 31mM D-glucose (HG) without ARI and with 5, 10, 15μM of zopolrestat/ml respectively. [a] vs HG, p= 0.01. [b] vs HG+ 5μM, p= 0.04. [c] vs HG+10μM, p= 0.004. (B) The bar graph presents the mean of NFκB-DNA binding activity in unstressed cells (NG) with and without ARI. The densito-metric quantitation of the bands obtained from 3 experiments are in means of arbitrary units ± SE.

To investigate the treatment with ARI on the hyperglycaemia -induced NFκB-DNA binding activity, isolated PBMCs from patient with diabetes as well as the NC were classified into three groups. The first was a control group (NG) grown with no hyperglycaemia stress. The second was stressed with 31 mM D-glucose without zopolrestat. The third was stressed with hyperglycaemia and 10 μM of zopolrestat/ml (HGZ). All groups were incubated at 37° C for 5 days. Figure 3.22 (A) is an autoradiograph, which represents the DNA-binding activity of NFκB protein in the three groups. The phospho-imager quantitation showed that zopolrestat reduces hyperglycaemia -induced NFκB-DNA binding activity.

In the NC group (n=5), there was no significant effect of the zopolrestat on hyperglycaemia -induced NFκB binding activity (0.4 ± 0.04 vs 0.6 ± 0.09, p=ns) whilst a significant inhibitory effect was found in patients with diabetes (n= 32) (0.35 ± 0.05 vs 0.7 ± 0.05, p=0.000002) (Table 3.23). The inhibition ratio was significantly decreased in patients with diabetes compared with NC (0.4 ± 0.05 fold vs 0.6 ± 0.09, p= 0.04) (Table 3.23).

The patients were categorized into two groups according to the type of complications: 15 with complications (DR, DN and DNU) and 17 with no complications (SD + DC). The quantitation showed significant inhibition of NFκB-DNA binding activity by zopolrestat in patients with or without complications (0.25 ± 0.07 vs 0.7± 0.07 and 0.4 ± 0.06 vs 0.8 ± 0.06 respectively, $p < 0.05$) (Table 3.23). Further, the (fold) was significantly reduced in patients with complications compared to those patients without complications (0.3 ± 0.06 vs 0.5 ± 0.05, p= 0.01) (Table 3.23).

(A)



(B)

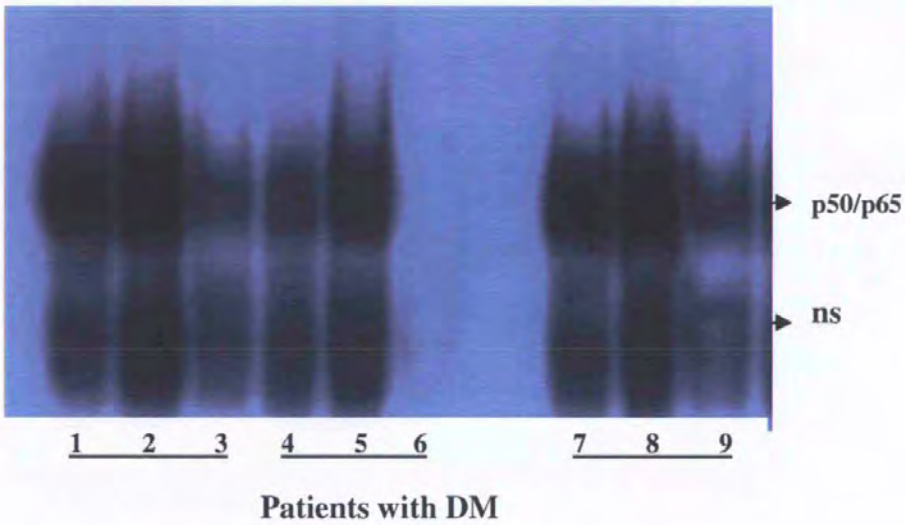


Figure 3.22: ARI-inhibited hyperglycaemia -increased NFκB-DNA activity

Autoradiographs show the NFκB-DNA binding activity in response to a concentration of 31 mM D-glucose (HG) with and without ARI treatment. Each three lanes present NG, HG and HG with ARI respectively for each individual. (A): Zopolrestat (10μM/ml) and HG-induced NFκB-DNA binding activity. (B): Sorbinil (5 μM/ml) and HG-induced NFκB-DNA binding activity. Lanes 1-9 for three patients with diabetes. ns=non-specific band (free protein).

	NC (n=5)	DM (n= 32)	All with comp (n=15)	All without comp (n=17)
HG	0.6 ± 0.09	0.7 ± 0.05	0.7± 0.07	0.8 ± 0.06
HGZ	0.4 ± 0.0.04	[a] 0.35 ± 0.05	[b] 0.25 ± 0.07	[c] 0.4 ± 0.06
HGZ/HG (Fold)	0.6 ± 0.09	[d] 0.4 ± 0.05	[e] 0.3 ± 0.06	0.5 ± 0.05

Table 3.23: Zopolrestat and hyperglycaemia-increased NFκB-DNA binding activity in patients with diabetes and normal healthy controls

The densito-metric quantitation of the bands obtained from each group of patients. Data present the mean of the AU ± SE. hyperglycaemia-stressed cells with zopolrestat show a significant decrease in NFκB binding activity compared with hyperglycaemia -stressed PBMCs without zopolrestat. DM= all patients with diabetes, HG= hyperglycaemia (31mM), HGZ= HG-stressed PBMCs +10μM/ml zopolrestat and NC= normal healthy controls.

[a] vs HG in all patients with DM, p= 0.000002

[b] vs HG in all patients with complications, p= 0.0008

[c] vs HG in all patients without complications, p= 0.0008

[d] vs NC, p= 0.04

[e] vs all without comp, p= 0.01

To confirm whether the effect of the ARI was specific the hyperglycaemia -stressed PBMCs were treated with a second class of ARI, sorbinil to study whether it has the same effect on the hyperglycaemia- induced NFκB activation. The dose of sorbinil was adjusted to 5 μM. NFκB-DNA binding activity in PBMCs of three patients was inhibited in response to treatment with sorbinil compared with hyperglycaemia -stressed cells without sorbinil (0.01 ± 0.02 vs. 0.2 ± 0.05, p= 0.001) (Figure 3.22 (B). However, sorbinil was used for a confirmatory purpose and it was not investigated in patients' subgroups.

3.2.3.2 ARI and hyperglycaemia -induced HSF-1-DNA binding activity

The effect of the zopolrestat on the hyperglycaemia -increased HSF-1-DNA binding activity was investigated in 29 patients with diabetes. Five of the patients had TIDM and 24 had TIIDM. The characteristics of the patients are shown in Table 3.24.

Overall, the HSF-1-DNA binding activity was lower in response to treatment of hyperglycaemia -stressed PBMCs with zopolrestat compared with the hyperglycaemia-stressed PBMCs without zopolrestat. Figure 3.23 is an autoradiograph, which shows the HSF-1-DNA binding activity in unstressed-PBMCs, hyperglycaemia -stressed PBMCs with 31 mM D-glucose, and HGZ-stressed PBMCs. In patients with diabetes, the phospho-imager quantitation revealed a significant inhibition in HSF-1-DNA binding activity in response to treatment with zopolrestat compared with hyperglycaemia-stressed PBMCs without zopolrestat (0.3 ± 0.05 vs 0.58 ± 0.05, p= 0.001) (Table 3.25).

	DC (n= 1)	TIDM-SD (n=4)	TIIDM-comp (n=14)	TIIDM-SD (n= 10)
Male: Female	0:1	0:4	8:6	8:2
Age at onset	6	21.25 ± 4	56 ± 4	66± 10
Duration	32	10 ± 5.8	5 ± 3	5 ± 2

Table 3.24: characteristics of patients included in the study of zopolrestat and hyperglycaemia -induced HSF-1-DNA binding activity

A total number of 29 patients included in the study of hyperglycaemia and HSF-1-DNA binding activity in response to the treatment with zopolrestat. DC = those patients with TIDM without microvascular complications after 20 years duration of diabetes. TIDM-comp & TIIDM-comp those patients with TIDM or TIIDM with nephropathy, overt neuropathy, and retinopathy. SD = those patients with diabetes for 10 years or less without complications. NC = normal healthy controls. Data is shown as means of years ± s.d.

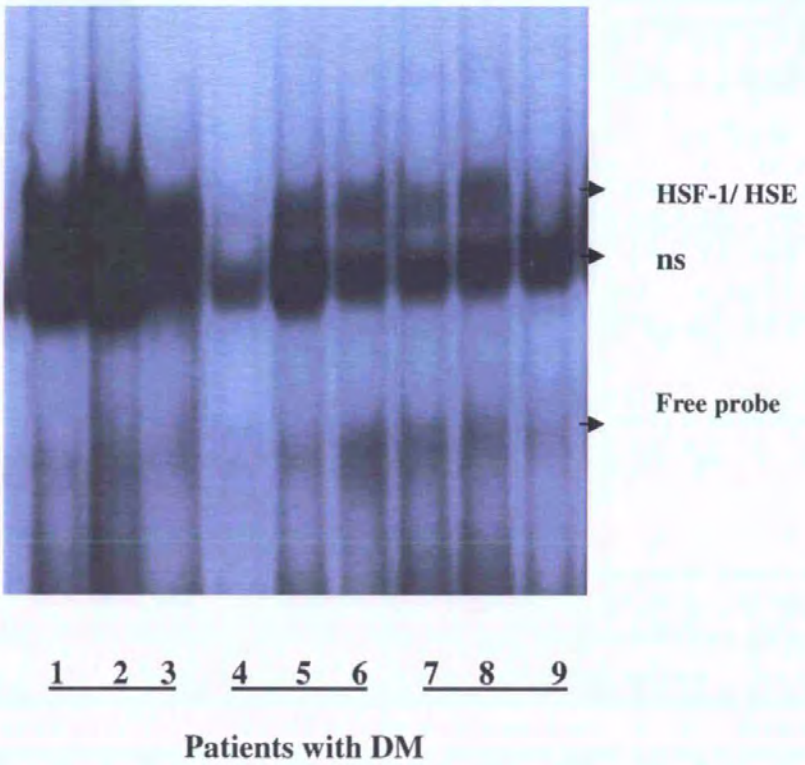


Figure 3.23: HSF-1-DNA binding activity in human PBMCs

An autoradiograph shows the HSF-1-DNA binding activity in PBMCs stressed with hyperglycaemia with and without zopolrestat. Each three lanes present normoglycemia, hyperglycaemia (31mM D-glucose), hyperglycaemia-stressed PBMCs +10 μM/ml zopolrestat DM=diabetes mellitus. ns =non-specific band (free protein). HSF-1/HSE is a complex of HSF-1 protein with the responsive element (GAA).

	Total DM (n= 29)	All comp (n=14)	All no comp (n= 15)
HG	0.58 ± 0.05	0.7 ± 0.08	0.4 ± 0.05
HGZ	[a] 0.3 ± 0.05	[b] 0.37 ± 0.08	0.3 ± 0.05
HGZ/ HG (folds)	0.6 ± 0.3	0.55 ± 0.07	0.7 ± 0.08

Table 3.25: Zopolrestat and HSF-1-DNA binding activity

The table shows AU quantitation of HSF-1-DNA binding activity in hyperglycaemia-stressed PBMCs with and without zopolrestat. DM=diabetes mellitus, all comp = patients with either TIDM or TIIDM with complications. All no comp= patients with either TIDM or TIIDM without complications. HG= hyperglycaemia (31mM), HGZ= HG-stressed PBMCs +10μM/ml zopolrestat. Densito-metric units of HSF-1-DNA binding activity were compared with t-test. ANOVA test was used to compare HGZ/HG (fold). Results are in means ± SE.

[a] vs HG in all patients with diabetes, p= 0.001

[b] vs HG in patients with complications, p=0.003

When patients were divided according to the presence of complications, the HSF-1-DNA binding activity was significantly lower in hyperglycaemia-stressed PBMCs with zopolrestat in patients with complications (n= 14) (0.37 ± 0.08 vs 0.7 ± 0.08, p= 0.003) but not in those without complications (n=15) (0.3 ± 0.05 vs 0.4 ± 0.05, p=ns). However, no difference in the folds was observed between patients with and without complications (Table 3.25).

3.2.4 Mitogen activated protein kinase p38 in cell lines

Hep G2 and E 6.1 cells were stressed with high D-glucose concentration of 31 mM and grown in the recommended medium for 5 days at 37° C. Whole cellular proteins were extracted by SDS-buffer. Western blot was performed and autoradiographs were visualised by phosphor-imager soft ware program (Figure 3.24). The p38 was two-four folds increased in response to hyperglycaemia stress. AU quantitation was 0.25 ± 0.02 in hyperglycaemia stressed-Hep G2 vs 0.15 ± 0.02 in unstressed Hep G2, p= 0.045. Whilst E6.1 cells have shown a significant increase in p38 expression in response to hyperglycaemia compared with controls (0.2 ± 0.05 vs 0.05 ± 0.05, p= 0.02) (Figure 3.25).

Hep G2 and E 6.1 cells were stressed with a concentration of 110 mM NaCl for 16 hours in the recommended medium and washed by PBS and grown for a complete time of 5 days at 37° C. Whole cellular proteins were extracted by SDS-buffer and Western blot was performed (Figure 3.24). Results have shown that HO stress did not induce p38 either in Hep G2 or E6.1 cells. AU quantitation was 0.1 ± 0.02 vs 0.15 ± 0.02 in HO stressed-Hep G2 compared with unstressed Hep G2. In E6.1 cells, HO stress did not induce MAPK p38 expression compared with controls (0.1 ± 0.05 vs 0.05± 0.05) (Figure 3.25).

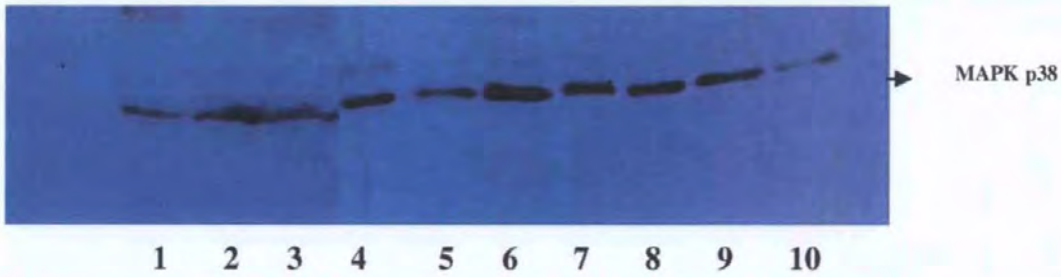


Figure 3.24: Western Blot assay and MAPK p38 expression

The autoradiograph depicts the binding of equal amounts of whole nuclear protein extracts from Hep G2 and E6.1 cells to phospho-active antibody of p38 (1:1000).

Lanes 1,2: HepG2 control, Lane 3, 4: HO-stressed HepG2, Lanes 5,6: hyperglycaemia-stressed HepG2 Lane 7: E6.1 cell control, Lanes 8, 9: hyperglycaemia-stressed E6.1 cell and lane10: HO-stressed E6.1.

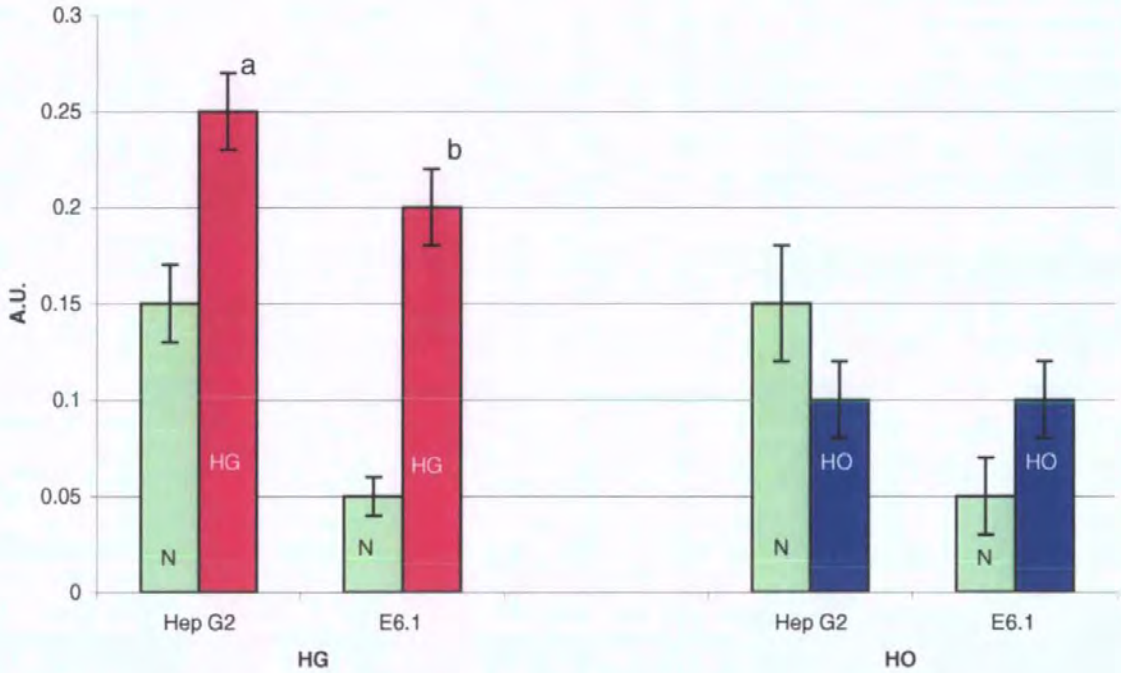


Figure 3.25: Mitogen activated protein kinase p38 in cell lines

To investigate MAPK p38 in response to hyperglycaemia, Hep G2 and E6.1 cell lines were stressed with 31 mM D-glucose for 5 days at 37 ^{o} C. For HO study, cells were stressed with 110 mM NaCl for 16 hrs then washed with PBS and grown in the recommended medium to complete incubation time of 5 days. N= normal control cells, HG = hyperglycemis, and HO= hyperosmolar stress. The bar graph demonstrates the means of arbitrary units (AU) quantitation ± SE.

[a] vs Hep G2 controls, p= 0.045

[b] vs E6.1 controls, p= 0.02

Chapter 4

DISCUSSION

4.1 Genotyping

This is the first study to look at the NFκB (4q24) and HSP70-A2 (14q22) genes in patients with TIDM. The results suggest that the A10 allele of the NFκB gene and H3 and H7 alleles of the HSP70-A2 gene are risk markers for the susceptibility to TIDM, whilst the A14 allele of the NFκB gene and H1 and H5 alleles of the HSP70-A2 gene may be protective markers and may reduce the risk of developing TIDM.

The polymorphism of the NFκB gene is located within the 3`UTR while that of the HSP70-A2 gene is located within the 5`UTR, suggesting that these polymorphisms may modify expression of the genes. It has previously been shown that polymorphisms in the non-coding regions of IL-6 (Fishman et al., 1998) and IFN-γ (Pravica et al. 1999) genes are linked to their expression. The regulatory machinery for a gene is different. For instance, enhancer elements have been found up to 50 Kb from the coding region and these can directly affect the expression of the genes (Rim et al., 1998). Consequently, the results of this study could be attributed to differences in the expression of the NFκB and HSP70 genes. They may determine the direction and magnitude of the cell response to an external stress and whether it is channelled towards apoptosis, inflammation or necrosis (Figure 4.1).

There is currently very little information on the mechanisms of islet β-cell death during the pre-diabetic period in man. It remains to be determined whether the (CA) polymorphism of NFκB and the (AAATA) polymorphism of the HSP70-A2 are associated with β-cell damage and the pathogenesis of TIDM.

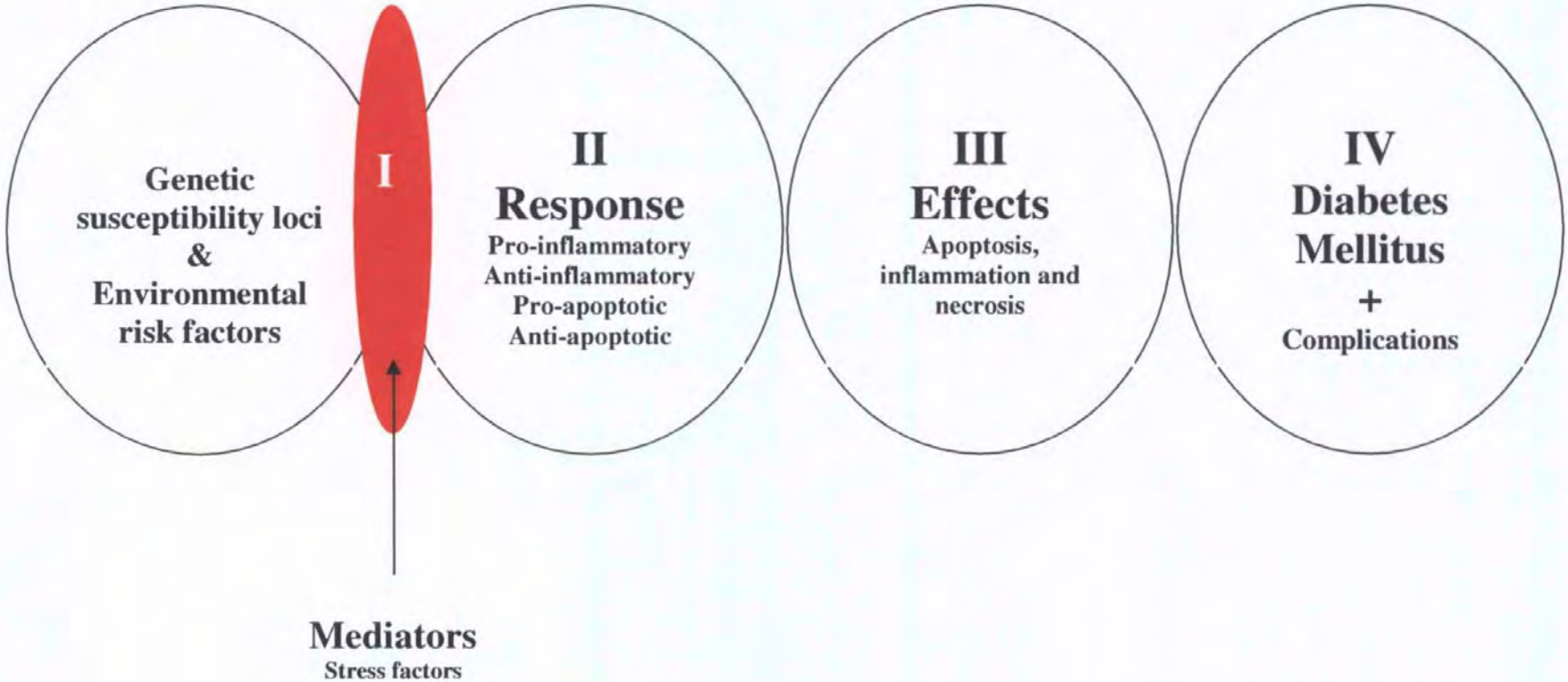


Figure 4.1: Algorithms of the course of diabetes mellitus and its late complications

Genetic and environmental factors could direct and magnify the effect of signals (mediators), which contribute to the development of the disease.

The association of the A10 allele of the NFκB gene with susceptibility to TIDM in this study may be linked with enhanced rate of transcription compared with the protective allele (A14). NFκB has both proinflammatory and anti-inflammatory effects (Tak et al., 2001) and it is possible that the A10 allele is associated with proinflammatory responses whilst the A14 allele is anti-inflammatory (Figure 4.2). This might be related to variable amounts of NFκB protein, which would have a detrimental effect allowing intracellular proteins and other molecules to be exposed to the immune system thereby, provoking an autoimmune response. It is possible that the polymorphism of the NFκB gene may affect the binding affinity of the p65/p50 dimer with the response element. It may also affect the efficacy of the p65 subunit (the functional unit of the NFκB dimer). However, further studies are needed to investigate whether this polymorphism linked to the NFκB-DNA binding activity and the p65 expression.

The (CA) polymorphism of the NFκB gene has been suggested to be a useful marker in inflammatory diseases (Ota et al., 1999). The investigation carried at part of this thesis was the first study of NFκB gene polymorphisms with susceptibility to TIDM (Hegazy et al., 2001). Recently, in Denmark, Gylvin et al. have investigated the same microsatellite marker (CA) in highly selective multiplex families. However, no association was observed with TIDM in this Danish population (Gylvin et al., 2002). It is well known that multiplex family studies do not always replicate the association in sporadic populations of non-MHC genes with susceptibility to TIDM (Davis et al., 1994, Hashimoto et al., 1994). This could be due to sampling, ethnic, environmental variation, or due to combination of all these factors. This is also possible to be due to an incomplete (low) penetrance between the genotypes and phenotype of TIDM in family studies.

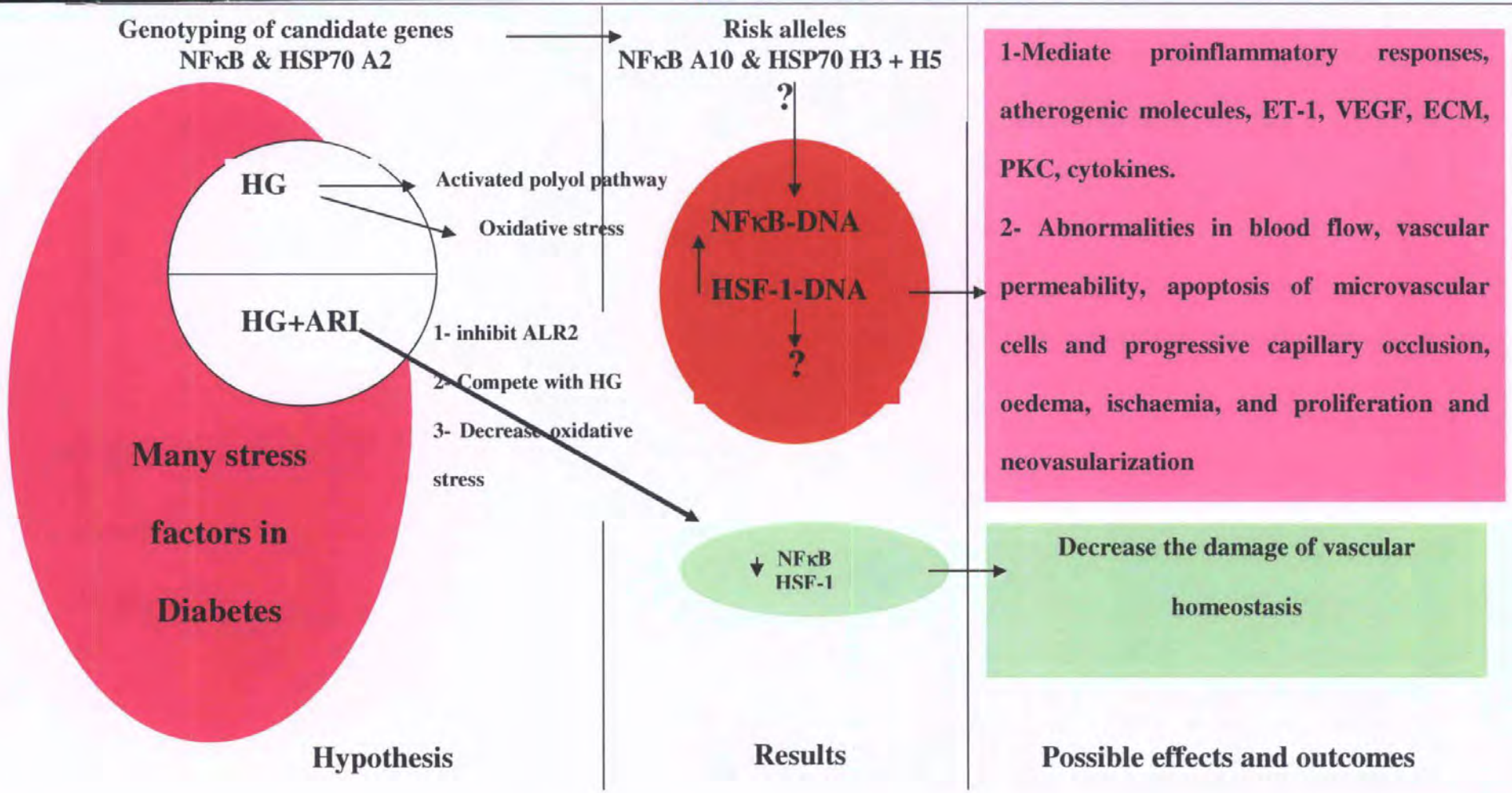


Figure 4.2: Illustration of hypothesis and results of the study

The study included genotyping of the candidate genes and investigating the role of hyperglycaemia (HG) with and without aldose reductase inhibitor (ARI) on the transcription factors-DNA binding activity.

? = Unknown further effects, which might need further studies.

With respect to HSP70-A2, the association of H3 and H7 with the susceptibility to TIDM may be explained in two ways. Firstly, these markers could be associated with decrease in the level of HSP70 leading to a deficiency in the cytoprotective mechanism, which may in turn accelerate β-cell autoimmune destruction. It has been suggested that HSPs in general and HSP70 in particular are a protective line of defence, which has been correlated to tissue damage (Bellmann et al., 1995; Eizirik, 1996; Burkart et al., 2000). Secondly, HSP-70 and HSF-1 are known to have an auto-regulation mechanism (Morimoto 1993; Schett et al., 1998; Schafer et al., 2000). It is possible that the risk alleles H3 and H7 of the HSP70 gene are associated with alteration of the HSF-1-DNA binding activity. HSF-1 may bind to the specific response element on proinflammatory genes, including cytokines, which contribute to β-cell destruction. Conversely the H1 and H5 alleles may direct HSF-1 to bind to the anti-inflammatory genes (Figure 4.2). This may be related to variable amounts of HSP protein, which would have a detrimental effect on the autoimmune response.

Previous studies have shown that MHC-linked HSP70 gene linked with susceptibility to TIDM in British Caucasoid population (Caplan et al., 1990). However, the following studies have revealed contradictory results in different populations (Pociot et al., 1993; Kawaguchi et al., 1993; Chuang et al., 1996). These studies and our study suggest that HSP genes may have susceptible loci to TIDM.

In this study, the risk markers of both NFκB and HSP70-A2 genes are associated with patients with TIDM compared with non-diabetic healthy controls. However, when patients were classified according to the presence or absence of microvascular complications, no difference was observed in the distribution of risk alleles between DC group and any of the complications groups.

The duration of the disease, age at onset, sex or age of the patients with TIDM could help to define subtypes of TIDM. However, no association was observed between these factors and the risk alleles of either NFκB or HSP70-A2 genes. This was the first time that NFκB and HSP70-A2 loci have been investigated to the susceptibility to TIDM and its late microvascular complications. More studies are needed to confirm these results with further examination of the molecular mechanisms and the protein expression underlying these findings. This may provide an insight into the pathogenesis of diabetic complications. However, several candidate genes have been previously suggested to confer susceptibility to TIDM and its microvascular complications but they have yielded inconclusive results and require further confirmation.

Our results revealed that the microsatellite (CAAA) 5 on the 5` SORD gene is not polymorphic. That has been confirmed by sequence analysis. The results suggest that screening for this polymorphism is unlikely to be a useful tool for risk assessment. In addition, no evidence of association was obtained between PKC-β gene T (+126) C polymorphism on exon 10 and TIDM or any of its late complications. Similarly, the same marker has been investigated previously in patients with TIDM with nephropathy and no association was observed (Araki et al., 2000). However, genotyping of one polymorphism on any gene is not enough to confirm the presence or absence of risk marker to the disease.

4.2 NFκB-DNA binding activity in patients with diabetes

In diabetes, there are a variety of different stressors such as hyperglycaemia, HO, oxidative stress, AGEs, hypoxia cytokines and growth factors. All of these stress factors either alone or in combination have the potential to induce the proinflammatory and heat shock responses. This study focused on the role of uncontrolled hyperglycaemia in the development of diabetic microvascular complications (Figure 4.2). The hypothesis was that uncontrolled hyperglycaemia might influence the transcriptional regulation of many genes. This study examined whether NFκB and HSF-1 activity is increased in the cells of patients with diabetes, whether this activity is responsive to hyperglycaemia and whether the response is polyol-dependent.

Elevated D-glucose concentration (31mM) significantly increased NFκB-DNA binding activity in PBMCs from patients with diabetes but not in those from the NC population (Figure 3.12). This result suggests that PBMCs of patients with diabetes were pre-stressed with hyperglycaemia and other stress factors in diabetes for long time. Therefore, more risk factors in diabetes magnified the effect of hyperglycaemia stress and induced NFκB activation. It is known that the effect of hyperglycaemia remains for a period of time even after the treatment with hypoglycaemic drugs (DCCT, 2000; Sheetz et al., 2002). Hyperglycemic memory may cause functional alteration such as formation of AGEs, stimulation of growth factors and enhanced proinflammatory processes. These factors induce NFκB activity. Previous studies have shown that NFκB up regulates VEGF and AGEs (Marumo et al., 1999; Tanaka et al., 2000).

Hyperglycaemia induces NFκB-DNA binding activity in most cell types. In this study, hyperglycaemia induced a significant increase of NFκB-DNA binding activity in Hep G2 cells but not in E6.1 cells (Figure 3.10).

NFκB has also been found to be active in response to hyperglycaemia in VSMC (Yerneni et al., 1999) and retinal pericytes but not retinal endothelial cells (Romeo et al., 2002). Therefore, these findings suggest that NFκB is implicated in the pathogenesis of endothelial dysfunction in diabetes. It is also possible that hyperglycaemia-induced polyol pathway in diabetes induces NFκB activation in PBMCs from patients with diabetes since hyperglycaemia itself did not induce significant activation in PBMCs from healthy individuals (Figure 3.10). The polyol pathway is implicated in the pathogenesis of microvascular complications in diabetes (Yabe-Nishimura 1998; Nishikawa et al., 2000a; b; Brownlee 2001). Both hyperglycaemia and the polyol pathway are important sources of oxidative stress, which induce NFκB activation (Williamson et al., 1993; Mohamed et al., 1999; Obrosova et al., 1999). Therefore, hyperglycaemia and diabetic-induced polyol pathway may induce their diverse effects through NFκB activation.

Hyperglycaemia-induced NFκB-DNA binding activity was pronounced in patients with TIDM (Figure 3.12). It is well known that the proinflammatory cytokines such as TNF-α and IL-1 are implicated in the pathogenesis of TIDM and they are upregulated by NFκB (Green et al., 1999; Kukreja et al., 1999; Eizirik et al., 2001 a and b; Cardozo et al., 2001). This may explain the higher activation of NFκB in patients with TIDM. Results showed a significant increase in hyperglycaemia-induced NFκB-DNA binding activity in PBMCs from patients with diabetic microvascular complications. No difference was observed between patients with TIDM-comp and those patients with TIIDM-comp (Figure 3.13). The result suggests that the role of NFκB in the underlying molecular mechanisms of microvascular complications is similar in both types of diabetes. Clinically, most of patients with diabetic microvascular complications have hypertension, long duration of the disease with uncontrolled blood glucose levels. These are major risk factors, which induce endothelial dysfunction and contribute to the development of diabetic microvascular

diseases (DCCT, 1993; 1995; Rossing et al., 1996; Weidmann et al., 1997; UKPDS 33, 1998; Gabir et al., 2000; Perin et al., 2001; NIH, 2002).

Hypertension potentially increases the mechanical load onto VSMC (Donnelly, 2000; Calles-Escandon et al., 2001) and triggers a number of biochemical changes including NFκB (Brand et al., 2001) and HSP (Pockley et al., 2000). In this study, most of the patients with microvascular complications had TIDM for more than 20 years. Therefore cells were exposed for a long period to cumulative hyperglycaemia, which induces the proinflammatory response and initiates endothelial dysfunction, which in turn induces more tissue damage. Microvascular diseases are associated with endothelial dysfunction. EC activation is associated with transcriptional induction of ET-1, E-selectin, VCAM-1, ICAM-1 and cytokines (Pieper et al., 1997; Guha et al., 2000; Quehenberger, 2000), most of these factors are up regulated by NFκB. Previous studies have also shown that NFκB activation is linked to increased MCP-1 and monocytes infiltration (Rovin et al., 1995). Therefore, the results suggest that NFκB is implicated in the pathogenesis of late diabetic complications. Further data analysis has revealed a significant positive correlation between the duration of diabetes and hyperglycaemia-induced NFκB- DNA binding activity in our population (Figure 3.14 (A)).

In this study, patients with TIIDM developed microvascular complications with a mean of 5 years after diagnosis. This may be due to strong hyperglycaemia exposure or due to other environmental factors such as insulin resistance, obesity, and hyperlipidaemia that could accelerate the development of microvascular complications in TIIDM. However, the intensity of hyperglycaemia as well as the duration of the disease is a major risk factor to diabetic complications (DCCT, 1993; Gabir et al., 2000). The results showed that hyperglycaemia-induced NFκB was positively but not significantly correlated with HbA_{1c} levels (Figure 3.14 (B)). However, another study did find a significant correlation between protein activity and HbA_{1c} levels in patients with TIDM (Hofmann et al., 1998).

Interestingly, no increase in NFκB- DNA binding activity was observed in response to 31mM D-glucose in patients who had TIDM for a mean duration of 31.8 ± 6.8 years and have remained free of microvascular complications. The results suggest that glucose alone is not enough to lead to the development of microvascular complications and support the role of genetic factors. Those patients may be genetically protected. It is possible that they have the protective allele of any of the genes, which contribute to the development of diabetic microvascular complications such as ALR2 and VEGF. It is known that these factors could modulate the NFκB activity (Marumo et al., 1999; Ramana et al., 2002& 2003).

In this study, there was no association between the identified risk markers of the NFκB gene and patients with diabetic complications while hyperglycaemia-induced NFκB-DNA binding activity was pronounced in PBMCs from those patients. The results suggest that the proteins-DNA binding activity is unlikely to be linked with the investigated polymorphisms. Therefore, it is possible that increased glucose levels interferes with IκB phosphorylation and stimulate its degradation or it might enhance the p65 subunit activity. More studies are needed to investigate the expression of IκB and p65 in response to hyperglycaemia and their relation with NFκB-DNA binding activity. Hyperglycaemia-induced NFκB- DNA binding activity was significantly increased in patients with DR and DNU but not DN. Supporting these observations; it has previously been shown that NFκB activity was increased in retinal cells in response to hyperglycaemia (Romeo et al., 2002). However, a previous study also showed raised NFκB-DNA binding activity in the PBMCs of TIIDM patient with DN; although in this instance the cells were not cultured in vitro (Hofmann et al., 1999). It is shown that polyol pathway and its derivatives including oxidative stress are active in lens and neural tissues (Mizuno et al., 1999; Obrosova et al., 1999; 2001). The polyol pathway may induce its devastating effects in those tissues through the activation of the proinflammatory NFκB.

4.3 HSF-1-DNA binding activity in patients with diabetes

Hyperglycaemia induced HSF-1-DNA binding activity in PBMCs from patients with diabetes but not in those of the NC group (Table 3.20). The protein activity was pronounced in patients with microvascular complications (Figure 3.19) suggesting that increased flux through the polyol pathway in diabetes in general and in diabetic complications in particular may induce HSF-1-DNA binding activity. The polyol pathway induces redox and osmolarity imbalances, previous studies have shown that both factors can increase HSF-1-DNA binding activity (Jacquier-Sarlin et al., 1996; Caruccio et al., 1997). Hyperglycaemia may also have a direct effect on the HSF-1-DNA binding mechanism since hyperglycaemia induced the protein-DNA binding activity in E6.1 lymphocytes in this study (Figure 3.16). However there is no published data to support this observation.

Hyperglycaemia-stressed PBMCs showed a significant increase in HSF-1 binding activity compared with unstressed cells from patients with TIDM (Table 3.20). The results showed that both NFκB and HSF-1-DNA binding activity was more pronounced in patients with TIDM. These observations suggest that TIDM as an autoimmune disease may affect cell viability and therefore magnify the response to stress factors. The increase of HSF-1-DNA binding activity in diabetes may be linked to the level of HSPs, which are known to play a role in the aetiology of TIDM such as HSP-60, HSP-65, and HSP-70 (Elias et al., 1990; 1995; Caplan et al., 1990; Pociot et al., 1993; Birk et al., 1996; Figueredo et al., 1996; Yamagishi et al., 2001). Further investigation is needed to study whether any relation between HSF-1-DNA binding activity and the expression of these proteins. However, high glucose concentration induced the activation of both factors in PBMCs isolated from patients with diabetes along with the cell lines. Therefore we believe our results correlate

with increased NFκB and HSF-1 in circulating mononuclear cells in patients with poorly controlled diabetes. Similarly, the activation of these factors has been described in other inflammatory diseases such as RA, Alzheimer's disease and arteriosclerosis (Schett et al., 1998; Bijur et al., 1999; Brand et al., 2001).

The results showed a highly significant positive correlation between the NFκB-DNA binding activity and that of HSF-1. This result suggests that HSF-1 may have proinflammatory effects. It might enhance the activation of NFκB. Previous studies have shown that heat shock response can modulate cellular proinflammatory signalling through their phosphorylation of the IκB gene (Malhotra et al., 2002), activation of IκB means an activation of NFκB and its subsequent proinflammatory effects. Therefore, it is possible that HSF-1 together with NFκB activation may accelerate the development of late diabetic microvascular complications through transcription of proinflammatory genes such as cytokines, adhesion molecules, and growth factors.

Phosphorylation of IκB and subsequent translocation of NFκB into the nucleus is auto-regulated by the level of NFκB and usually terminates within a short time (Baeuerle et al., 1991 & 1996; Barnes et al., 1997; Ghosh et al., 1998). HSF-1-DNA binding activity is also auto-regulated by HSP70 (Morimoto 1993; Schafer et al., 2000; Kim et al., 2000). In diabetes, chronic hyperglycaemia and activated polyol pathway may induce persistent transcription factor activation, which may disturb the auto-regulation mechanisms of these factors. Prolonged activation of the transcription factors in vivo may induce expression of adhesion molecules. Such molecules recruit inflammatory cells and initiate angiopathy (Guha et al., 2000; Calles-Escandon et al., 2001).

4.4 Aldose reductase inhibitor and transcription factors-DNA binding activity

In this study, it remains unclear whether the protein activation observed in response to high glucose levels is derived from the direct effect of hyperglycaemia or is dependent on the generation of other hyperglycaemia-induced products such as excessive polyol pathway flux and its subsequently biochemical products. However, the data presented here indicate that hyperactive polyol pathway plays an important role since ARIs such as zopolrestat and sorbinil reduced the DNA-binding activity of both NFκB (Table 3.23) and HSF-1 (Table 3.25) in hyperglycaemia-stressed PBMCs isolated from patients with diabetes. The effect was more pronounced in patients with microvascular complications.

Hyperglycaemia may contribute to the pathogenesis of diabetic complications via rapid increases in ALR2 transcription and expression. ALR2 is found in many tissues susceptible to diabetic complications such as kidney, nerve cells, the eye, and cells lining blood vessels (Yabe-Nishimura 1998). It catalyses the NADPH-mediated conversion of glucose to sorbitol, which escapes slowly from cells and can cause tissues damage. In addition, it has a pro-oxidant effect. Studies have shown that the expression of cytoplasmic anti-oxidant genes decrease in response to excess glucose in patients with diabetes (Ceriello et al., 2000; Hodgkinson et al., 2003). These observations detect the detrimental effect of excess polyol pathway flux on the induction of antioxidant enzymes in response to high glucose. Since exposure to hyperglycaemia has been shown to result in excess ROS generation that is largely polyol pathway dependent, it may be that excess ROS interferes with induction of protective antioxidant genes. This likely reflects the increase in NFκB and HSF-1 binding activity in cells exposed to high glucose is most likely part of a generalized response to oxidative stress and redox imbalance. The results also suggest that ARI (zopolrestat) may reduce the production of oxidative stress, which induces both NFκB and HSF-1 (indirect effect). ALR2 activity may be linked with an activation of the

transcription factors NFκB and HSF-1 as a part of signalling pathways. Studies have shown that the promoter region of the ALR2 gene contains binding sites for many transcription factors including NFκB (Aida et al., 1998; Fazzio et al., 1999; Iwata et al, 1999), it is also possible that there is a binding site for HSF-1. Therefore, ARIs may have a direct inhibitory effect on the binding sites of NFκB and HSF-1; this in turn reduces the proteins-DNA binding activity. It is also possible that the ARI (zopolrestat or sorbinil) competes with the effect of hyperglycaemia on the proteins activity. With respect to NFκB, ARI could interfere with NFκB pathway by reducing the IκB phosphorylation; therefore it will prevent its degradation. It is previously shown that anti-inflammatory drugs negatively modulate NFκB pathway by specific inhibition of IκB phosphorylation (Peirce et al., 1996). Furthermore, ARI could interfere with hyperglycaemia-induced phosphorylation of the functional subunit (p65). Studies have shown that ALR2 is involved in mediating mitogenic and apoptotic signalling (Ramana et al., 2002& 2003). It has also been shown that ALR2 activity links to an increase in VSMC growth and proliferation (Kasuya et al., 1999; Ruef et al., 2000). So ALR2 is a good target for potential medicines for diabetic complications. Inhibition of ALR2 has been shown to prevent the pathological changes related to diabetic complications in several target tissues (Yabe-Nishimura 1998).

However, the effect of ARI might be more useful than the specific inhibition of either NFκB or HSF-1 since specific inhibition may disturb the functional balance. It is known that NFκB has both pro and anti-inflammatory as pro and anti-apoptotic (Taylor et al., 1998; Yamamoto et al., 2001). So specific inhibition of NFκB may inhibit the proinflammatory but in the same time it will block the anti-inflammatory. [During this study, the NFκB and HSF-1-DNA binding activity has been investigated in LPS-stressed human umbilical vein endothelial cells (HUVEC). LPS is a strong stimulator for

NFκB activity (Schulze-Osthoff et al., 1997). The treatment with specific NFκB inhibitor (SN50) revealed a highly significant inhibition of the NFκB. This result indicates that SN50 has a specific inhibition on the NFκB-DNA binding activity since it blocked the binding activity of NFκB but not HSF-1-DNA (Figure A.1 and Table A.3)].

The results suggest that pharmacological modulation of NFκB and HSF-1-DNA binding activity could be useful as potential therapeutic targets to slow the progress of long-term diabetic microvascular diseases. Inhibition of NFκB pathway by both aspirin and salicylate has been shown to prevent leukocyte recruitment (Peirce et al., 1996) and NFκB inhibition reduced the progress of RA (Bondeson et al., 2000) and β cell apoptosis (Heimberg et al., 2001).

Metabolic flux through the polyol pathway is very sensitive and it is important to define the degree of inhibition needed for maximum efficacy for maintaining cytosolic coenzyme balance. Despite recent clinical, experimental and pharmacological data has shown that ARIs slow the progression of diabetic complications, no ARI is on the worldwide market (Cameron et al., 1997; Oates et al., 1999; Mizuno et al., 1999; Obrosova et al., 1999, 2001; Okayama et al., 2002). It is shown that inhibition of polyol pathway has recently yielded a dose dependent efficacy on nerve structure and function (Cameron et al., 1997; Mizuno et al., 1999; Obrosova et al., 2001). However, there was no detectable impact on retinal or renal dysfunction (Juhl et al., 1997; Mcauliffe et al., 1998). On the other side, clinical trials have been disappointing because treatment with ARI (zopolrestat) produces short term optimistic therapeutic approach (Arezzo et al., 1996). In addition, many ARIs (sorbinil and tolrestat) have an inadequate safety and efficacy (Pfeifer et al., 1997; Kubo et al., 1999). So, more understanding of the natural history and molecular mechanism of diabetic microvascular complications are needed to demonstrate that slowing the progress of diabetic complications is not rapid reversal of symptoms.

4.5 Cell lines

The protein assay study was mainly conducted to investigate NFκB and HSF-1-DNA binding activity in response to hyperglycaemia in PBMCs from patients with TIDM. However, Hep G2 and E6.1 cell lines were used for optimising and supportive investigation, which was difficult to perform on patients due to limited amounts of cells. In this study, NFκB and HSF-1-DNA binding activity was significantly activated by hyperglycaemia. The proteins activity seems to be a cell dependent mechanism (Figure 3.10& 3.16 respectively). However, distinct forms of stress can differentially trigger the protein activation. When cells were simulated with 110 mM NaCl, no activation was observed in NFκB-DNA binding activity in any of the cell types (Figure 3.11). Supporting this result, recently, an in vitro study has shown inhibition of NFκB activation in response to hypertonicity in smooth muscle cells (Pingle et al., 2003). These observations indicate that hypertonic solutions may prove useful as anti-inflammatory agents. On the other side, hyperglycaemia and HO induced HSF-1 DNA binding activity in E6.1 cells but not Hep G2 (Figure 3.16 and 3.17 respectively). There is no published data to support these observations. However, the results showed that the mode of the proteins activation is different in response to HO stress, supporting that signal and cell type can generate qualitative differences in gene expression (Hill et al., 1995).

The transduction of extra-cellular signals to the nucleus often involves phosphorylation cascades that allow rapid transmission and amplify the signal by activating multiple factors. It is previously shown that MAPK p38 is involved in the activation pathways of both NFκB and HSF-1 (Haire et al., 1988; Schulze-Osthoff et al., 1997; Kim et al., 1997). Results have shown that p38 expression was significantly increased in response to 31mM D-glucose in both Hep G2 and E6.1 cells. The expression of p38 was linked but not

correlated with NFκB activation in Hep G2 cells and with HSF-1 activation in E6.1. Previous studies have shown that MAPK p38 is implicated in the pathogenesis of diabetic microvascular complications (Schaffler et al., 1998). Therefore, these findings suggest that MAPK p38 may contribute to the development of diabetic complications through the activation of these factors.

MAPK p38 is viewed as an osmotic response element (Igarashi et al., 1999; Karin 2001). However, in this study the HO stress of 110mM NaCl did not induce p38 expression in either Hep G2 or E6.1 cells. This observation suggests that under normal conditions the protein has a specific functional role that could change due to the disturbance of homeostasis within the cell. However, in vitro studies have a time-limited course from hours to days that may induce a transient response, while the chronicity of stress in the intact organism could produce a sustained activation.

4.6 Methodological aspects of the project

At the beginning of the study, the plan was to follow the genotyping of the NFκB and HSP-70A2 genes by an investigation of transcription factor-DNA binding activity and the protein expression of NFκB (p65) and HSP-70 in PBMCs of patients with TIDM with and without microvascular complications. However, for practical reasons and limited flexibility the initial plan of investigation was changed to include cell line and patients with TIIDM to allow the completion of the study.

Patients and design of the studies

This study is a case-control study. Matching between normal and patients populations has been proposed to improve the power of the study. In the genotyping study, 100% British Caucasoid patients with TIDM were included. The combination of genotype information with functional data gives important information about the role of certain susceptibility loci. Therefore, the binding activity of the transcription factors NFκB and HSF-1 was investigated in relation to identified susceptibility alleles in few numbers of patients but the results were inconclusive. This was a disadvantage in this study. Larger patients numbers may have been an advantage, however, the recruitment of patients was difficult to complete this stage. It was also difficult to genotype same DNA samples for all the candidate genes due to lack of samples and degradation of DNA. Most of samples were used in other studies.

The protein assay study was conducted to investigate NFκB and HSF-1-DNA binding activity in response to hyperglycaemia in PBMCs of patients with TIDM with and without microvascular complications. However, 35% of the patients included were TIIDM to complete the study of treatment with zopolrestat on the hyperglycaemia-induced proteins activity. The pathogenesis of TIDM is different from that of TIIDM however; it is suggested that the underlying molecular mechanisms of microvascular complications in both types are similar (DCCT, 1993; UKPDS 33, 1998; Calles-Escandon et al., 2001).

Patients with either TIDM or TIIDM of short duration are included in this study as patients with diabetes mellitus irrespective of the duration of the disease. Patients with TIDM of short duration without microvascular complications present 8.3% and 11% of patient's population in the genotyping study of HSP70-A2 and protein assay study respectively. They have been included as an unbiased sample of patients with TIDM and act as controls

for diabetes itself while other diabetic subgroups such as nephropaths, retinopaths, and neuropaths have all been specifically chosen for their complication phenotype. Sometimes, the frequency of the polymorphisms seems the same in normal controls as a complication group, but short duration patients give a better idea of association with the autoimmune disease itself. However, data analysis of HSp70-A2 alleles and genotypes excluding this group did not affect the association of the identified alleles in either patients or normal control populations.

With respect to the protein assay study, approximately 24% of patients had short duration diabetes (11% with TIDM without microvascular complications and 13% with TIIDM with microvascular complications). This group showed that the duration of the disease as an environmental risk factor is implicated in the pathogenesis of micovascular diseases in diabetes as well as hyperglycaemia.

Summary and Conclusions

1- A strong association between polymorphisms in close proximity to the NFκB (4q24) and HSP70 A2 (14q22) genes and susceptibility to TIDM but not with the long- term microvascular complications of this disease. At the present time, the influence of these microsatellites on the expression of either NFκB or HSP70 proteins is unknown.

2- Hyperglycaemia induces a marked increase in the NFκB and HSF-1-DNA binding activity, which was more pronounced in patients with TIDM, those patients with microvascular complications in particular.

3- HSF-1-DNA binding activity correlates with NFκB-DNA binding activity in patients with diabetes.

4- NFκB-DNA binding activity was correlated with the duration of the disease.

5- The ARIs, zopolrestat and sorbinil reduce the hyperglycaemia-induced transcription factors binding activity–particularly in diabetes that may either be competing with hyperglycaemia or, interfere with the binding mechanism of the transcription factor.

6- The transcription factor-DNA binding activity may be cell and stress specific mechanism.

Further studies

The A10 allele of the NFκB gene, H3 and H7 alleles of the HSP70-A2 gene were identified as risk markers of TIDM in general population. Further, future efforts are aimed to confirm the association of these markers in a family-based study. During this study, the number of patients with TIDM with microvascular complications was relatively small and limited for practical reasons. It remains possible that with large numbers of patients to study these polymorphisms may be shown to be related to the presence or absence of microvascular complications rather than to diabetes itself.

Gene's transcription is a complicated mechanism. The binding of transcription factor to DNA (response element) is the start point of the transcription mechanism. It either inhibits or assists RNA polymerase in initiation and maintenance of transcription. In this study, investigation has shown that high glucose concentration increased NFκB-DNA binding activity in PBMCs of patients with TIDM with microvascular complications. However, the expression of p65, the functional part of the NFκB heterodimer in response to hyperglycaemia is not clear. Future work is now required to ascertain whether the hyperglycaemia-induced NFκB-DNA binding activity is combined with an increase in the p65 expression in human PBMCs.

The down-regulation of NFκB activity in PBMCs needs to be clarified. It could be difficult to find a treatment which does not completely inhibit the NFκB-DNA binding activity. Inhibition of polyol pathway by ARI has shown a promising effect on the protein activity. Treatment of PBMCs with ARI (10 μmol zopolrestat) reduced but did not block the effect of hyperglycaemia on NFκB-DNA binding activity. However, more studies are needed to investigate the effect of different classes of polyol pathway inhibitors or

specific NFκB inhibitors on NFκB-DNA binding activity in human PBMCs.

Further, it is a point of interest to investigate the phosphorylation of IκB in response to hyperglycaemia with and without treatment by ARI, since IκB is the main regulator of NFκB activation. Different techniques such as Western Blot and immuno-cytochemistry will be used to investigate the phosphorylation of proteins for comparison purposes and more accuracy.

APPENDIX

A.1 Re: HSP-70 A2 results

	Age at Onset (years)		
	<10	10-20	>20
Genotype	(n=57)	(n=63)	(n=73)
	%	%	%
	Male: Female	Male: Female	Male: Female
H1/X	0.02	0.05	0.03
	0:1	3:0	1:1
H3/X	0.3	0.1	0.3
	9:11	1:6	6:17
H5/X	0.08	0.1	0.1
	0:5	2:6	5:5
H7/X	0.3	0.4	0.3
	1:14	16:10	18:6
X/X	0.3	0.3	0.2
	4:12	9:10	11:3

Table A.1: Frequency (%) of the most common HSP70-A2 genotypes with respect to age at onset of T1DM and gender

Patients were classified into three groups according to the age at onset (AAO) of T1DM: <10,10-20, >20 years. No difference was found in the frequency of the genotypes with either AAO or gender.

A.2 Specific-NFκB inhibitor and transcription factors -DNA binding activity

A.2.1 Human Umblical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were derived from a newborn, male donor. Cells were obtained from Totam Biologicals, Northampton, UK No: C-003-5C.

A.2.2 Preparation of medium for culture

The HUVECs were grown in medium 200, which supplemented with low serum growth supplement (LSGS). Cells, medium and LSGS were supplied from Totam Biologicals, Northampton, UK. Medium 200 contains essential and non-essential amino acids, vitamins, trace minerals, and inorganic salts and dose not contains antibiotics, antimycotics, hormones, growth factors, or proteins. The HUVECs were stressed with LPS (specific stimulant for NFκB activation). Furthermore, LPS-stressed HUVECs have been treated with SN-50 (specific-NFκB inhibitor) and sodium arsenite (Na As), which induce the heat shock response and is suggested to inhibit NFκB-DNA binding activity. Table A.2 shows the stress conditions and the duration of exposure. Cells were stressed and incubated for 5 days at 37 ^{O}C and 5% CO2.

Stress	Concentration	Time
N		5 days
Na As	0.1 μM	30 min
SN50	50μg/ml	30 min
LPS	10 μg/ml	18 hrs

Table A.2: Stress conditions and the duration of exposure of the growing cells

Cells were stressed according to the type of experiments, grown in the recommended medium and incubated for 5 days at 37 ^{O}C and 5% CO2. N= control (unstressed cells). NaAS = sodium arsenite, SN50 = specific-NFκB inhibitor, and LPS = lipopolysacharride.

A.2.3 Results

Figure A.2 is an autoradiograph shows the NFκB and HSF-1 DNA binding activity in HUVEC. LPS induced more than two fold increase in the NFκB-DNA binding activity (2.5 fold) (Table A.3). When LPS- stressed HUVEC were treated with SN-50 (100 μg/ml), there was a significant inhibition of the NFκB-DNA binding activity compared with LPS-HUVEC with no SN-50 (0.09 ± 0.008 vs 0.13 ± 0.008, p=0.025). Also, Na As reduced the NFκB-DNA binding activity in LPS-stressed HUVEC (0.02 ± 0.008 vs 0.13 ± 0.008, p= 0.0008). LPS induced HSF-1-DNA binding activity compared with unstressed HUVEC (p= 0.017) (Table A.3). No inhibition was observed in the HSF-1-DNA binding activity in LPS-stressed HUVEC with SN-50.

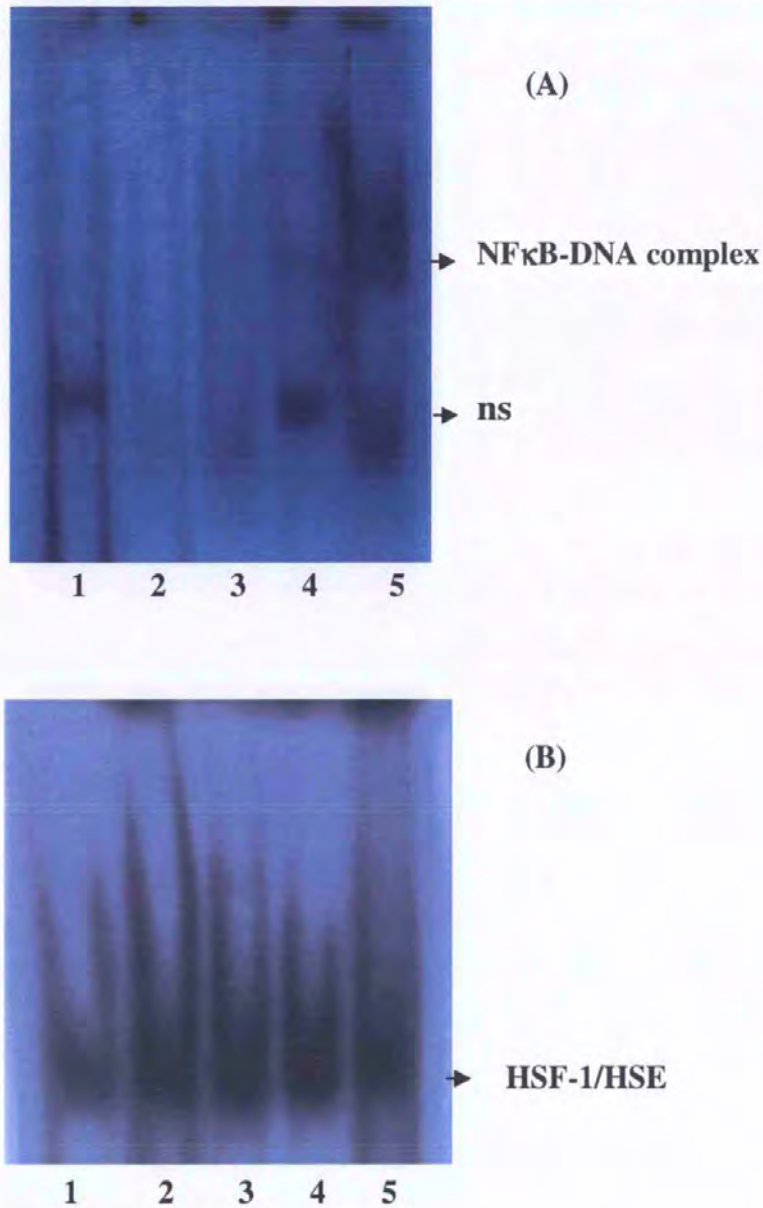


Figure A.1: Autoradiograph shows the NFκB and HSF-1-DNA binding activity in HUVEC

(A) NFκB-DNA binding activity, lane 1: LPS-stressed HUVEC with SN50 (NFκB inhibitor), lane 2: LPS-stressed HUVEC+ NaAS, lane 3, unstressed HUVEC, lane 4& 5: LPS-stressed HUVEC. (B) HSF-1-DNA binding activity, lane 1: unstressed HUVEC, lanes 2, 3, 4: LPS-stressed HUVEC+ NS 50 (100, 75, 50 μg/ml respectively), and lane 5: LPS-stressed HUVEC. NaAS = sodium arsenite and LPS = lipopolysacharride. ns= non-specific bands.

Stress	NFκB-DNA binding activity	HSF-1 DNA binding activity
N	0.05 ± 0.008	0.29 ± 0.01
LPS	*0.13 ± 0.008	♦0.37 ± 0.01
LPS+ SN 50	**0.09 ± 0.008	0.39 ± 0.01
LPS+ NaAs	***0.02 ± 0.008	

Table A.3: Results of EMSA for NFκB and HSF-1-DNA binding activity

HUVEC cells were stressed with LPS with or without NFκB inhibitors. Cells were incubated at 37 ^{0} C for 5 days. Experiments were repeated for 3-4 times. The results are the means ± SE. N= control (unstressed cells). NaAS = sodium arsenite, SN50 = specific-NFκB inhibitor, and LPS = lipopolysacharride.

*vs HUVEC controls, p= 0.008, **vs LPS-stressed HUVEC, p= 0.025,

*** vs LPS-stressed HUVEC, p= 0.0008.

♦ vs HUVEC controls, p= 0.017.

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1. **Hegazy DM**, Yang BM, Oates PJ, Millward BA, Demaine AG. Aldose Reductase Inhibitor Reduces the Hyperglycaemia-Induced Increase in Nuclear Factor Kappa B Binding Activity in Cells from Patients with Diabetes Mellitus (Submitted, Diabetes, August 2003).

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5. **Hegazy DM,** Yang BM, Millward BA and Demaine AG Study of Polymorphism of the Heat Shock Protein 70-A2 Gene in Type 1 Diabetes and its Late Microvascular Complications (In Submission).

Conferences attended and work presented

1. **Hegazy DM,** Yang BM, Oates PJ, Millward BA and Demaine AG. American Diabetes Association, 63RD Scientific Sessions. Poster presentation with abstract: Aldose Reductase Inhibitor Reduces the Hyperglycaemia-Induced Increase in Nuclear Factor Kappa B Binding Activity in Cells from Patients with Diabetes Mellitus. New Orleans, USA. 13-17 June 2003.

2. **Hegazy DM**, Yang BM, Oates PJ, Millward BA and Demaine AG. Diabetes UK. Annual Professional Conference. Oral Presentation: Aldose Reductase Inhibitor Reduces the Hyperglycaemia-Induced Increase in Nuclear Factor Kappa B Binding Activity in Cells from Patients with Diabetes Mellitus. Glasgow, UK. 19-21 March 2003.

3. **Hegazy DM**, Yang BM, Millward BA and Demaine AG. Diabetes UK, Annual Professional Conference. Poster presentation with abstract: An Investigation of Polymorphism of Heat Shock Protein -70 Association in Patients with Type 1 Diabetes and Its Late Microvascular Complications. ICC, Birmingham, UK. 13-15 March 2002.

4. **Hegazy DM,** Yang BM, Millward BA and Demaine AG. Renal Association Autumn Meeting with the British Association for Paediatric Nephrology, poster presentation with abstract: Polymorphism of Heat Shock Protein –70 association in Type 1 Diabetes and its Late Microvascular Complications. Royal College of Physicians, London UK, 20-21 September 2001.

5. **Hegazy DM,** O'Reilly DA, Yang BM, Hodgkinson AD, Millward BA and Demaine AG. American Diabetic Association Annual Meeting, poster presentation with abstract: NFκB Polymorphisms and Susceptibility to Type 1 Diabetes. Philadelphia USA, 22-26 June 2001.

6. **Hegazy DM,** O'Reilly DA, Yang BM, Hodgkinson AD, Millward BA and Demaine AG. Anglo-Danish- Dutch- Diabetic-Group (ADDDG) Meeting, oral presentation: NFκB Polymorphisms and Susceptibility to Type 1 Diabetes. Denmark, 15-18 May 2001.

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Membership

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Aldose reductase inhibition reduces the hyperglycemia-induced increase in nuclear factor kappa B binding activity in patients with diabetes mellitus

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ABSTRACT

Diabetes is associated with considerable metabolic abnormalities leading to oxidative stress and redox imbalance within the cell. The redox-sensitive transcription factor, nuclear factor kappa B (NFκB) is activated in patients with microvascular complications. Gel-shift assays were used to investigate the NFκB DNA binding activity in peripheral blood mononuclear cells (PBMCs) of 67 patients with diabetes 45 with type 1 and 23 with type 2 diabetes mellitus (T1DM and T2DM) and 10 normal healthy controls (NC). PBMC were exposed to high glucose (31mM D-glucose, HG) for 5 days. NFκB activity was more than two-fold higher in PBMC exposed to HG compared to normal glucose (NG). The increased activity was more pronounced in PBMC from patients with diabetes compared with NC (2.1 ± 0.9 vs 1.6 ± 0.4, p=0.01). The binding activity was significantly higher in cells from T1DM patients with microvascular disease compared to those with none after 20 years duration of diabetes (uncomp) (2.2 ± 0.8 vs 1.9 ± 0.6 p=0.04). When the PBMCs were cultured in the presence of 10 μM of the aldose reductase inhibitor (ARI) zopolrestat there was a highly significant reduction in NFκB-DNA binding activity (0.1-1 fold, p= 0.000006). Similar results were obtained using sorbinil, a structurally distinct ARI. This was more pronounced in PBMC from patients compared to the NC group (0.4 ± 0.2 vs 0.7 ± 0.3, p= 0.04). In conclusion, these results suggest that inhibition of the polyol pathway can directly influence diabetes-induced NFκB activation. Although the underlying biochemical mechanisms for this response remain to be elucidated, these data suggest that HG-induced activation of redox-sensitive NFκB results from cytoplasmic redox alteration resulting from enhanced metabolic flux through the polyol pathway.

INTRODUCTION

Chronic exposure to diabetes inevitably leads to diabetic microvascular complications in a proportion of patients with type 1 as well as type 2 diabetes mellitus (T1DM and T2DM respectively). Whilst the underlying mechanisms responsible for the development of diabetic microvascular complications have still to be elucidated there is compelling evidence for the involvement of oxidative stress and the polyol pathway [1-6].

Aldose reductase (AKR1B1 or ALD2) is the first and rate-limiting enzyme of the polyol pathway and reduces glucose to sorbitol in a NADPH dependent reaction; sorbitol is then metabolised to fructose by the enzyme sorbitol dehydrogenase and the co-factor NAD^+. Increased flux through the polyol pathway due to diabetes has been linked with a network of metabolic perturbations including increased osmotic stress, reductive stress and oxidative stress [5-7). Although there is in vitro evidence in some cell types to suggest that ALR2 (along with other aldo-ketoreductases) may play a role in cell defence against noxious insults [8], it has become clear that ALR2 and members of the aldo-keto reductase family are involved in mediating cytokine-initiated mitogenic signalling and apoptosis in a variety of cell types including vascular smooth muscle cells (VSMC)[9], lens epithelial cells [10], 3T3 fibroblasts [11] and inner retinal neurons [12].

Many of the cytokine responses will involve the activation of NFκB, a redox-sensitive transcription factor NFκB whose activation is thought be triggered by oxidative stress [13-15]. NFκB promotes the expression of over 150 target genes including those coding for cytokines such as tumour necrosis factor-α (TNF-α), and interleukin-1β (IL-1β); chemokines, adhesion molecules and endothelin-1 (ET-1) [14-17]. Previous studies have shown increased activity of NFκB in patients with T1DM as well as T2DM who have microvascular disease [18-22]. Further, the promoter region of ALR2 contains an NFκB response element and the gene can be activated directly by TNF-α [9,23].

The aim of this study was to investigate the link between aldose reductase and NFκB activity using peripheral blood mononuclear cells (PBMC) obtained from patients with diabetes and exposing them to hyperglycaemia in the presence or absence of two structurally distinct aldose reductase inhibitors (ARI).

MATERIALS AND METHODS

Subjects

Caucasoid patients with either T1DM or T2DM (defined by the National Diabetes Data Group, [24]) were recruited from the Diabetic Clinic, Derriford Hospital, Plymouth, UK). Local ethical committee approval was obtained. The criteria for defining the presence of microvascular complications has been defined previously [25]. Briefly, uncomplicated patients with T1DM have had T1DM for at least 20 years but remain free of retinopathy, microalbuminuria, proteinuria, and overt neuropathy. Patients with short duration diabetes (SD) have had diabetes for less than 10 years and remain free of nephropathy, retinopathy or neuropathy. Patients with complications have the following:

Diabetic nephropathy: persistent proteinuria (urine Albustix positive on at least three consecutive occasions over 12 months or three successive total urinary albumin excretion rates more than 0.5 g/24h) in the absence of haematuria or infection on midstream urine samples. The presence of nephropathy is always associated with diabetic retinopathy.

Retinopathy: defined as having more than 5 dots or blots per eye, hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy and maculopathy or vitrous haemorrhage. None of these patients had microalbuminuria or protienuria. The clinical characteristics on of the patients are shown in Tables 1 and 3.

Preparation of peripheral blood mononuclear cells (PBMC)
The fresh whole blood (20ml) was collected into 5% ethylenediaminetetraacetic acid (EDTA) Vacuainers (Becton Dickinson, Oxford, UK). PBMCs were isolated using Histopaque according to the manufacturer's instructions. A concentration of 0.5x 10^6 cells were grown in culture flasks (200ml) containing 20 ml of RPMI-1640 (Invitrogen, Paisley, UK) with 20µl of phytohemagglutinin (PHA) (Sigma Aldrich, UK). According to the type of the experiment, cells were grown under normal conditions (NG) or, high glucose conditions (HG) where the medium was supplemented with 28 mmol/L D-glucose. For the ARI studies, the PBMC were grown in the presence of 10 µM of either zopolrestat or sorbinil (Pfizer, Groton). To investigate the dose effect of zopolrestat on NFκB-DNA binding activity the PBMC were exposed to either NG or HG and divided into four flasks containing either 0, 5, 10, or, 15 µM of zopolrestat. To confirm the inhibitory effect of zopolrestat, PBMCs were stressed with 10 µg/ml of lipopolysacharride (LPS) for 18 hrs which is known to be a strong activator of NFκB. The PBMCs were incubated at 37° C with 5% CO2 for 5 days and then harvested.

Nuclear Protein Extraction
The PBMCs were harvested by centrifugation. The cell pellets were lysed by adding 300µl of buffer A containing 15mM of HEPES pH7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM dithiothreiotol, 0.5mM phenylmethylsulfonyl fluoride (PMSF), 1µg/ml each of leupeptin and pepstatin, 0.01 U/ml aprotinin) before incubating on ice for 15 min. The lysate was centrifuged at 12000 rpm for 10 min, resuspended in 50µl of buffer C (containing 15mM HEPES (pH 7.9), 20% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.5mM iodoacetamide, 0.2mM EDTA, 0.5mM PMSF, 1µg/ml each of leupeptin and pepstatin, and 0.01U/ml of aprotinin) and incubated on ice for 10 min. Then the lysate was centrifuged at 13000 rpm for 3 min. The supernatant containing the nuclear protein was kept at –80° until required for GSA. The concentrations of the extracts were determined by Coomassie protein assay reagent (Pierce, Chester, UK) following the manufacturers instructions. A standard curve using ? human serum albumin ? was established by measuring the Coomassie protein assay reagent in a spectrophotometer at OD600 that was then used to determine protein concentrations of the extracts.

Gel Shift Assay
A 5% non-denaturing polyacrylamide vertcial gel was prepared and left to set overnight at room temperature. 10 µg of nuclear protein was mixed with 2µl of gel shift binding buffer, incubated at room temperature for 10 min, mixed with 1µl of radiolabelled oligonucleotide probe and incubated at room temperature for 20 min. The probe used was NFκB consensus oligonucleotide: 5` TGA GGG GAC TTT CCC AGG C 3` (Promega, Delta House, Southampton, UK) labelled with [γ^{32}P] ATP by using T4 polynucleotide kinase. At the end of the incubation, 1µl of loading buffer was added and the mixture loaded on to the gel and electrophoresed in 0.5x TBE at 100v for 2-3 hrs. The gel was then transferred onto filter paper, dried and exposed to X-ray film for 18 hrs at -80°C. Autoradiography was performed and a phosphor-imager software program used for quantify the bands (Bio-Rad Multi-Analyst, Hertfordshire, UK).

Statistical Analysis
The intensity of the bands was expressed as means ± SD with each sample run in triplicate. The fold change in binding activity in cells exposed to high glucose compared to normal glucose was calculated for each sample. Statistical analysis was performed using student's *t*- test. p value less than 0.05 was considered to be significant.

RESULTS

Ten normal healthy controls and sixty-seven patients (45 with T1DM) were investigated for HG-induced NFκB-DNA binding activity. The characteristics of the patients are shown in Table 1. In cells from normal controls and diabetics exposed to high glucose the activity of NFκB was increased two-fold when compared to cells cultured under normal condition (normals controls, 0.2 ± 0.08 vs 0.5 0 ± 0.08 and diabetics, 0.5 ± 0.07 vs 0.3 ± 0.07, p = 0.004)(Table 2). This fold increase in binding activity was significantly higher in patients compared to the normal healthy controls (2.1 ± 0.9 vs 1.6 ± 0.4, p= 0.01) When the patients were separated according to their type of diabetes, there was a significant increase in binding activity between the patients with T1DM and normal controls but not with those with T2DM (2.2 ± 0.8 vs. 1.6 ± 0.4, p= 0.001 and 1.7 ± 0.9 vs. 1.6 ± 0.4, p=ns respectively)(Table 2). When the patients with T1DM were separated according to either the presence or, absence of microvascular complications, the former had a significant increase in binding activity compared to the latter (2.4 ± 0.8 vs 1.9 ± 0.6, p= 0.04). However, this difference was not apparent with patients with T2DM with complications (n=11) and vs 2.1± 1.1, p=ns respectively) (Figure 3). No difference was observed between the other groups.

When the tissue culture media was supplemented with an ARI there was a marked and consistent reduction in NF-κB binding activity in a dose-dependent manner. (Figure 1). A further 34 patients and 5 normal controls were studied for the effect of ARI on NFκB binding activity (Table 3). Zopolrestat, reduced the HG-induced protein binding activity by more than 50% compared to HG-stressed cells without zopolrestat (0.3 ± 0.1 vs 0.7 ± 0.4, p= 0.000006). PBMCs cultured in normal media in the presence or absence of zopolrestat showed no difference in the protein binding activity (0.3 ± 0.1 vs 0.3 ± 0.2, p= ns). When the PBMCs were stimulated with the NFκB activator LPS, there was a marked increase in NFκB-DNA binding activity that was inhibited by zopolrestat (data not shown).

The reduction of NFκB activity by zopolrestat was greater in patients when compared to normal controls (0.4 ± 0.2 vs 0.7 ± 0.3, p= 0.04). When the patients were categorised according to the presence or absence of complications, NFκB activity was found to be significantly reduced by zopolrestat in those with complications compared to either patients to normal controls (0.3± 0.1 vs 0.7 ± 0.3, p= 0.03). Whilst the reduction of NFκB activity in the uncomplicated patients was less marked, this was still significantly different from the normal controls (0.5 ± 0.2 vs 0.7 ± 0.3, p= 0.04) (Table 4). Similar results were obtained in PBMC from normal controls exposed to HG in the presence of sorbinil, a second, structurally distinct ARI (0.2±0.05 vs 0.01±0.02, p=0.001).

DISCUSSION

This study examined whether NFκB activity is increased in the cells of patients with diabetes, whether this activity is responsive to hyperglycaemia and whether the response is polyol-dependent. In our study, PBMCs from patients with diabetes had threefold higher NFκB binding under normal glucose conditions compared to cells from normal controls and normal healthy controls cultured with a high glucose concentration demonstrated an increase of NFκB-DNA binding activity. Previous studies have shown that NFκB-DNA binding activity was increased in response to elevated glucose in cultured porcine VSMCs [26] and that activity was correlated to the glycaemic control in PBMCs of patients with T1DM [19].

In the present study, PBMCs from patients with T1DM had a higher increase in glucose-induced NFκB activity than PBMCs from patients with T2DM and normal healthy controls. This effect was more apparent in PBMCs from patients with microvascular

complications than in those without or normal controls. A previous study also showed raised NFκB-DNA binding activity in the PBMCs of T2DM patient with diabetic nephropathy, although in this instance the cells were not cultured in vitro [20]. We found a clear increase in NFκB activity in response to high glucose, and this was prevented by treatment with two structurally distinct ARIs. Another ARI, epalrestat, has been shown to inhibit platelet-derived growth factor [27] and inhibition of the polyol pathway causes decreased TNF-α-induced NFκB-DNA binding activity in the neointima of rat carotid arteries and cultured VSMCs [9]. In the present study we have to our knowledge demonstrated for the first time that inhibition of the polyol pathway can have a marked effect on NFκB-DNA binding activity in the human cells, including patients with or without microvascular complications. These findings together with those recently reported in cell lines and other species [6,7,9,10] strongly suggest that increased flux through the polyol pathway causes activation of the pro-inflammatory transcription factor NFκB.

We have previously shown that PBMC of patients with nephropathy have increased expression of ALR2 and SORD genes following exposure to high glucose when compared to cells from patients with no complications after 20 years of diabetes. The induction is tightly linked to the Z-2 'susceptibility' marker in the promoter region of ALR2 [28]. In addition, PBMC of patients with nephropathy have decreased expression of cytoplasmic anti-oxidant genes in response to excess glucose, whilst those from patients with no complications have a marked increase in expression of these genes [29,30]. All of these disturbances in gene expression can be ameliorated by inhibition of the polyol pathway [28,29]. These observations are consistent with a detrimental effect of excess polyol pathway flux on the induction of key antioxidant enzymes in response to high glucose. Since exposure to HG has been shown to result in excess ROS generation that is largely polyol pathway dependent [31,32], it may be that excess ROS interferes with induction of protective antioxidant genes.

This likely reflects The increase in NFκB binding activity in cells exposed to high glucose is most likely part of a generalized response to oxidative stress and redox imbalance [32].

In conclusion, we have shown that NFκB activity is elevated in cells that have been exposed to high glucose. The effect was particularly apparent in patients with T1DM and microvascular complications and could be reversed by treatment with two structurally distinct ARIs.

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Table 1: Clinical Characteristics of the Patients

	Sex (M/F)	Age at onset of diabetes(Ys)	Duration of diabetes (Yearss)
Uncomplicated (n= 7)	2:5	8.3 ± 4.8	31.8 ± 6.8
T1DM –Comp (n= 28)	21:7	26.8± 15.5	29.8 ± 8.9
T1-SD (n=8)	2:6	26.3 ± 16.3	8.2 ± 4.2
T2-Comp (n=14)	8:6	56 ± 4	5 ± 3
T2-SD (n= 10)	8:2	66 ± 10	5 ± 2
Normal controls (n=10)	4:6		
Total	**45:32**		

Legend Table 1: A total number of 67 patients and 10 normal controls were included in this study. Patients were sub-grouped according to the presence or absence of microvascular complications and type of diabetes. The table presents the classification according to sex, age at onset and duration of diabetes. Data are presented in means ± SD.

High Glucose and NFκB-DNA binding activity

M ± SE	Normal controls (n=10)	Diabetics (n= 67)	T1DM (n=43)	T2DM (n=24)	Uncomp (n=7)	T1-Comp (n=28)	T2-Comp (n= 14)	Short Duration (n= 18)
NG	0.1± 0.08	0.3± 0.07	0.2 ± 0.08	0.5± 0.1	0.2 ± 0.1	0.1 ± 0.09	0.5 ± 0.2	0.5 ± 0.1
High glucose (HG)	0.2 ± 0.08	0.5 ± 0.07	0.3 ± 0.08	0.7 ± 0.1	0.4 ± 0.1	0.2 ± 0.09	0.7 ± 0.2	0.7 ± 0.1
HG/NG (fold)	* 1.7 ± 0.2	2.1± 0.1	**2.2 ± 0.1	1.7± 0.2	1.9 ± 0.3	***2.5 ± 0.1	2.0± 0.2	1.5 ± 0.2

Table 2: High Glucose and NFκB-DNA binding activity in PBMC from Patients with Diabetes

Patients were sub-grouped according to the presence or absence of microvascular complications and type of diabetes. Results are in means of folds (± SE).

* vs all patients, p= 0.01, and vs patients with T1DM, p= 0.003

** vs those patients with T2DM, p= 0.02

*** vs Normal Controls, p= 0.005, vs those patients with Uncomplicated, p=0.047, and vs those patients with Short Duration, p= 0.00008

Patient Characteristics for Study of Effect of Zopolrestat on High glucose –induced NFκB-DNA binding activity

	Uncomp (n= 3)	T1DM-Comp (n=1)	T1DM-Short duration (n=4)	T2DM-comp (n=14)	T2DM-SD (n= 10)	Normal Control (n= 5)
Sex (m/f)	1:2	1: 0	0:4	8:6	8:2	4:1
Age at onset (Years)	26.5 ± 0.7	7	22 ± 9.5	56 ± 4	66± 10	
Duration (Years)	15.5 ± 4.9	35	9	5 ± 3	5 ± 2	

Table 3: Characterisation of patients included in the study of zopolrestat and HG-increased NFκB -DNA binding activity

A total number of 32 patients were studied. Uncomp = those patients with T1DM with no microvascular complications after 20 years duration of diabetes. T1DM-Comp and T2DM-Comp those patients with either T1DM or T2DM and nephropathy and retinopathy. SD = those patients with diabetes for 10 years or less with or without diabetes. AAO= Age at onset of diabetes. Data is shown as mean ± SD.

Mean ± SE	Normal controls (n=5)	Diabetes (n= 32)	Patients with complications (n=15)	Patients without complications (n=17)	ANOVA
High glucose (HG)	0.6 ± 0.2 suggest use 2	0.7 ± 0.05	0.7± 0.07	0.8 ± 0.06	
HG+ ARI	decimal s for these data	0.35 ± 0.05	0.25 ± 0.07	0.4 ± 0.06	
	0.4 ± 0.2				
Fold	0.6 ± 0.09	0.4 ± 0.05	* 0.3 ± 0.06	0.5 ± 0.05	0.008

Table 4: Zopolrestat inhibits the high glucose increase in NFκB-DNA binding activity in patients with diabetes and normal controls

Results are in mean of folds (± SE).

* vs normal controls, p= 0.03 & vs all without comp, p= 0.01

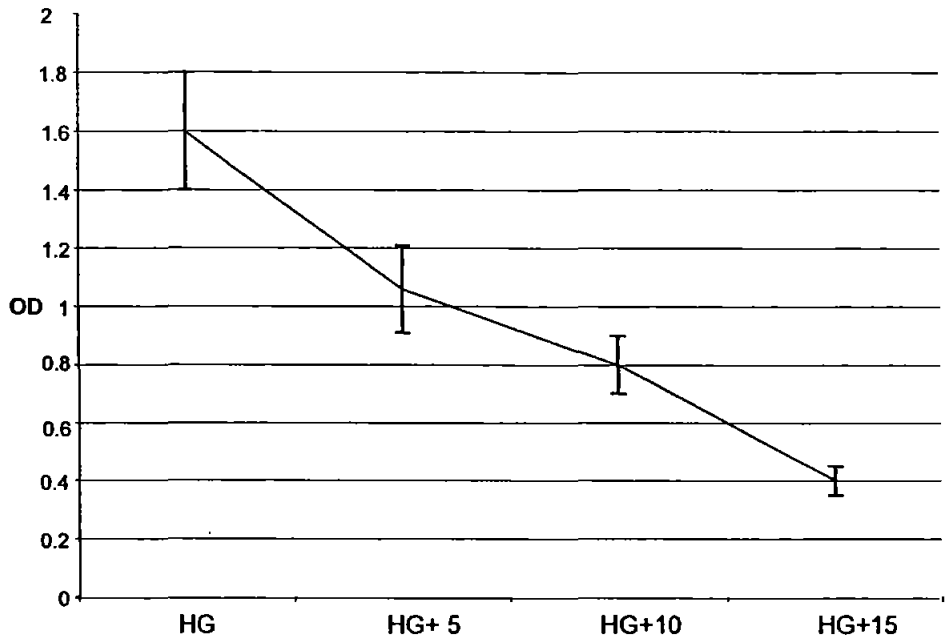

Figure 1: Zopolrestat reduced the high glucose-increased NFκB-DNA binding activity in a dose-dependent fashion

HG= HG-stressed cells without ARI, HG+5, 10, 15= HG with 5, 10, 15 μM of zopolrestat, respectively. Data are shown in means ± SD

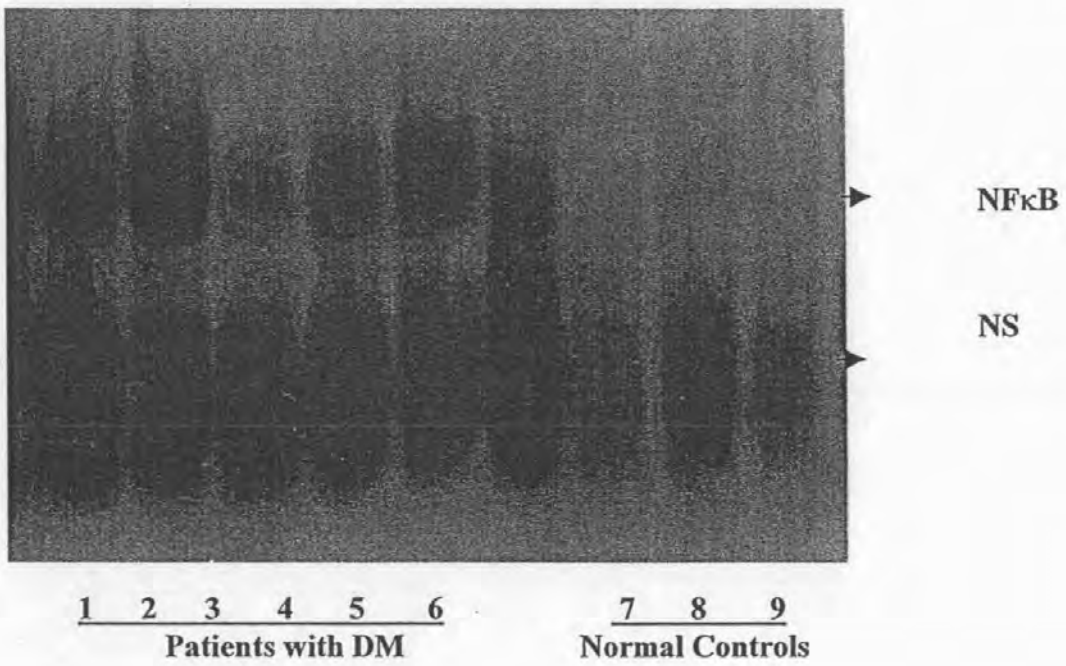

Figure 2: High glucose-induced increase in NFκB-DNA binding activity is inhibited by an aldose reductase inhibitor

Lanes 1, 4 and 7 represent PBMCs from 3 separate subjects grown under normal glucose conditions. Lanes 2, 5 and 8 represent the PBMCs grown under high glucose conditions and 3, 6 and 9 represent the PBMCs grown under high glucose and 10 μM of zopolrestat. Lanes 1-6 for two patients with diabetes and lanes 7-9 correspond to a normal control. NS= non-specific band.

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ABSTRACT BOOK

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798-P

Aldose Reductase Inhibition Reduces the High Glucose Induced Increase in Nuclear Factor Kappa B Binding Activity in Cells from Patients with Diabetes Mellitus

DOHA HEGAZY, BINGMEI YANG, PETER OATES, BEVERLEY MILLWARD, ANDREW DEMAINE. *Plymouth, Devon, United Kingdom; Groton, CT*

Long term exposure to diabetes is associated with metabolic abnormalities such as redox imbalance and oxidative stress that have been linked to the development of microvascular and macrovascular complications. Previous studies have shown that the redox-sensitive transcription factor, nuclear factor kappa B (NFκB) is activated in patients with microvascular complications. We investigated the NFκB binding activity in peripheral blood mononuclear cells (PBMC) of 67 caucasoid patients with diabetes (45 with type 1 and 22 with type 2 diabetes mellitus, T1DM and T2DM) and 10 normal healthy controls (NC) by gel shift assays. PBMC were exposed to high glucose (31mM D-glucose, HG) for 5 days. The binding activity was more than two-fold higher in PBMC exposed to HG compared to basal glucose (11mm D-glucose, BG). The increase was more pronounced in diabetic patients compared with NC (2.1± 0.9 vs. 1.6± 0.4, p=0.01). In addition, T1DM patients with microvascular disease had a significant increase in the binding activity compared to those with no complications after 20 years of the disease (2.2± 0.8 vs 1.9± 0.6, p=0.04). When the PBMC were cultured in the presence of 10μM of the aldose reductase inhibitor zopolrestat or, sorbinil there was a highly significant reduction in NFκB binding activity (0.1 -1.0 fold, BG vs.HG, p=0.000006). The reduction was more pronounced in patients compared to NC (0.4± 0.2 vs. 0.7± 0.3, p=0.04). In conclusion, these results suggest that inhibition of the polyol pathway can influence high glucose-induced NFκB activation. The underlying mechanisms for this response are largely unknown but suggest that flux through the polyol pathway and consequent redox changes play a key role in the activation of NFκB.

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ABSTRACT BOOK

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1004-P

Nuclear Factor κB Polymorphisms and Susceptibility to Type 1 Diabetes

DOHA M. HEGAZY, BINGMEI YANG, ANDREA D. HODGKINSON, ANN MILLWARD, ANDREW G. DEMAINE. *Plymouth, Devon, United Kingdom*

Nuclear factor kappa B (NFκB) is a member of the rel-family proteins and is an important transcription factor that is involved in the response to oxidative stress and inflammation. Target genes include those for cytokines as well as HLA molecules. Recent studies suggest that it may be involved in the development of diabetic microvascular complications. A highly polymorphic (CA) dinucleotide repeat microsatellite has been identified in the 5′-upstream region of the NFκB gene. The aim was to investigate whether this polymorphic region was associated with susceptibility to type 1 diabetes or, its late complications. Genomic DNA was extracted from the peripheral blood of 217 Caucasoid patients with type 1 diabetes and 111 normal healthy controls. Polymerase chain reaction was used to amplify the 5′promoter region containing the repeat region. Amplified fragments were separated by gel electrophoresis and visualised using image analysis. 18 alleles (A1-A18) were identified. There was a highly significant decrease in the frequency of allele A14 (146 base pair) in the patients compared to the normal controls (0.03 vs 0.28 respectively, $\chi2=79.8$, $p<0.000001$). In contrast, the frequency of the A10 allele was significantly increased in the patients compared to the normal controls (0.17 vs 0.02, $\chi2=32.8$, $p<0.00001$). More than 70 genotypes were identified. A8/A14 was the most common genotype in the normal controls but was found in only 1/217 patients (12.6% vs 0.5% $\chi2=22.1$ $p<0.000002$) Similarly, the A14/A14 genotype was present in 9.9% of the normal controls but was absent from the patients ($\chi2=19.3$, $p<0.00001$). There was no association with microvascular complications, age at onset or gender of the patient. In conclusion, these results demonstrate that the NFκB gene may play a role in the susceptibility to type 1 diabetes.

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NFκB Polymorphisms and susceptibility to type 1 diabetes

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Nuclear factor kappa B (NFκB) is an important transcription factor that is involved in the response to oxidative stress and inflammation. Recent studies suggest that it may be involved in the development of diabetic microvascular complications. A highly polymorphic (CA) dinucleotide repeat microsatellite has been identified in the regulatory region of the NFκB gene. The aim of this study was to investigate whether this polymorphic region was associated with susceptibility to type 1 diabetes, or its late complications. Genomic DNA was extracted from the peripheral blood of 217 patients with type 1 diabetes mellitus (T1DM) and 111 normal healthy controls. In our population 18 alleles (A1-A18) were identified. There was a highly significant decrease in the frequency of allele 146 bp (A14) in type 1 diabetes (0.03) compared with the normal controls (0.28) ($\chi^2 = 79.8$, $Pc = 0.00001$). In contrast, the frequency of the allele 138 bp (A10) was significantly increased in patients with type 1 diabetes (0.17) compared with the normal controls (0.02) ($\chi^2 = 32.8$, $P < 0.00000$). These results demonstrate that the NFκB gene may play a role in the susceptibility to type 1 diabetes: individuals with the A10 allele may be more likely to develop diabetes compared with the A14 allele. Genes and Immunity (2001) **2**, 304–308.

Keywords: *type 1 diabetes; hyperglycaemia; nuclear factor kappa B; diabetic complications*

Introduction

Type 1 diabetes mellitus (T1DM) is caused by the autoimmune destruction of the pancreatic beta (β) cells in the islets of Langerhan. It is well known that genetic as well as environmental factors are implicated in the pathogenesis of this disease. The genes coding for certain major histocompatibility complex (MHC) HLA class II alleles are strongly associated with susceptibility to T1DM in Caucasoid populations.[1–3] The HLA susceptibility alleles make a major contribution to the genetic component of T1DM, however, non-HLA loci including insulin and certain cytokine genes may also play an important to this disease.[4–6]

Nuclear factor kappa B (NFκB) is a member of the rel-family proteins and is an eukaryotic transcription factor that is intricately involved in mediating the immune response.[7–10] The gene coding for NFκB is located on chromosome 4q24 and promotes the expression of over 150 target genes including those coding for cytokines such as tumour necrosis factor-α, and interleukin-1β; chemokines, adhesion molecules as well as increased expression of HLA molecules. It is also apparent that many viruses have NFκB binding sites in their genome thereby inducing increased viral transcription by the cell's own defence system.

Inhibitor-κB proteins (IκB) inhibit NFκB by trapping it in the cytoplasm. Phosphorylation of IκB by kinases allows activation of the NFκB, which translocates into the nucleus and binds DNA motifs.[8] Activation of NFκB and subsequent release of cytokines occurs through a redox-sensitive mechanism[11,12] and oxidative stress is the main inducer for activation of the pathway. NFκB plays a role in protecting cells from apoptosis by upregulating the synthesis of anti-apoptotic gene products.[13] Long-term exposure to hyperglycaemia is an important risk factor for developing diabetic microvascular complications: neuropathy, nephropathy, and retinopathy.[14,15]

Several studies have demonstrated that chronic hyperglycaemia causes metabolic abnormalities such as oxidative stress, hypoxia, advanced glycated end products (AGEs) and changes in the NADH/NAD$^+$ ratio may contribute to the development of these complications.[16–18] The oxidative stress and AGEs produced by hyperglycaemia are an important source for free radical oxygen intermediates (ROIs); these free radicals are responsible for accelerating the vascular complications due to their inflammatory, necrotic and ischaemic effects. Recent studies have suggested that the increased activation of NFκB is associated with the development of diabetic microvascular complications in those patients with either T1DM or T2D.[19–22] It has previously been shown that increased activity of NFκB is present in the peripheral blood mononuclear cells isolated from patients with diabetic nephropathy.[20] Hyperglycaemia has also been shown to activate NFκB in porcine vascular smooth muscle cells.[21] These studies suggested that NFκB might be involved in the pathogenesis of diabetic microvascular complication. The gene coding for aldose reductase (ALR2), the first and rate limiting of the polyol pathway has been also shown to have a NFκB binding site. Recently, a polymorphic dinucleotide (CA) repeat has been identified in close proximity to the coding region of the human NFκB gene[23] at 4q24 (108 111 682–108 111 727

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reverse strand-Human Genome Project (HGP) Working Draft at UCSC Blat search). To our knowledge this polymorphism has not previously been studied in either patients with T1DM or, other autoimmune disease.

The aim of our study was to investigate the (CA) dinucleotide repeat polymorphism in a large group of patients with T1DM with, and without microvascular complications.

Results

In our population, 18 alleles (A1-A18) of the (CA) repeat microsatellite in the regulatory region of the NFκB gene were detected. The size of the alleles ranged from 120 to 154 base pairs (bp) (A1-A18) (Figure 1). The observed and expected frequency of the alleles was similar in both the patient and normal control populations and therefore conformed to the Hardy–Weinberg equation.

The frequency of the alleles in the patients and controls are shown in Table 1. The A8 and A14 alleles were the most prevalent in the normal controls (19.8% and 28.4% respectively). In contrast, in patients with T1DM there was a highly significant decrease in the frequency of both the A8 as well as the A14 allele compared to the normal controls (6.2% *vs* 19.8%, $\chi^2 = 26.75$, $P < 0.0000002$, $Pc = 0.0000034$ and 3.9% *vs* 28.4%, $\chi^2 = 79.80$ $P < 0.000001$, $Pc = 0.000017$ respectively). In patients with T1DM, there was a highly significant increase in the frequency of the A10 (138 bp) allele compared with the normal controls (17.5% *vs* 2.7% respectively, $\chi^2 = 32.8$, $P < 0.000001$, odds ratio =9.4).

More than 70 genotypes were identified in our population. Table 2 shows the frequency of the common genotypes. In the normal control population, the most common genotype was A8/A14 but this was only found in one of the 217 patients with T1DM (12.6% *vs* 0.5%, $\chi^2 = 22.1$, $P < 0.000002$, $Pc = 0.00014$). The second most prevalent genotype in the normal control group was A14/A14 and this was completely absent from the patient group (9.9% *vs* 0.0% $\chi^2 = 19.3$, $P < 0.00001$, $Pc = 0.00007$). Similarly, the A8/A11 genotype was found in 6.3% of the normal controls but was absent from the patient population ($\chi^2 = 11.1$, $P < 0.0008$). However, this difference was not significant after correction of the *P*-value ($Pc = 0.06$). In the patient group, the A9/A10 genotype was the most common genotype and was present in four of the 111 normal control subjects (12.0% *vs* 3.6%, $\chi^2 = 5.2$, $P < 0.02$, Pc = NS). The A10/A11 genotype was found in 9.2% of the patients but was absent from the normal control group $\chi^2 = 9.3$, $P < 0.002$, Pc = NS).

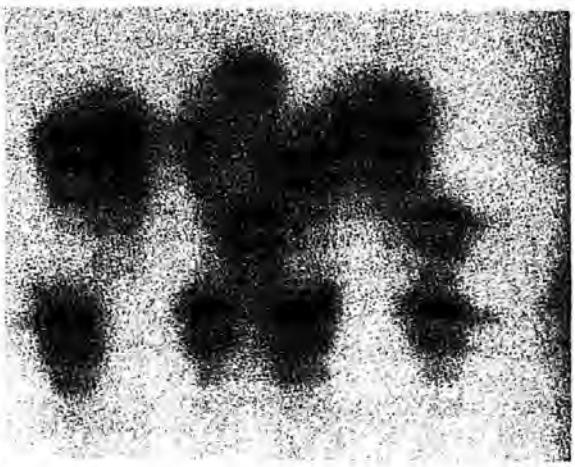

Figure 1 Autoradiograph is showing some of the CA dinucleotide repeat polymorphisms in the human NFκB gene. Lane 1: A8/A14, Lane 2: A13/A14, Lane 3: A8/A8, Lane 4: A11/A11, Lane 5: A8/A13, Lane 6: A14/A14, Lane 7: A13/A14, Lane 8: A8/A11.

Table 1 The frequency (%) of NFκB microsatellite alleles in patients with type 1 diabetes and normal controls

Alelle	*Type 1 diabetes (n = 434)*	*Normal controls (n = 222)*
A1	0.0 (0)	0.45 (1)
A2	1.61 (7)	0.45 (1)
A3	1.15 (5)	0.0 (0)
A4	6.22 (27)	0.0 (0)
A5	4.84 (21)	0.0 (0)
A6	5.76 (25)	0.0 (0)
A7	5.76 (25)	0.45 (1)
A8	6.22 (27)	19.82 (44)*
A9	14.98 (65)	9.91 (22)
A10	17.51 (76)	2.70 (6)**
A11	10.60 (46)	9.91 (22)
A12	7.60 (33)	2.25 (5)
A13	5.30 (23)	11.26 (25)
A14	3.92 (17)	28.38 (63)***
A15	6.68 (29)	7.21 (16)
A16	1.61 (7)	5.41 (12)
A17	0.23 (1)	0.90 (2)
A18	0.0 (0)	0.90 (2)

Note: Allele frequencies are expressed as percentage of the total number of chromosomes. The numbers in parentheses is the number of chromosomes. *A8 $P < 0.0000002$, $Pc < 0.000003$ in the normal controls *vs* patients subgroups. **A10 $P < 0.00000$ in the patients subgroups *vs* normal controls. ***A14 $P < 0.000001$, $Pc = 0.000017$ in normal controls *vs* subgroups of type 1 diabetes.

Table 2 The frequency (%) of NFκB genotypes in patients with type 1 diabetes and normal controls

Genotypes	*Type 1 diabetes (n = 217)*	*Normal controls (n = 111)*
A8/A9	3.23 (7)	4.50 (5)
A8/A11	0.00 (0)	6.31 (7)
A8/A14	0.46 (1)	12.61 (14)
A9/A10	11.98 (26)	3.60 (4)
A9/A15	4.15 (9)	0.90 (1)
A10/A11	9.22 (20)	0.00 (0)*
A14/A14	0.00 (0)	9.91 (11)**
X/X	70.96 (154)	62.16 (69)

The table shows the frequency of genotypes with prevalence <3%. X = all other genotypes. *The A10/A11 genotype is the most common in patients with type 1 diabetes. **The A14/A14 genotype is prevalent with the normal controls.

Table 3 Frequency (%) of the most common NFκB genotypes with respect to age at onset of type 1 diabetes and gender

	Age at onset (years)		
	<10	*10–20*	*>20*
Genotype	(n = 42) M:F	(n = 68) M:F	(n = 107) M:F
A9/A10	4.8 0:2	13.2 4:5	14.0 8:7
A10/A11	16.7 1:6	8.8 2:4	6.5 4:3
A10/X	14.3 2:4	16.2 7:4	12.2 5:8
X/X	64.2 15:12	61.8 20:22	67.3 34:38

The patients were classified into three groups according to the age at onset (AAO) of type 1 diabetes: <10, 10–20, >20 years.

Table 3 shows the frequency of the NFκB genotypes with respect to age at onset of T1DM and gender. The patients were categorised into <10, 10–20 or >20 years age at onset of diabetes. There were no significant differences in the frequency of the genotypes with age at onset although the A9/A10 genotype was found in only 4.8% of those diagnosed before the age of 10 years compared to 13.2% and 14.0% in the 10 to 20-year and >20-year age at onset respectively. There were no significant differences in the frequency of the NFκB genotypes with gender. Table 4 shows the frequency of the common NFκB alleles with respect to the presence or absence of diabetic microvascular disease after 20 years duration of diabetes. Whilst A9 was more common in those with either diabetic microvascular disease compared to those with no complications after 20 years of diabetes, these differences were not significant. All the other frequencies of the alleles were similar between the patient subgroups.

Table 4 Frequency (%) of the most common NFκB alleles with respect to presence or absence of diabetic microvascular complications

Allele	*DC* (n = 76)	*DN* (n = 96)	*DNU* (n = 110)	*DR* (n = 152)
A10	19.8 (15)	17.7 (17)	16.4 (18)	17.1 (26)
A9	9.2 (7)	16.7 (16)	16.4 (18)	15.8 (24)
A11	11.8 (9)	10.4 (10)	9.1 (10)	11.2 (17)
X	59.2 (45)	55.2 (53)	58.1 (64)	55.9 (85)

DC, those patients with no nephropathy, retinopathy or overt neuropathy after 20 years duration of diabetes; DN, those patients with nephropathy; DNU, those patients with overt neuropathy; DR, those patients with retinopathy. There were no significant differences in the frequency of the NFκB alleles between any of the patient groups.

Discussion

To our knowledge, this is the first study of NFκB polymorphisms in either an autoimmune or inflammatory disease. The results suggest that the A10 allele may contribute to the susceptibility of T1DM whilst those individuals with the A14 allele have a reduced risk of developing T1DM. This polymorphism is located in close proximity to the coding region of the NFκB gene. It has previously been shown that polymorphisms in the non-coding regions of cytokine genes can influence expression[24,25] whilst enhancer elements up to 50 kb from the coding region can directly effect expression of transcription factors.[26] Consequently, the results described here could be attributed to differences in the expression of the NFκB gene. At the present time, nothing is known about the influence of this microsatellite on the expression of the NFκB gene, or regulatory sequences that may be located nearby. This may determine the direction and magnitude of the response of a cell to an external stress and whether it is channelled towards either apoptosis or, inflammation and necrosis. There is currently very little information on the mechanisms of islet β-cell death during the pre-diabetic period in man. It is possible that the A10 allele is associated with pro-inflammatory and anti-apoptotic responses whilst conversely, the A14 allele is anti-inflammatory and pro-apoptotic. This might be related to variable amounts of NFκB protein. In the case of the anti-apoptotic response, too much protection of the cell perhaps because of its increased abundance would prevent apoptosis but would cause increased necrosis. This would have a detrimental effect by allowing intracellular proteins and other molecules to be exposed to the immune system thereby provoking the autoimmune response.

Recent studies have shown that increased activation of NFκB is significantly increased in patients with either T1DM or, T2D and micro vascular disease.[19–22] In this study we have found no association with the NFκB allele or genotypes with either the presence or absence of diabetic microvascular complications in those patients with T1DM. It is possible that an association may exist with microvascular disease in those patients with T2DM. There is no information at the present time on the functional role of the A10, A14 or other polymorphisms in the regulatory region of this gene. It is possible that these polymorphisms may be related to function perhaps by altering the efficiency of its transcription under different types of stress. Activation of NFκB occurs at the protein rather than the genetic level although prolonged chronic exposure to stress such as hyperglycaemia would lead to increase abundance of the transcription factor within the cell. IκB may also play an important role in enabling the translocation of the transcription factor to the nucleus.

In conclusion, we have a strong association between a polymorphism in close proximity to the NFκB gene and susceptibility to T1DM but not with the long-term micro vascular complications of this disease. Previous studies have found no evidence for linkage of 4q24 with susceptibility to T1DM. However, these genome screens used highly selective multiplex families whilst in this study the majority of cases are sporadic cases. Further, the multiplex family studies were not always able to replicate the association of non-MHC genes with susceptibility to T1DM across or even within similar populations. It

Table 5 Clinical characteristics of the patient subgroups

Patient subgroup	*DC (n = 38)*	*DN (n = 48)*	*DNU (n = 55)*	*DR (n = 76)*	*NC (n = 111)*
Male:Female	14:24	22:26	26:29	37:39	58:53
Age at onset of diabetes (years)	17.8 ± 10.7	16.4 ± 10.4	19.1 ± 11.3	18.1 ± 10.5	
Duration of disease (years)	29.2 ± 10.5	34.5 ± 11.5	31.5 ± 10.8	32.1 ± 11.7	

DC, those patients with no microvascular complications after 20 years duration of diabetes; DN, those patients with diabetic nephropathy; DNU, those patients with overt diabetic neuropathy; DR, those patients with diabetic retinopathy; NC, normal healthy controls; data are shown as mean ± s.d.

remains a possibility that the polymorphism is related to the presence or absence of microvascular complications rather than diabetes itself. Further studies are now required to determine whether there is any relationship between the NFκB alleles and function.

Materials and methods

Subjects

Two hundred and seventeen British Caucasoid patients with T1DM with or without complications and 111 normal healthy controls (NC) were studied. All the patients had attended the Diabetic Clinic, Derriford Hospital, Plymouth, UK. The patients were classified according to their microvascular complications as follows:

Uncomplicated diabetic controls (DC n = 38): these patients have had T1DM for at least 20 years but remain free of retinopathy, microalbuminuria (defined by the Diabetes Control and Complications Trial as 15–30 mg/l), proteinuria, and overt neuropathy.

Nephropathy patients (DN n = 48): these patients have had T1DM for at least 10 years with persistent proteinuria (urine Albustix positive on at least three consecutive occasions over 12 months or three successive total urinary albumin excretion (UAE) rates more than 0.5 g/24 h) in the absence of haematuria or infection on midstream urine samples. The presence of nephropathy was always associated diabetic retinopathy.

Retinopathy patients (DR n = 76): these patients had retinopathy defined as more than five dots or blots per eye, hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy and maculopathy or vitrous haemorrhage. None of these patients had microalbuminuria or protienuria.

Neuropathy patients (DNU n = 55): these patients had overt neuropathy defined as clinical evidence of peripheral or autonomic neuropathy. The criteria for inclusion in this category have previously been published.[27]

Normal control samples were consisted of 80 cord bloods collected sequentially after normal obstetric delivery from the labour ward, Derriford Hospital. The remaining samples were obtained from normal healthy schoolchildren (n = 15) and adults (n = 16). The clinical characteristics of the patients and control populations are shown in Table 5.

Extraction of genomic DNA and analysis of the microsatellite polymorphism

DNA was extracted from peripheral blood collected into EDTA-containing tubes using Nucleon II DNA extraction kit (Tepnel Life Science PLC, Manchester, UK) following the manufacturer's instructions. Briefly, the samples were lysed and deproteinised before extraction with chloroform. Finally, DNA was isolated by ethanol precipitation and dissolved in 500 μl of double distilled water.

The (CA)n region was amplified by polymerase chain reaction (PCR) using primers[23] (NF(B 1 1F) 5′-CTTCAG TATCTAAGAGTATCCT-3′ (108 111 762 4q24 forward HGP-UCSC) and (NFκB 1 1R) 5′-CAAGTAAGAC TCTACGGAGTC-3′ (108 111 625 4q24 reverse HGP-UCSC). The forward primer was labelled with [^{32}Pγ] dATP by T4 polynucleotide kinase (Amersham Pharmacia, UK Limited). Fifty to 100 ng of genomic DNA was used for amplification in a volume of 30 μl with a hot start for 4 min at 94°C. Then 30 cycles were performed at 94°C for 30 sec, at 53.5°C for 2 min, at 72°C for 30 sec. The resulting fragments and a molecular weight ladder were electrophoresed through 6% denaturing polyacrylamide gels at 1800 W for 3 h. The dried gels were autoradiographed at −80°C for 18 h. The films were visualised for allelic and genotypic identifications.

Statistical analysis

The frequencies of alleles and genotypes were calculated by Statgraphics software (Statistical Graphics Corp, USA, 1996). The comparison between patient's subgroups and control subjects was made by 2 × 2 contingency tables using the χ^2 test. The P values were corrected for the number of comparisons made (Pc) using the Bonferroni inequality method,[28] and Pc values of <0.05 were considered to be significant.

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