

The effects of the adipocyte-secreted proteins resistin and visfatin on the pancreatic beta-cell

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**A thesis submitted in partial fulfilment of the requirements of
the University of Wolverhampton for degree of
Doctor of Philosophy.**

July 2009

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Abstract

Adipose tissue secreted proteins (adipokines) have been proposed to form a link between obesity and type 2 diabetes (T2D). Resistin and visfatin are two adipokines which have been previously suggested as having roles in the pancreatic islet. The aim of this study was therefore to investigate the regulatory role of the adipokines resistin and visfatin in the pancreatic beta-cell. In order to do this, pancreatic β -cell lines from rat (BRIN-BD11) and mouse (β TC-6) were used to study the effect of exogenous incubation with physiological and pathological concentrations of resistin and visfatin on diverse elements of beta-cell biology including cell viability, gene expression and insulin secretion. In addition to this the expression levels of these two adipokines was also measured in the beta-cell. PCR array analysis showed that resistin and visfatin treatment resulted in significant changes in the expression of key beta-cell specific genes. Interestingly, both resistin and visfatin are highly expressed in the beta-cells. This suggests that the roles of these adipokines are not confined to adipose tissue but also in other endocrine organs. Resistin treatment significantly increased viability of the beta-cells at physiological concentrations however there was no increase with the elevated pathological concentrations. Resistin at elevated concentrations decreased insulin receptor expression in the beta-cells however there was no significant effect at lower concentrations. Both physiological and elevated resistin concentrations did not have any effect on glucose stimulated insulin secretion. Incubation of visfatin induced phosphorylation of insulin receptor and the intracellular signalling MAPK, ERK1/2. Visfatin treatment at 200ng/ml also significantly increased insulin secretion. These effects were replicated by incubation of beta-cells with the product of visfatin's enzymatic action, nicotinamide mononucleotide and were reversed by visfatin inhibitor FK866. Visfatin treatment at low concentrations did not have any effect on cell viability however the elevated concentrations resulted in a decline. These data indicate that both resistin and visfatin potentially play important roles in beta-cell function and viability and that they form a significant link between adipose tissue and the pancreatic islet in type 2 diabetes.

Acknowledgements and dedication

Firstly, I would like to thank my supervisory team, Dr Simon Dunmore, Dr James Brown and Dr James Vickers for their invaluable advice, patience, support and guidance over the last couple of years. I would also like to thank Professor Chimay Anumba (Pennsylvania State University, USA), Professor Sergey Mikhalovsky and Dr Louba Mikhalovska (University of Brighton, UK) who have supported me and facilitated all that I ever needed to succeed. I am also indebted to Dr Jim Wicks and PrimerDesign UK Ltd for the training and financial assistance towards the Real-time RT-PCR assays.

I would like to thank my colleagues Donna Brown, Elisabeth Goessl, Harpreet Dibra, Mary Holton, Xiaoxia Guo, Dr Angel Armesilla, Dr Sarah Brown, Dr Sarah Jones, and Dr Weiguang Wang for their input, help, critique, assistance with several aspects of this work and above all friendship. Additional thanks go to Dr Angela Williams and Keith Holding for their invaluable technical support.

I would also like to thank my friends Carol and Paul Erfemeijer, Christine Audi, Claire Anumba, Edwina and Abok Ager, Elizabeth Mutsoli, Ezekiel and Rachel Chinyio, Jagjiwan Khela, Joyce Adokwu, Joseph Moturi, Marie Tsado, Mereza Ogango, Pascal Kaudu, Peter Muriu and Tokunbo Ogundana for their motivation and friendship especially during the last three years.

Lastly but not least I want to thank my family; my mum and dad, my uncle Apollo, Francis and Joel, my aunt Roselyn, Ruth and Sarah; and my siblings for their love and support.

Finally, I would like to thank two special friends Don and Jocelyn Blair who have been incredible and tremendously great in their support and love. It is to you two that I dedicate this work

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Abbreviations

AC	Adenylate cyclase
ADP	Adenosine 5'-diphosphate
Akt/PKB	Akt/protein kinase B
AMV	Avian Myeloblastosis Virus Reverse transcriptase
ATP	Adenosine 5'-triphosphate
BAT	Brown adipose tissue
BSA	Bovine Serum Albumin
BRIN-BD11	Electrofusion cell line of RINm5F cells with New England Deaconess Hospital rat pancreatic islet cells
HBSS	Hanks Balanced salt solution
Ca^{2+}	Calcium ions
$[Ca^{2+}]_i$	Intracellular calcium
cDNA	Complementary DNA
Cl^-	Chloride
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocytes
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal related protein kinase
FCS	Foetal calf serum
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GSK3	Glycogen synthase kinase 3
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IgE	Imunoglobulin E
IL-1 β	Interleukin-1beta
IL-6	Interleukin-6
IL-8	Interleukin-8
JNK	Jun kinase
K _{ATP}	ATP-activated K ⁺ (K _{ATP}) channels
MAPK	Mitogen Activated Protein Kinase
mRNA	Messenger RNA
NaCl	Sodium Chloride
NF- κ B	Nuclear factor-kappa B
NMN	Nicotinamide mononucleotide
p38-MAPK	p38 isoform of Mitogen-activated protein kinase
PBS	Phosphate buffered saline
PI3K	Phosphotidylinositol 3-kinase (PI3-kinase)
PIP2	Phosphatidyl inositol bisphosphate (PIP2)
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
SH3	Src homology 3
SMCs	Smooth muscle cells
TBS-T	Tris-Buffered Saline Tween-20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TMB	3, 3', 5, 5' – Tetramethyl Benzidine
TNF- α	Tumor necrosis factor-alpha
WAT	White adipose tissue

Chapter 1

Introduction

1.1. Diabetes: pathology and epidemiology

Diabetes mellitus (commonly referred to as diabetes) describes a group of metabolic diseases of multiple aetiology characterized by high blood glucose levels resulting from defects in insulin secretion, action or both. The most easily recognized symptoms of diabetes are thirst, polyuria, blurring of vision, hyperglycaemia, glycosuria, and ketoacidosis (Mayfield, 1998).

The two common forms of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D) which are both caused by a combination of genetic and environmental risk factors. T1D is an autoimmune disease resulting from selective destruction of insulin-producing pancreatic β -cells by autoreactive T helper 1 (Th1) cells and their mediators and is often characterised by absolute insulin deficiency (Atkinson and Maclaren, 1994). The process leading to T1D appears to start when the immune system recognizes and attacks proteins on the surface of the β -cells, possibly mistaking them for antigenic matter belonging to an invading organism. The immune response results in the T-lymphocytes flooding into the pancreatic islets and causing an inflammation known as insulinitis. Once enough β -cells have been destroyed, the symptoms of diabetes begin to appear.

Patients suffering from T1D have to inject insulin to compensate for the absolute insulin deficiency caused by this aberrant autoimmune response. Currently available therapies do not closely mimic the normal function of the β -cell, which precisely adjusts the rate of insulin secretion in response to biological needs. Type 1 diabetics thus have to constantly monitor their glucose levels and inject insulin whenever required. Lack of proper control can result in either hypoglycaemia which can cause a coma or hyperglycaemia which increases the risk of developing diabetic complications such as blindness, kidney failure and amputations (Cowie *et al.*, 1989; Icks *et al.*, 1997).

In comparison, T2D is a more complex metabolic disorder characterized by obesity, impaired β -cell function, increased endogenous hepatic glucose output and insulin resistance in target tissues (Leahy, 2005). T2D is the most common metabolic disease

worldwide (Zimmet *et al.*, 2001). The incidence of T2D is increasing worldwide primarily due to increases in the prevalence of a sedentary lifestyle, consumption of high-calorie diets and obesity (Tuomilehto *et al.*, 2001). Between 2000 and 2010 the prevalence of T2D is projected to rise by 47% to over 215 million affected worldwide (Amos *et al.*, 1997). Furthermore, the problem is no longer restricted to the ageing population with increasing number of children and young adults developing diabetes (Fagot-Campagna *et al.*, 2001). The Asian / Pacific region is projected to have the largest population of people diagnosed with diabetes in the world accounting for 46 percent of the global burden of diabetes (Coughlan *et al.*, 1997).

About 90% of cases of T2D are associated with obesity. In relation to this, approximately 197 million people worldwide are thought to have impaired glucose tolerance (IGT), most commonly due to obesity and the associated metabolic syndrome (Dunstan *et al.*, 2002). This number is expected to increase significantly to 420 million by 2025. (Hossain *et al.*, 2007). Various measures of being overweight, such as relative weight, body mass index, and body fat distribution, have repeatedly been shown to be risk factors for T2D (Colditz *et al.*, 1995). Not only does this trend have significant implications for the individuals concerned in terms of developing complications associated with the disease, but there are also significant health cost for society and governments at large. Lifestyle changes, while desirable, have proven difficult to achieve. Thus, a better understanding of the molecular mechanisms underlying insulin resistance will be required to combat the epidemics of T2D and cardiovascular disease that are fuelled by obesity-associated insulin resistance.

Although T2D is thought of as a polygenic disease, most of the progress in treatment to date has been in identifying genes that cause several uncommon forms of diabetes with monogenic inheritance. The most common form of monogenic diabetes is maturity-onset diabetes of the young (MODY) syndrome, causing autosomal dominant non-insulin dependent diabetes appearing before the age of 25 years and diabetes due to mutations in mitochondrial DNA (Vaxillaire *et al.*, 2006). Neonatal diabetes mellitus (NDM) is another form of monogenic diabetes, usually defined as overt diabetes diagnosed during the first 6 months of life (Hamilton-Shield, 2007).

In humans, mutations in specific genes lead to the various forms of MODY. Mutations of the glucokinase (GCK) gene lead to the manifestation of MODY-2 (Hattersley *et al.*, 1992), with the remaining five types of MODY resulting from defects in genes encoding for transcription factors: Hnf-4 α (MODY-1), Hnf-1 α (MODY-3), Pdx-1 (MODY-4), Hnf-1 β (MODY-5), and NeuroD1 (MODY-6) (Yamagata *et al.*, 1996; Horikawa *et al.*, 1997; Stoffers *et al.*, 1997). Many novel mutations found in the MODY genes are constantly being reported in different population groups. Despite an impressive number of landmark achievements and breakthroughs in diabetic research, many questions about the causes of diabetes and its chronic complications remain unanswered.

Previous studies have also implicated cytokines as important mediators in the destruction of pancreatic beta-cells and impaired function leading to both T1D and T2D (Arnush *et al.*, 1998, Oshima *et al.*, 2006). The proinflammatory cytokines interleukin (IL)-1 β , either alone or in combination with tumour necrosis factor-alpha (TNF- α) or interferon-gamma (IFN)- γ have been shown to elicit dramatic negative effects on pancreatic beta (β) cells in vitro (Alba *et al.*, 2004; Ou *et al.*, 2002).

1.2. Factors influencing the prevalence of T2D

It is now well established that T2D results from the interaction between a genetic predisposition and behavioural and environmental risk factors (Gerich, 1998). Previous studies have shown that first degree relatives of individuals with T2D are about 3 times more likely to develop the disease than individuals without a positive family history of the disease (Flores *et al.*, 2003; Gloyn *et al.*, 2003, Hansen, 2003). This together with other studies provide compelling evidence that genetic factors make a major contribution to the development of T2D and helps to illustrate that the disease is in part inherited. Environmental factors, especially diet, physical activity, and age, interact with genetic predisposition to affect disease prevalence. However despite their major role, the genetic causes of the most common forms of T2D remain elusive and the genetic basis of the disease has yet to be identified.

The growing prevalence of T2D and cardiovascular disease in Western societies is known to be linked to excess adiposity (Kahn *et al.*, 2000, Porte *et al.*, 1998) with the past decade witnessing a parallel rise in the incidence of obesity and T2D among both children and adults (Flegal *et al.*, 2002). Evidence from several studies indicates that obesity and weight gain are linked to an increased risk of T2D (Ford *et al.*, 1997, Resnick *et al.*, 2000) and intentional weight loss associated with a reduction in the risk and rate of developing T2D (Will *et al.*, 2002). Perhaps the most sobering observation regarding the obesity epidemic is the fact that obesity generally confers a significantly increased rate of mortality when compared with individuals of normal body weight (Jazet *et al.*, 2003).

It has now been demonstrated that T2D can be prevented, or at least delayed, by both pharmacological and non-pharmacological interventions, such as lifestyle modification including diet, exercise and therapeutic agents including metformin and pioglitazone (Pax *et al.*, 1997, Tuomilehto *et al.*, 2001, Knowler *et al.*, 2002, Chiasson *et al.*, 2002, Buchanan *et al.*, 2000). Metformin is a mild insulin-sensitizing agent which improves hyperglycemia primarily through its suppression of hepatic glucose production via AMPK activation (Kirpichnikov *et al.*, 2002) while pioglitazone works by activating peroxisome proliferator-activated receptors-gamma (PPAR- γ) (Krentz AJ and Friedmann PS, 2006). Diet has also long been believed to be an important risk factor for T2D. Some randomised trials have consistently shown, increased physical activity and weight loss are efficient approaches for the control and prevention of T2D (Alberti *et al.*, 2007, Hu *et al.*, 2001).

1.3. The beta cell function: insulin secretion, signalling and glucose homeostasis

1.3.1. Beta cell biology

Pancreatic islet β -cells function mainly to secrete insulin in response to changes in circulating levels of glucose and amino acids in the blood (Weyer *et al.*, 1999). Normal glucose homeostasis requires the finely tuned orchestration of insulin secretion by pancreatic β cells in response to subtle changes in blood glucose levels. Glucose tolerance is determined by the balance of insulin secretion and insulin action (Lillioja *et al.*, 1988). Any alteration in β -cell function has profound impact on glucose homeostasis with excessive secretion of insulin liable to cause hypoglycaemia, and insufficient insulin secretion ordinarily resulting in hyperglycaemia.

Insulin is a small protein hormone, with a molecular weight of about 6000 Daltons composed of two chains held together by disulfide bonds and synthesized in significant quantities only in beta-cells in the pancreas. Since the discovery of the insulin gene sequence (Bell *et al.*, 1980), several patients with insulin gene mutations have been identified. Some of the mutations have been shown to affect insulin processing by inhibiting conversion of proinsulin to insulin (Chan *et al.*, 1987; Warren-Perry *et al.*, 1997) while others produce normally processed insulin with subnormal biological activity e.g. reduced affinity of insulin for its receptor (Nanjo *et al.*, 1986).

1.3.2. Insulin secretion

Insulin is secreted primarily in response to elevated blood glucose concentrations after which it causes reduction of hepatic glucose output (via decreased gluconeogenesis and glycogenolysis) and increases the rate of glucose uptake, primarily into striated muscle and adipose tissue (Hedeskov, 1980; Straub *et al.*, 2002). Glucokinase is the key rate-limiting enzyme in glucose metabolism in β -cells and has been convincingly shown to be the glucose sensor for glucose-stimulated insulin secretion (Matschinsky

et al., 1993). In addition to glycaemic control, insulin is a pleiotropic hormone that also influences all aspects of carbohydrate, protein, and lipid metabolism where it exerts its effects by increasing lipid synthesis in liver and fat cells, and attenuating fatty acid release from triglycerides in fat and muscle (Vernon *et al.*, 1991; Kersten, 2001; Hansmannel *et al.*, 2006). Because muscle lacks glucose 6-phosphatase, glycogenolysis in muscle does not provide glucose for release into the blood (Bresolin *et al.*, 1989). However, when insulin levels are low, muscle exports amino acids and other substrates to the liver for gluconeogenesis.

Glucose stimulates insulin secretion by generating, triggering and amplifying signals in the beta-cells (Henquin, 2000). Glucose-stimulated biphasic insulin secretion involves at least two signalling pathways: the K_{ATP} channel-dependent (Thams *et al.*, 2005) and K_{ATP} channel-independent pathways (Westerlund *et al.*, 2001). In the first instance, insulin secretion is induced by glucose entry into β -cells, and its metabolism leads to the generation of metabolic coupling factors including ATP (Doliba *et al.*, 2007). Enhanced glucose metabolism increases the cellular adenosine triphosphate / adenosine diphosphate (ATP/ADP) ratio. This induces closure of ATP-dependent potassium ion channels and membrane depolarization which, in turn, leads to activation and opening of voltage-dependant calcium ion (Ca^{2+}) channels. This resultant increase in Ca^{2+} entry and intracellular Ca^{2+} as well as the increase in ATP induces the movement of insulin-containing large dense core vesicles to the plasma membrane, their docking and their fusion to release the peptide hormone. The K_{ATP} channel-independent pathways augment the response to increased intracellular Ca^{2+} by mechanisms that are currently unknown.

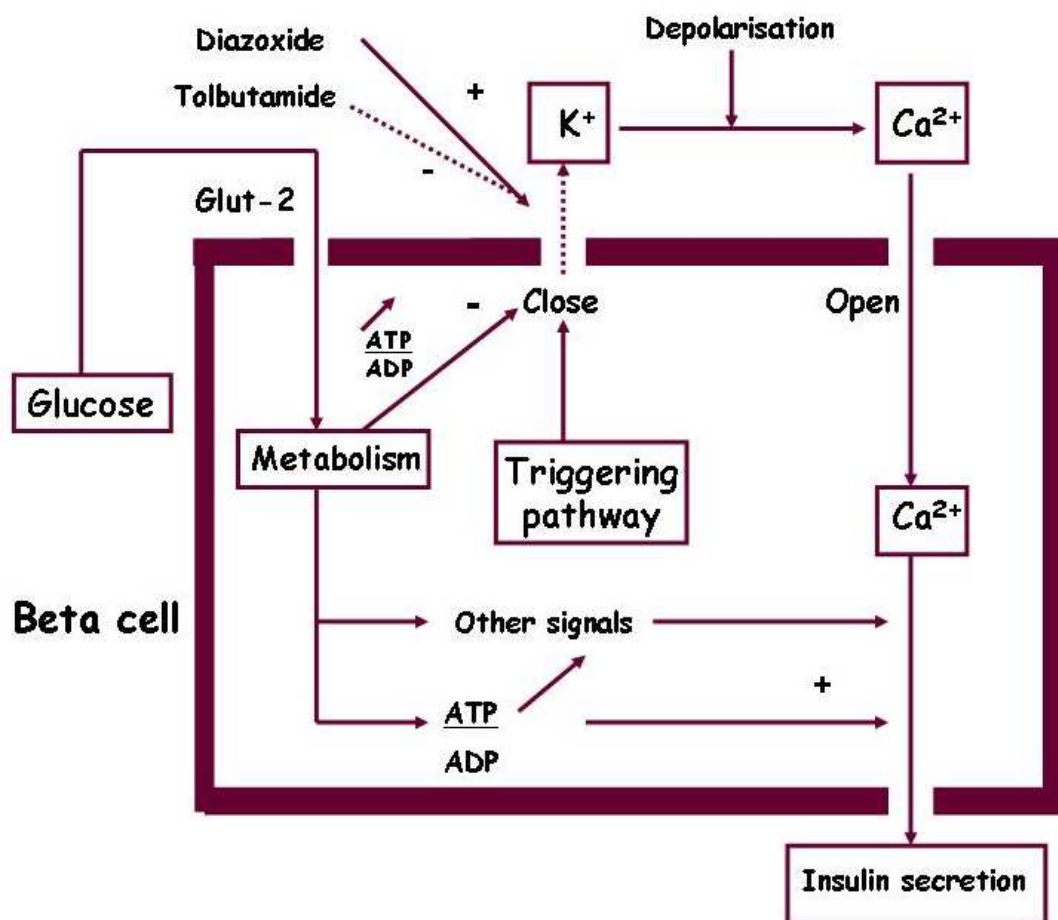


Figure 1.0 Schematic representation of glucose stimulated insulin secretion in the pancreatic β cells. Glucose enters the β -cells by GLUT-2 glucose transporter and the intracellular glucose is subsequently metabolized to produce ATP. Elevation of ATP/ADP ratio induces closure of cell surface K^+ channels resulting in depolarization and opening of voltage-gated calcium channels. This facilitates extracellular Ca^{2+} influx into the β -cell. A rise in free cytosolic Ca^{2+} triggers the exocytosis of insulin. **Key:** + = stimulation; - = inhibition; ADP = adenosine diphosphate; ATP = adenosine triphosphate; Glut-2 = glucose transporter 2
Figure adapted from Henquin, 2000.

The cells work in a feedback loop in maintaining a narrow range of blood glucose levels. In the first instance; as previously illustrated, hyperglycaemia signals the β -cells to produce insulin and suppress glucagon, which switches off glucose production from the liver and increases glucose uptake in muscle, fat, and liver. This results in

normoglycaemia. Conversely, hypoglycaemia signals the β -cells to decrease insulin secretion and increase glucagon, which stimulates glucose production from the liver. In non-diabetics where β -cell function is intact, the rate of hepatic glucose production equals the rate of glucose disposal, however in the presence of β -cell dysfunction resulting in inadequate insulin secretion, hepatic glucose production exceeds glucose disposal, and fasting hyperglycaemia ensues (Porte, 1991)

1.3.3 Insulin signalling and transduction pathways

Insulin rapidly elicits its biological response by inhibiting hepatic glucose production and stimulating peripheral glucose utilization. Various signalling pathways and feedback mechanisms are involved in regulating β -cell function and insulin's ability to maintain whole-body glucose homeostasis. Insulin action is initiated at the point when it binds to the extracellular domain of the insulin receptor resulting in a cascade of signalling events which culminate in translocation of the major insulin responsive glucose transporter 4 (GLUT-4) from intracellular vesicles to the plasma membrane (Olefsky *et al.* 1990). The critical function of the insulin receptor in glucose homeostasis has been confirmed in insulin-receptor knockout mice which have a defect in glucose sensing and die shortly after birth with severe hyperglycaemia, ketosis and reduced beta cell mass, despite hyperinsulinemia (Kulkarni *et al.* 1999). In addition to this, individuals have been identified with rare genetic defects in the insulin receptor that influence expression, insulin binding, and tyrosine kinase activity which result in severe insulin resistance (Krook *et al.*, 1996; Taylor *et al.*, 1998)

The insulin receptor is a heterotetrameric transmembrane protein composed of two extracellular α subunits and two β subunits linked together by disulphide bonds and a member of the tyrosine kinase receptor family. The insulin receptor is expressed in almost all mammalian tissues, with the highest concentration found in target tissues of insulin: skeletal muscle, adipose tissue and liver (Cheatham *et al.*, 1995). Skeletal muscle is the largest tissue by mass regulated by insulin, and is responsible for over 80% of insulin-stimulated glucose disposal (Zurlo *et al.*, 1990; Zierath *et al.*, 2000).

Insulin binds to the extracellular α subunits of the receptor, transmitting a signal across the plasma membrane. The α -subunits contain the insulin binding sites whereas the β -subunits are transmembrane proteins containing the tyrosine kinase domains.

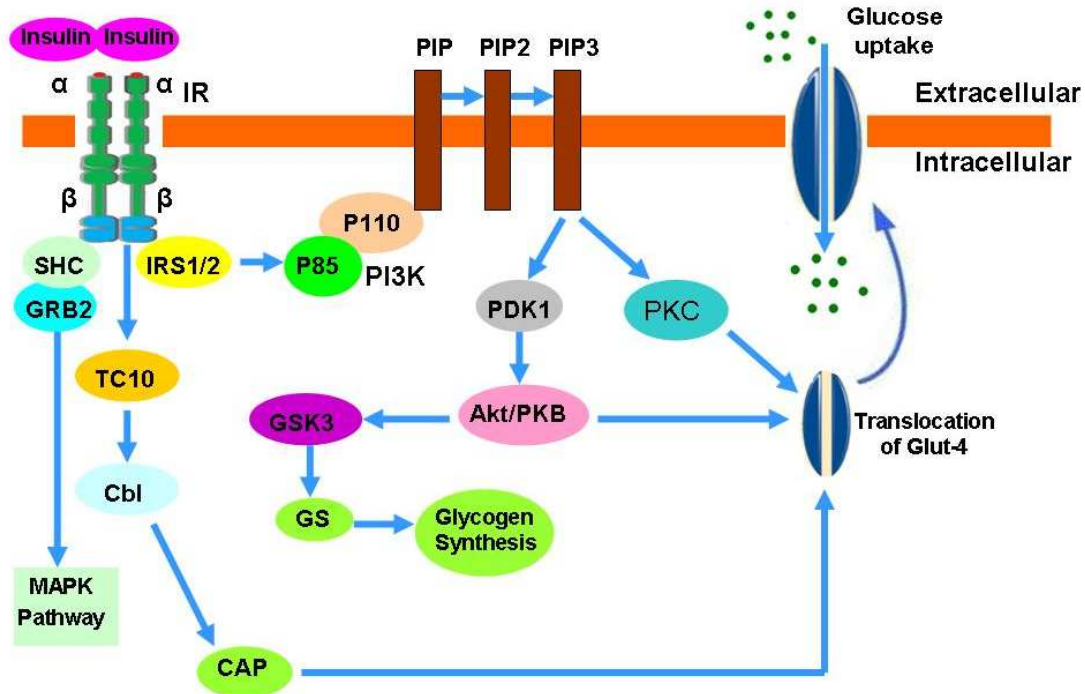


Figure 1.1 The insulin signalling system responsible for the action of insulin in glucose homeostasis. This is achieved through binding of insulin to its receptor and subsequent interaction of the insulin receptor with IRS-1/2 and other substrate molecules. This leads to eventual GLUT-4 translocation and glucose uptake into the cells. **Key:** Cbl=c-Cbl proto-oncogene, GS=glycogen synthase, Glut-4=glucose transporter-4, GRB2= growth factor receptor bound protein 2, IR=insulin receptor, IRS=insulin receptor substrate, PI3K=phosphatidylinositol 3-kinase, PDK= PIP₃-dependent kinase, PIP= phosphatidylinositol triphosphate, PKC=protein kinase C, SH2= Src homology 2

The critical initial steps in insulin signalling include phosphorylation of scaffolding proteins and activation of phosphatidylinositol 3-kinase (PI3). PI3 kinase forms a complex with various growth factor receptors hence it has been implicated in the signalling mechanism of growth receptors that act through tyrosine kinase. The

presence of PI3 kinase in non-proliferating cells such as platelets and neutrophils suggests it has an additional role (Traynor-Kaplan *et al.*, 1989; Kucera and Rittenhouse, 1990; Parker and Waterfield, 1992).

The tyrosine kinase domain of the insulin receptor located on the cytoplasmic portion of the β -subunit once activated undergoes conformational change resulting in rapid auto-phosphorylation and docking of insulin receptor substrate (IRS) proteins such as IRS-1, IRS-2, IRS-3 and IRS-4 (Kaburagi *et al.*, 1999). Other substrates include growth factor receptor bound protein 2 (Grb-2) - associated binder-1 (Gab1), p60^{dok}, the c-Cbl proto-oncogene (Cbl), adaptor protein with pleckstrin homology (PH) and Src homology 2 (SH2) domains (APS) and 3 isoforms of SH2 domain-containing alpha-2 collagen-related protein (Shc) (Virkamäki *et al.*, 1999). Tyrosine phosphorylation of these proteins creates recognition sites for both Src homology (SH2) binding and phosphotyrosine binding (PTB) domains of several downstream effector proteins.

Tyrosine phosphorylation of IRS-1 leads to recruitment and activation of phosphatidylinositol-3-kinase (PI3K) through the SH2 domain of the p85 subunit of PI3 kinase (Baker *et al.*, 1992). This then generates intracellular lipids such as phosphatidylinositol triphosphate PI(3,4,5)P₃, that activate PIP₃-dependent kinase (PDK) that, in turn, phosphorylate and activate Akt / protein kinase B (PKB), which is necessary for stimulation of glucose transporter molecules. Akt is a proto-oncogene encoding a serine-threonine kinase (also known as PKB or RAC-PK) (Bos, 1995). Activated Akt phosphorylates and regulates components of the glucose transporter 4 (GLUT-4) complex, protein kinase C (PKC) isoforms, and GSK3, all of which are critical in insulin-mediated metabolic effects (Czech *et al.*, 1999, Ueki *et al.*, 1998). The best understood mechanism of IRS-protein-mediated signalling is the binding of SH2 domain-containing signaling molecule PI3-kinase by tyrosine phosphorylation sites on IRS proteins.

The ability of insulin to recruit the insulin-responsive glucose transporter GLUT-4 from an intracellular compartment to the cell surface in muscle and adipose tissue accounts for the majority of insulin's effect to stimulate glucose uptake in the body (Kainulainen *et al.*, 1994; Dombrowski *et al.*, 1998). GLUT-4 proteins are contained

in intracellular vesicles which are predominantly localized to a perinuclear compartment in the basal state. The glucose transporter proteins catalyses the rate-limiting step for glucose uptake and metabolism in skeletal muscle and other insulin target tissues. After insulin stimulation, the GLUT-4 containing vesicles are translocated to the plasma membrane (Pessin *et al.*, 1999). As circulating insulin levels decline, the GLUT-4 transporters return to their interior vesicles. Key to terminating the signal cascade is Protein tyrosine phosphatases (PTPs). These proteins have a prominent role in the control of insulin receptor (IR) signalling in that they dephosphorylate the IR and its substrates and thus serve to inactivate the IR and terminate signalling (Chen *et al.*, 1997).

1.4. Beta cell failure and T2D

T2D results from various pathological processes: the first instance being reduced β -cell mass, progressive decline of β -cell function, chronic insulin resistance and impaired insulin secretion (Kudva *et al.*, 1997). However, the mechanisms controlling the interplay of these impairments are unclear. Defects in glucose-stimulated insulin secretion and an eventual decrease in β -cell mass are important factors in the development and progression of T2D (Kahn, 2001). The primary cause of T2D is presently unknown.

Insulin resistance is traditionally assessed by insulin's ability to promote normal glucose metabolism, however its physiological role is much broader, and includes the metabolism of all three macronutrients (carbohydrates, lipids, and proteins) as well as cellular growth. Insulin resistance is a key etiological factor for T2D and plays a role in numerous other metabolic disorders including hypertension and atherosclerosis (Ferri *et al.*, 1999; Nigro *et al.*, 2006)

At the molecular level, this resistance can occur anywhere in the insulin signalling pathway, from receptor binding to downstream signalling events. Insulin sensitivity alone has long been recognized to be an important factor determining the magnitude of the insulin response to β -cell stimulation (Kahn *et al.*, 1993). An increased metabolic demand for insulin due to insulin resistance (resistance to the effects of

insulin on glucose uptake, metabolism, or storage) in several tissues usually precedes the development of hyperglycaemia. Over a long period of time, chronic insulin resistance of peripheral tissues often necessitates elevated concentration of insulin to activate the insulin receptor.

In order to maintain normoglycaemia, the beta-cells compensate for this increased demand by increasing its secretory capacity, β -cell mass or both (Bruning *et al.*, 1997, Polonsky, 2000). Compensation involves expansion of β -cell mass, enhanced insulin biosynthesis, and increased responsiveness of nutrient-secretion coupling. In rodent models of obesity without diabetes there is an adaptive increase in β -cell mass (Flier *et al.*, 2001) while other studies have suggested that the β -cell mass is also adaptively increased in non-diabetic obese humans (Kloppel *et al.*, 1985). This hyper-secretion of insulin by the pancreatic β -cells to compensate for insulin resistance often leads period of normal or near-normal glycaemia.

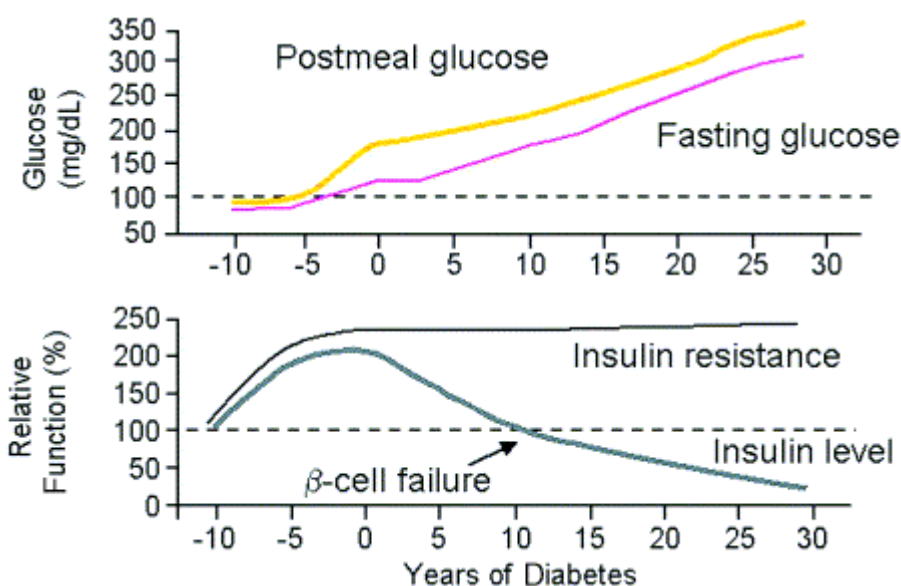


Figure 1.2 β -cell secretory response, compensatory insulin release and resultant β -cell failure. T2D is characterized by a progressive decline in β -cell function and chronic insulin resistance. The trend involves insulin resistance, compensatory hyperinsulinemia, β -cell dysfunction and decline in insulin secretion. Figure adapted from Bergenstal *et al.*, 2001.

However at some point, this period is followed by β -cell failure, partly due to the individual's inability to sustain the β -cell compensatory response. The exact cause of β -cell failure is not yet known. Failure of pancreatic β -cells to continue the increased compensatory insulin secretion that is sufficient to meet the metabolic demand often leads to T2D (Ferrannini *et al.*, 2005). These abnormalities result in reductions in insulin release in response to glucose, changes in pulsatile and oscillatory insulin secretion, increased rates of glucose release by the liver and kidney as well as decreased clearance from the circulation (O'Rahilly *et al.*, 1988; Polonsky *et al.*, 1988).

β -cell failure often results in elevated blood glucose level which can exert deleterious effects on β -cell function. The ensuing hyperglycaemia worsens the insulin resistance and further impairs β -cell function, thus creating a vicious circle (Skyler, 1998). Another consequence of prolonged exposure to hyperglycaemia is desensitization of the β -cells to glucose stimulation (Eizirik *et al.*, 1992). Decreased responsiveness to stimuli such as arginine or sulfonylureas in islets from type 2 diabetic patients have previously been reported and raises the question of whether glucotoxicity may also impede late steps in insulin secretion (Del Guerra *et al.*, 2005)

A number of factors have been suggested as possibly linking insulin resistance and beta-cell dysfunction in the pathogenesis of T2D. The likely mechanisms of early β -cell demise include oxidative stress, endoplasmic reticulum (ER) stress and glucolipotoxicity (Laybutt *et al.*, 2007; Poitout and Robertson, 2008; Xu *et al.*, 2009). T2D is also associated with marked impairment of mitochondrial function in β -cells with an increased expression of uncoupling protein-2 (UCP-2), leading to lower ATP levels, decreased ATP/ADP ratio in response to glucose, and reduced insulin secretion (Brown *et al.*, 2002; Anello *et al.*, 2005). The cause of damage to β -cells is thus likely to be multifactorial.

One of the major question in ongoing research is whether current forms of therapy can affect the decline in β -cell function in T2D. In humans, increased β -cell mass has been observed in the pancreas of obese compared with lean non-diabetic subjects (Butler *et al.*, 2003). Data from the United Kingdom Prospective Diabetes Study (UKPDS) indicated that there is a progressive deterioration in β -cell function over

time in T2D mellitus condition regardless of therapy allocation, insulin, chlorpropamide, glibenclamide, or metformin treatment (UKPDS-33, 1998; UKPDS-34, 1998). In this study, the pancreatic islet function was also found to be about 50% of normal at the time of diagnosis, independent of the degree of insulin resistance and a reduction in β -cell mass of about 60% was shown at necropsy.

The reduction of β -cell mass can be attributed to accelerated apoptosis and decreased neogenesis, however impaired β -cell function and possibly β -cell mass were found to be reversible, particularly at early stages of the disease. This can be explained by the fact that new islet formation and β -cell replication are normal in diabetic and obese individuals (Meier *et al.*, 2006; Butler *et al.*, 2007). In another UKPDS sub-study, Sulfonylureas were used and failed to stop the β -cell decline however metformin administration resulted in an improvement of 51% to 66% in β -cell function in the first year of therapy but a decrease to 38% at 6 years (UKPDS, 1995). This outcome shows that attempts to stimulate insulin secretion and improve insulin action with drug therapies were only temporarily helpful but were ultimately unable to prevent progressive β -cell dysfunction.

Based on these studies it is now clear that β -cell exhaustion, desensitization of the β -cells, lipotoxicity, amyloid deposition and a reduction in β -cell mass are major contributory factors to β -cell failure and the development of T2D. The data outlined above also shows that the major defect leading to a decrease in β -cell mass in T2D is increased apoptosis, while new islet formation and β -cell replication are normal. Individuals with T2D, whether lean or obese, have about a 50% decrease in β -cell mass. In this regard, therapeutic approaches designed to arrest apoptosis and improve insulin sensitivity could be significant in protecting the β -cells and also provide alternative physiological solution in the management of T2D. This approach could prove to be very effective in that it might actually reverse the disease to a degree rather than just palliate glycaemia.

1.5. The adipose tissue: role as an endocrine organ

1.5.1. Adipose tissue: Background and function

The adipose tissue is no longer considered to be a passive and inert tissue mainly devoted to energy storage, insulation and mechanical support but is emerging as an active endocrine organ participating in regulating whole-body physiology. Previously the adipose tissue has long been known to synthesize and store triglyceride in periods of nutritional abundance and hydrolyse and release non-esterified fatty acids (NEFA) when they are needed in response to fasting (Spiegelman *et al.*, 1993).

During adipose tissue development, genes that code for lipogenic and lipolytic enzymes that are involved in lipid transport are induced to carry out adipocyte function (Linhart *et al.*, 2001; Daval *et al.*, 2006). The activities of these enzymes in triacylglycerol biosynthesis and lipolysis are tightly controlled by nutritional and hormonal conditions e.g. feeding causes an induction whereas fasting causes suppression of lipogenic enzymes (Paulauskis *et al.*, 1989).

1.5.2. Types of Adipose tissue

Adipose tissue can be divided into 2 major types: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT represents the vast majority of adipose tissue in humans and is the physiological site of triglyceride storage during energy consumption and NEFA release when energy expenditure exceeds energy intake (Vázquez-Vela *et al.*, 2008). Adipose tissue also surrounds internal organs and provides some protection for these organs from jarring. The overall picture of the central functions of WAT has changed radically in recent years, with white fat now being viewed as a major endocrine organ, secreting both critical hormones and a number of protein factors in addition to fatty acids which play either a local or systemic role (Mohamed-Ali *et al.*, 1998).

Alternatively, BAT is a specialized adipose tissue depot which has a role in non-shivering thermogenesis and energy expenditure, particularly in small mammals and

human neonates (Gesta *et al.*, 2007). BAT transfers energy from food into heat especially through oxidation of fatty acids in mitochondria resulting in production of large amounts of heat. Its lipid reserves are depleted when the animal is exposed to a cold environment, and the colour darkens. In contrast to white fat, brown fat is richly vascularised and has numerous un-myelinated nerves which provide sympathetic stimulation to the adipocytes (Kawate *et al.*, 1994). Brown fat is most prominent in newborn animals. In human infants it comprises up to 5% of body weight, then diminishes with age to virtually disappear by adulthood

1.5.3. Adipose tissue formation and transcription regulators

There are a series of elaborate network of transcriptional events which regulate pre-adipocyte differentiation (adipogenesis) and adipocyte function and also coordinate expression of hundreds of proteins responsible for establishing the mature fat-cell phenotype. In the past decade transcriptional activation of adipocyte genes has been the focus of much research. Members of the CCAAT enhancer-binding protein (C/EBP- α , β and δ) and PPAR (PPAR- γ) families of transcription factors have been shown to be critical in directing adipocyte-specific gene expression and adipogenesis (Rosen *et al.*, 2002). Compelling evidence shows that C/EBP- α is required for adipocyte differentiation (Tang *et al.*, 2004). This is clearly illustrated in investigations with C/EBP α “knock-out” mice in which the adipose tissue fails to develop normally, accompanied by accumulation of triglyceride which is the hallmark of white adipose tissue (Wang *et al.*, 1995). The other transcription factor that has been implicated in adipose differentiation is sterol regulatory element-binding protein-1c (SREBP-1c), one of three SREBPs found in animal cells (Tontonoz *et al.*, 1993; Yokoyama *et al.*, 1993).

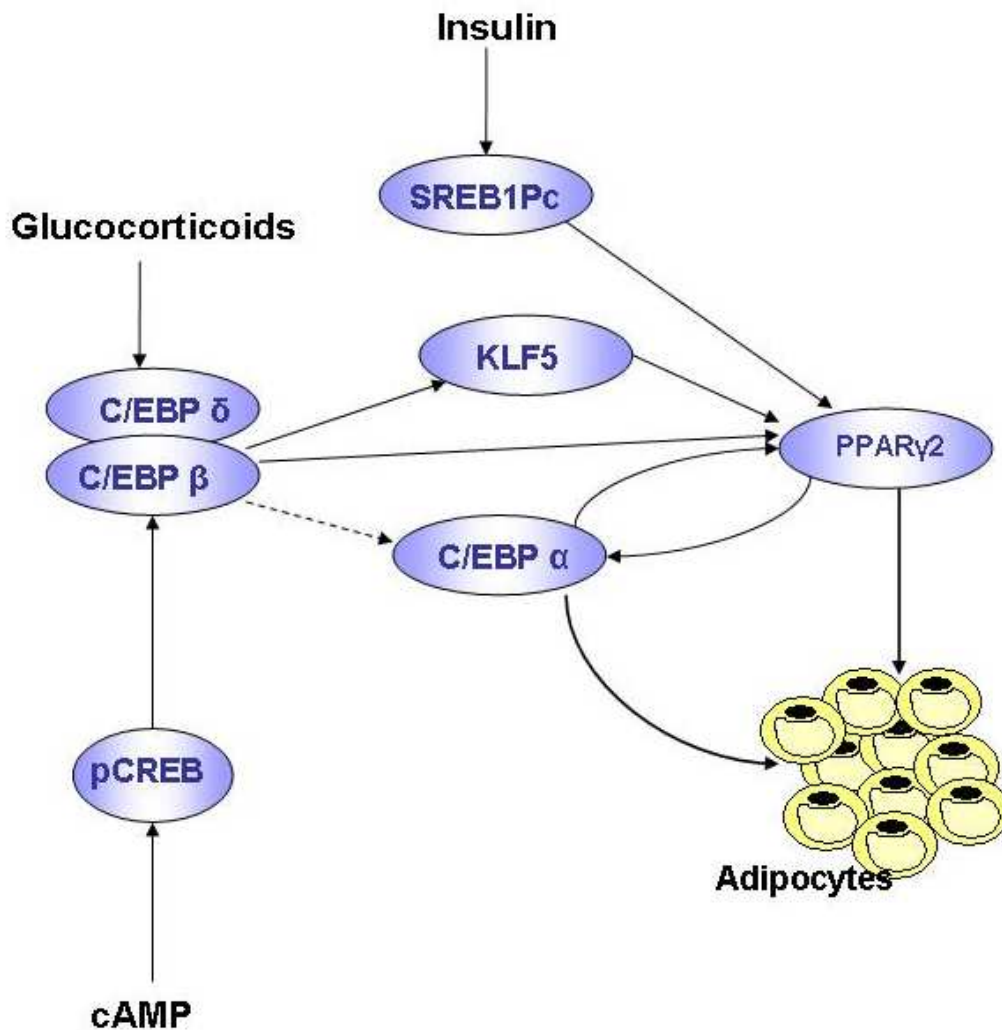


Figure 1.3 Transcription factors involved in adipocyte development. The major transcriptional factors involved in the adipogenic include proteins belonging to the CCAAT/enhancer binding protein family (C/EBP), peroxisome proliferator-activated receptor- γ (PPAR- γ) and adipocyte determination and differentiation dependent factor 1, also known as sterol regulatory element-binding protein 1 (SREB-1). These play a key role in the complex transcriptional cascade during adipocyte differentiation. Exposure of preadipocytes to adipogenic inducers e.g. insulin, glucocorticoids, agents that elevate cAMP and foetal bovine serum in cell culture, activates expression of several transcription factors that converge on PPAR- γ . PPAR- γ then induces C/EBP- α expression, and together, these factors oversee terminal adipogenesis

Adipose differentiation is accompanied by changes in cellular morphology, hormone sensitivity, gene expression and a dramatic accumulation of intracellular lipid. The ability to regulate specific key genes of lipogenesis or lipolysis could theoretically allow control of fat deposition. Troglitazone is one of the thiazolidinediones that activate the peroxisome proliferator activated receptor-gamma (PPAR- γ), which is expressed primarily in adipose tissues and offers new therapeutic possibilities for a variety of metabolic disorders.

1.5.4. Adipose tissue: endocrine and secretory function

In recent years, a line of evidence has demonstrated a much more complex function of adipose tissue as an endocrine organ that releases hormones into the blood stream to take part in physiological activities of the body with potential implication in insulin resistance, obesity and diabetes (Kim *et al.*, 2000). These adipocyte derived hormones are collectively called adipokines. One of the most important adipokines is leptin, a peptide hormone with numerous actions, including influences on energy homeostasis and neuro-endocrine and immune function. This remarkable understanding has helped to clearly define the role adipocytes play in regulation of metabolism, energy intake, and fat storage and possible secretory and endocrine function.

Adipose tissue secretory factors

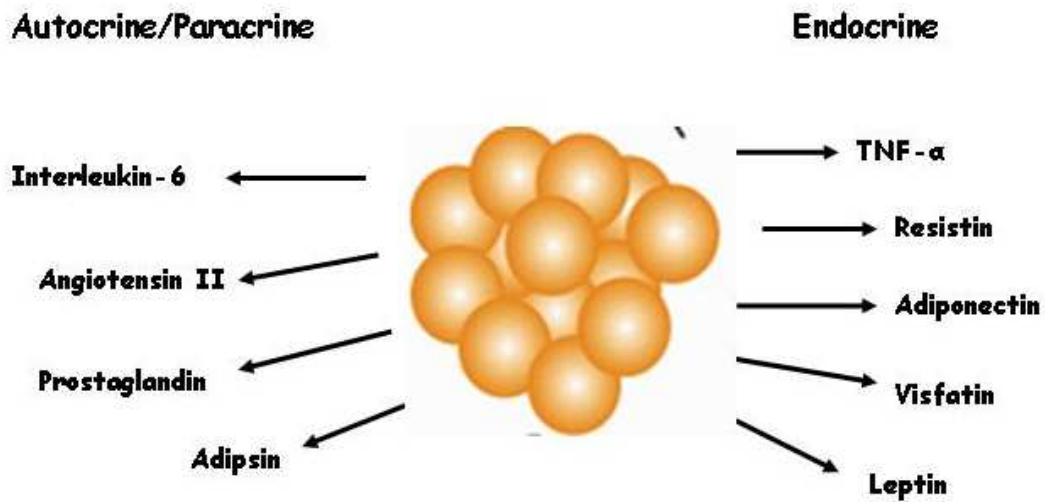


Figure 1.4 Biological functions of adipocytes. Previously, adipocytes were considered to be inert and passive reservoir for energy storage releasing fuel as fatty acids and glycerol in time of fasting or starvation. More recently it has become clear that adipocytes are highly active metabolic and endocrine organ that secrete important hormones, cytokines, vasoactive substances, and other peptides.

Adipose tissue was first suggested to have endocrine functions by Siiteri PK and co workers in 1987 who identified the ability of adipose tissue to interconvert steroid hormones. During this time adipose tissue was identified as a major site for the production of adipsin, an endocrine factor that is markedly down-regulated in rodent obesity (Flier *et al.*, 1987). Adipsin is a member of the serine protease gene family whose principal site of synthesis is the adipose tissue. Another group was able to show that adipsin is secreted and is present in the peripheral circulation (Cook *et al.*, 1987). In this regard, adipsin may be viewed as the first specific candidate for such a class of fat cell-derived regulatory molecules. The subsequent identification and characterization of leptin in 1994 transformed views of the tissue and of obesity and firmly established adipose tissue as an endocrine organ (Zhang *et al.*, 1994). Leptin has since been shown to have profound effects on appetite and energy balance and its

discovery has focused attention on the role of proteins secreted by adipose tissue. The recognition of leptin's important roles especially its effects on reducing food intake in both rodents and man stimulated the search for other proteins secreted by adipose tissue.

Over the past decade, enormous progress has been made in recognising the complex nature of the adipose tissue as a secretory organ as well as a site of the regulation of energy storage. Adipose tissue metabolism is extremely dynamic, and the supply of and removal of substrates in the blood is acutely regulated according to the nutritional state. Adipose tissue is highly specialized in its role as an endocrine organ and regulation of energy storage, fatty acid metabolism, and glucose homeostasis (Cornelius *et al.*, 1994). Genes involved in lipid metabolism and glucose homeostasis are prominently expressed in the fat tissue. These include: fatty acid synthase, the fatty acid binding protein aP2, lipoprotein lipase, phosphoenolpyruvate carboxykinase, malic enzyme, glyceraldehyde-3-phosphate dehydrogenase and Glut-4 (Moustaid *et al.*, 1991, Spiegelman *et al.*, 1983, Hunt *et al.*, 1986, Cornelius *et al.*, 1988 and Beale *et al.*, 1992).

The receptors for insulin, insulin-like growth factor 1 and adrenergic compounds are expressed in adipocytes (Reed *et al.*, 1980, Campfield *et al.*, 1995 and Fève *et al.*, 1990). This is quite significant to the adipose tissue due to their lipogenic and lipolytic activity. During times of increased food intake and/or decreased energy expenditure, surplus energy is deposited efficiently in adipose tissue in the form of neutral triglycerides under the mediation of key lipogenic enzymes. Excessive accumulation results in obesity. However, in times of nutritional deprivation and/or increase in energy expenditure requirements, the lipid reserves are usually released to provide fuel for energy generation. This latter process is mediated by lipases contained in the adipocyte which break down triglycerides into glycerol and fatty acids that are used in fatty acid oxidation liver, muscle, and BAT (Schweiger *et al.*, 2006)

The studies of animal models which completely lack adipose tissue have provided tremendous insights into the physiological role of adipose tissue (Shimomura *et al.*, 1998). Genetic manipulation to alter adipose depots has enabled functional dissection

of obesity and its metabolic sequel. An early model from Bruce Spiegelman's laboratory used transgenic mice in which an attenuated diphtheria toxin A chain gene was expressed specifically in adipose with the aim of reducing adiposity (Ross *et al.*, 1993). Transgenic mice with high-level of toxin expression resulted in newborn lethality accompanied by fatty fluid in the peritoneum with the mice dying after birth.

In addition to secretion and fat storage, the adipose tissue is emerging as a key regulator of many physiological functions including energy homeostasis, glucose and lipid metabolism. Knowledge of specific signalling pathways involved will be key to unravelling the primary causes of T2D and various metabolic diseases related to obesity.

1.6. Adipose tissue changes in obesity and link to T2D

1.6.1. Obesity, adipogenesis and diabetes

Obesity is an energy balance disorder in which nutrient intake chronically exceeds energy expenditure often characterized by an overgrowth of adipose tissue and excess storage of calories as triglyceride (Dvorak *et al.*, 1997). Obesity is associated with adipose cell hypertrophy, and more severe forms also show adipose cell hyperplasia (Kahn, 1992). Adipose tissue plays an important role in glucose homeostasis and affects insulin sensitivity in other tissues. The important endocrine function of adipose tissue is emphasized by the adverse metabolic consequences of both adipose tissue excess (obesity) and deficiency (malnutrition).

Epidemiological studies have shown that the risk for T2D and presumably insulin resistance rises as body fat content measured by body mass index (BMI) increases from the very lean to the very obese, implying that the amount of body fat has an effect on insulin sensitivity (Colditz *et al.*, 1990). The expansion of adipose cell number (hyperplastic growth) can occur at virtually any time in response to marked overfeeding (Ailhaud *et al.*, 1992). The prevention of obesity is thus very likely to reduce the incidence of T2D.

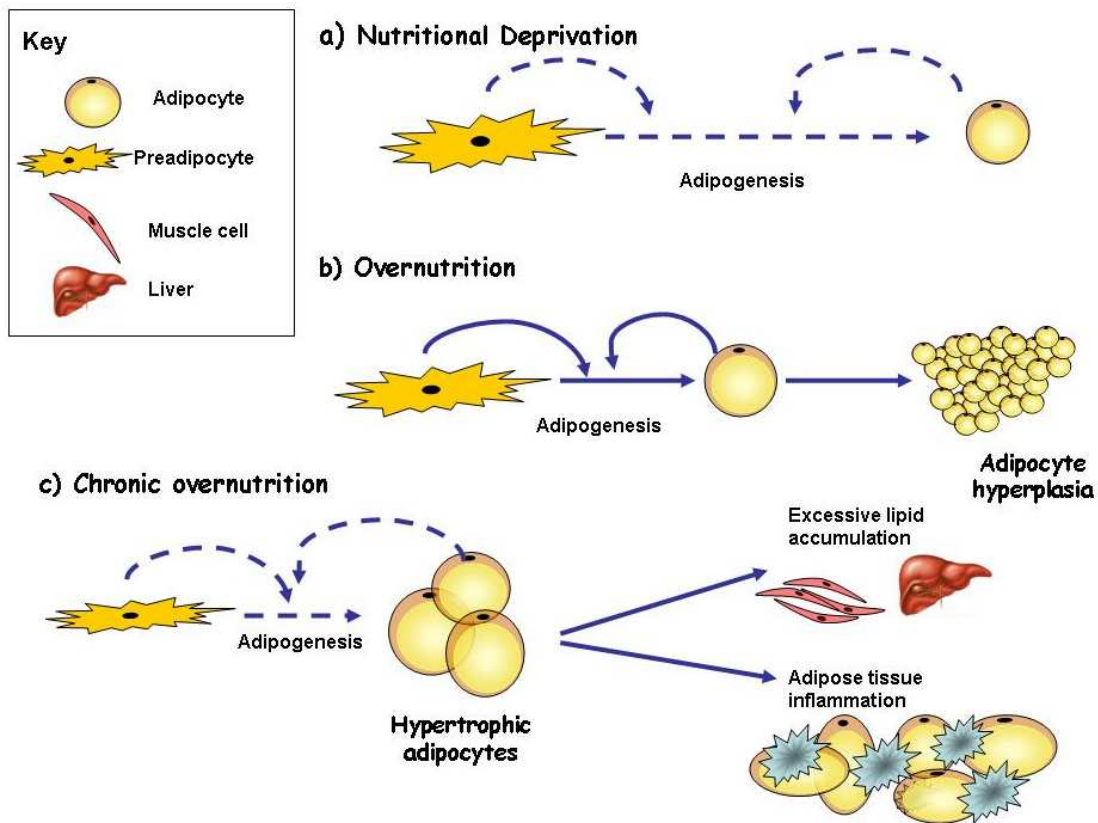


Figure 1.5 Role of nutrition and adipose tissue development. (a) Changes in the balance of diet e.g. conditions of nutritional deprivation induce anti-adipogenic signals emanating from both pre-adipocytes and mature adipocytes which restrain pre-adipocyte differentiation. (b) Continuous food intake especially high fat diet (overnutrition) results in hypertrophic adipocytes which secrete factors that stimulate preadipocyte differentiation. These include both positive adipogenic factors and factors that block negative adipogenic signals. (c). Disruption of local signalling networks during chronic over-nutrition leads to impaired adipogenesis and adipose expandability. This, in turn, results in adipocyte hypertrophy and dysfunction, and secondary adipose inflammation, in addition to ectopic lipid accumulation at extra-adipose sites (e.g. liver and muscle), which together, contribute to the pathogenesis of insulin resistance. Key: $\text{-----}\blacktriangleright$ = inhibition, $\text{-----}\blacktriangleright$ = stimulation

The World Health Organization currently defines ‘overweight’ as a BMI of 25–29.9 kg/m² and obese as a BMI >30 kg/m² (WHO Expert Committee 1995). Small adipocytes in lean individuals have been shown to promote metabolic homeostasis

while enlarged adipocytes as those found in obese individuals have been shown to recruit macrophages and release of a range of factors that predispose toward insulin resistance and also promote inflammation. A large number of studies have found that an increased body mass index correlates with increased plasma concentrations of inflammatory adipokines and acute phase proteins (Bastard *et al.*, 2000). This has re-defined the adipose tissue not only as a key component of the endocrine system, but also of the immune system.

1.6.2. Obesity and insulin resistance

Two complementary theories have been proposed to explain the pathophysiological link between obesity and insulin resistance. The first theory which relates to adipose tissue expandability proposes that when adipose tissue expands to its threshold level, its storage capacity becomes saturated (Virtue and Vidal-Puig, 2008). The capacity of adipose tissue mass to expand is the key determinant as to whether obesity leads to metabolic complications. As a result, it becomes less able to amass more fat. Once this stage is reached, excess fat is redirected towards other organs, such as the liver, pancreas or muscle. In these organs, lipid accumulation can be toxic and induce insulin resistance (Schrauwen, 2007)

The second hypothesis proposes that the excessive accumulation of fat in adipose tissue can alter the secretion pattern of adipokines (Weyer *et al.*, 2000; Trayhurn and Wood, 2004; Skurk *et al.*, 2007). Suggestive evidence shows that some of these adipokines e.g. leptin modulate insulin sensitivity not only in adipose tissue but also in other metabolically relevant organs, such as liver or muscle (Bastard *et al.*, 2002; Farooqi *et al.*, 2002). The patho-physiological link between the increase of fat mass, namely obesity, insulin resistance and cardiovascular complications is thus becoming clearer as the roles of these adipokines emerge.

1.6.3. Adipokines and T2D

Obesity is associated with a low-grade inflammation of WAT which can subsequently lead to insulin resistance and impaired glucose tolerance. In obesity, WAT is characterized by abnormal adipokine production and secretion of a wide range of inflammatory molecules which result in activation of some pro-inflammatory signalling pathways and induction of several biological markers of inflammation (Hotamisligil *et al.*, 1993, Fried *et al.*, 1998). The altered adipokine production in obesity has been shown to have local effects on WAT physiology but also systemic effects on other organs including the pancreatic beta-cells (Bastard *et al.*, 2002; Sartipy *et al.*, 2003).

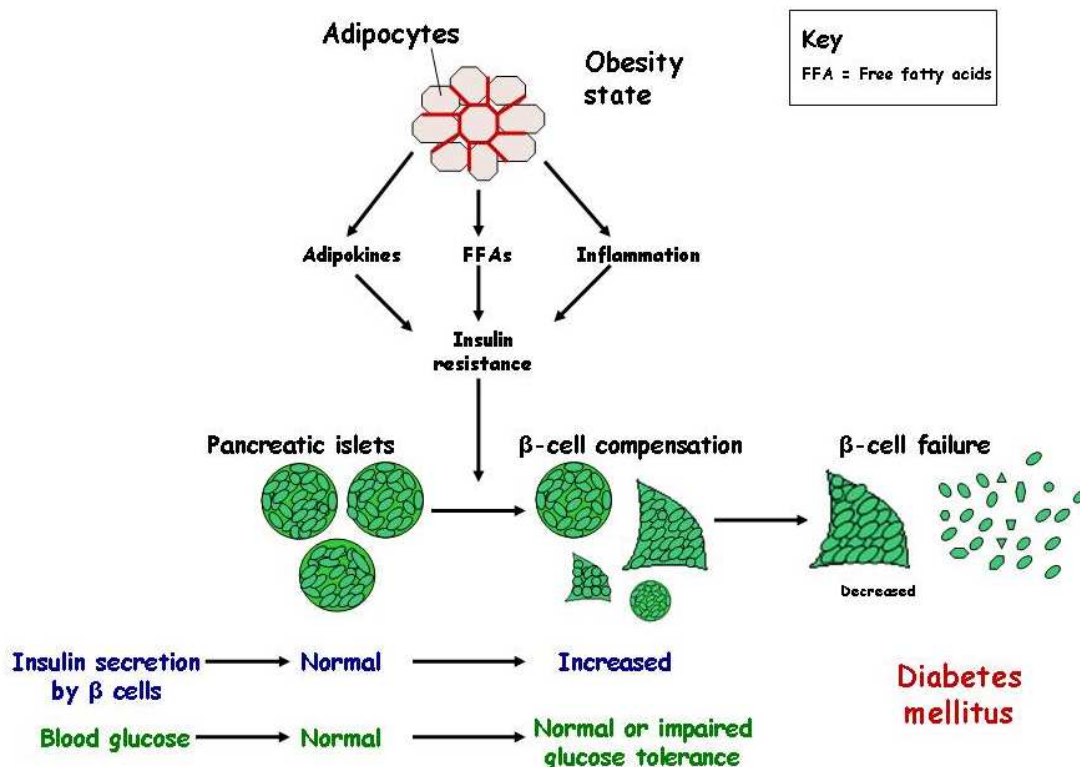


Figure 1.6 Adipose tissue secreted factors and β-cell function / failure.

Pathways leading to insulin resistance associated with obesity which are induced by adipokines, free fatty acids, and chronic inflammation in adipose tissue.

Figure adapted from Kasuga, 2006.

Inflammatory markers, such as C-reactive protein (CRP) and interleukin-6 (IL-6) have also been shown to be increased in obese individuals compared with lean subjects (Hotamisligil *et al.*, 1993). Insulin resistance involves a broad range of interactions and dysfunction in feedback mechanism that takes into account the interplay between nutrition, glucose, insulin and adipokines in various tissues of metabolic importance. Although there is a clear association between obesity in particular visceral obesity and the development of insulin resistance, the underlying mechanisms involved in obesity-related insulin resistance are complex and not fully defined.

Obese animals and humans have been shown to have characteristic over-production of pro-inflammatory adipokines e.g. tumour necrosis factor-alpha (TNF- α), IL-6 and resistin by WAT, and a deficiency in anti-inflammatory adipokines e.g. adiponectin (Kern *et al.*, 2003; Lihn *et al.*, 2003). Inflammation and adiposity cause insulin resistance by interfering with insulin signalling pathways. In mice, inflammatory serine 307 phosphorylation of IRS-1 interferes with insulin signalling (Qiao *et al.*, 2002). It has previously been reported that TNF- α can directly lead to insulin resistance by inducing serine phosphorylation of the insulin receptor, which inhibits insulin signalling (Hotamisligil *et al.*, 1994). It is thus clear that TNF- α plays a causal role in the insulin resistance of experimental animals since neutralization of TNF-alpha in obese fa/fa rats caused a significant increase in the peripheral uptake of glucose in response to insulin (Hotamisligil *et al.*, 1993). However, despite inflammatory activity in animal studies, neutralization of TNF- α activity has proved ineffective in ameliorating insulin sensitivity in diabetic patients (Ofei *et al.*, 1996).

Interleukin 6, a major pro-inflammatory cytokine, is produced in a variety of tissues, including activated leukocytes, adipocytes, and endothelial cells. Elevated concentrations of interleukin-6 are associated with development of T2D (Fernandez-Real *et al.*, 2000, Pradhan *et al.*, 2001). In vivo infusion of human recombinant IL-6 has been shown to induce gluconeogenesis, subsequent hyperglycaemia, and compensatory hyperinsulinemia (Stith *et al.*, 1994). Elevated levels of IL-6 can thus be used to predict the development of T2D illustrating the possible role for inflammation in diabetogenesis.

Adiponectin and leptin have each been demonstrated to increase rates of fatty acid (FA) oxidation thus decreasing muscle lipid content and also increase insulin sensitivity in lean and obese rodents (Steinberg *et al.*, 2002; Fruebis *et al.*, 2001). Adiponectin is a 30-kDa adipose-derived hormone that appears to play an important role in regulating energy homeostasis and insulin sensitivity (Yamauchi *et al.*, 2002). Adiponectin levels have been shown to be decreased in insulin-resistant states such as obesity and T2D (Berg *et al.*, 2001; Kubota *et al.*, 2002). Injection of adiponectin decreases plasma glucose levels by suppressing glucose production in the liver and injection of the globular domain of adiponectin decreases elevated fatty acid levels by oxidizing fatty acids in muscle (Berg *et al.*, 2001; Combs *et al.*, 2001; Fruebis *et al.*, 2001). Adiponectin-deficient mice develop premature diet-induced glucose intolerance and insulin resistance, and increased serum fatty acids (Kubota *et al.*, 2002; Maeda *et al.*, 2002). In contrast, transgenic overexpression of adiponectin in mice leads to improved insulin sensitivity, glucose tolerance, and lower serum fatty acids (Maeda *et al.*, 2002; Combs *et al.*, 2004). These observations suggest that adiponectin ameliorates insulin resistance in lipotrophic mice and T2D mice and may be a major insulin-sensitizing hormone secreted by adipose tissue. However, the physiological role of adiponectin in vivo is not yet clear because the conclusions have been primarily based upon gain of function experiments

1.6.4. Adipose tissue and enzyme malfunction in glucose homeostasis

Adipose tissue also plays an important role in glucose homeostasis and affects insulin sensitivity in other tissues. As previously illustrated, the GLUT-4 mediates insulin-stimulated glucose uptake in adipocytes and muscle by rapidly moving from intracellular storage sites to the plasma membrane. In obesity and T2D, GLUT-4 expression is decreased in the adipose tissue but preserved in the muscle (Shepherd *et al.*, 1999). The role of adipose GLUT-4 in glucose homeostasis has previously been determined. In one study, selective depletion of GLUT-4 in mice adipose tissue led to impaired glucose tolerance and insulin resistance but with preserved adipose mass. However unexpectedly, these mice developed insulin resistance in muscle (despite preservation of GLUT-4) and liver, manifested by decreased biological responses

(Abel *et al.*, 2001). This suggests that glucose transport in adipose tissue plays a vital role in glucose homeostasis and the selective down-regulation of GLUT-4 in the adipose tissue as seen in human obesity and T2D (Shepherd *et al.*, 1999) may contribute to insulin resistance in other insulin target tissues.

There is also clear evidence that the size and distribution of adipose stores influence metabolism in human disease. Obesity-induced insulin resistance is affected by the total amount of adipose tissue, localization and body fat distribution (Evans *et al.*, 1984) with weight gain ordinarily preceding insulin resistance, and weight loss improving it (Groop *et al.*, 1993). Surgical removal of visceral fat has also been shown to delay the onset of diabetes and improve the insulin effect on hepatic glucose production in Zucker Diabetic Fatty (ZDF) model of obesity and diabetes (Gabriely *et al.*, 2002). Understanding the mechanisms regulating adipose formation and function should provide valuable information in the fight to combat the growing incidence of obesity and related metabolic diseases.

There are huge gaps in the current knowledge about the mechanism of action of various adipokines. Resistin and resistin-like molecules (RELM) receptors have not yet been identified and major advances are still being made in the understanding of biological function of leptin. Ongoing research continues to shed light on the role of adipose tissue with new identification of adipokines being reported. A better understanding of neuronal input and potentially output from adipose tissue is vitally important especially in light of current realization that many metabolic phenomena in peripheral tissues are directly controlled through central neuronal circuits, particularly through central pathways of nutrient sensing.

1.7. Resistin and beta cell function

1.7.1. Resistin: Origin and structure

Resistin is a 12.5-kDa peptide hormone belonging to a novel class of cysteine-rich secreted proteins termed as the resistin-like molecules RELM and the FIZZ (found in inflammatory zone) (Steppan *et al.*, 2001). Mouse resistin is exclusively expressed in white adipose tissue while human resistin is mainly expressed in circulating mononuclear cells (Steppan and Lazar, 2004). The mouse resistin gene encodes a 114 amino acid polypeptide including a 20 amino acid signal sequence. Human resistin is a dimeric protein containing 108 amino acids (Holcomb *et al.*, 2000) and it is synthesized and secreted selectively from adipose tissue.

Resistin was discovered by three different independent groups using modern genomic approaches (Steppan *et al.*, 2001; Kim *et al.*, 2001; Holcomb *et al.*, 2000). Kim and co-workers used microarray analyses and were able to identify resistin as adipose secretory factor (ADSF) while Holcomb's group initially found resistin, which they termed 'FIZZ3', as an expressed sequence tag related to a protein they found to be induced during lung inflammation.

However it was Steppan and co-workers who were the first to demonstrate that both resistin mRNA and protein expression were markedly reduced in rodents treated with insulin-sensitizing thiazolidinedione in vitro, with the majority of subsequent in vivo data supporting these observations (Moore *et al.*, 2001, Way *et al.*, 2001; Steppan and Lazar, 2002). These reports suggest a potential mechanism for obesity-related insulin resistance and also shed light on a potential mechanism of action for thiazolidinedione. No effects of RELM α on metabolic control have yet been reported, though its homology with resistin suggests they may have related function.

1.7.2. Resistin: proposed biological function

Several cellular mechanisms and pathways of insulin resistance have been described. Serine phosphorylation of insulin receptor substrate-1 (IRS-1) has been shown to inhibit insulin action by blocking insulin receptor/IRS-1 interaction (Aguirre *et al.*, 2002). Fatty acid induced insulin resistance utilises this mechanism and is mediated by increased protein kinase C (PKC) and IRS-1 Ser (307) phosphorylation and decreased IRS-1 tyrosine phosphorylation (Yu *et al.*, 2002).

Resistin has been shown to produce insulin resistance and glucose intolerance when injected into normal mice (Steppan *et al.*, 2001) however the precise mechanisms by which resistin causes insulin resistance are still unclear. Defects in the insulin-signalling cascade to glucose uptake in skeletal muscle have been identified in people with T2D. Resistin has been proposed to induce insulin resistance by disrupting the insulin signalling pathways in insulin target tissues, however the mechanism by which resistin initiates and elicits signals is not well established.

Considerable debate exists regarding the role of resistin in the pathophysiology of insulin resistance in human and animals. Resistin has previously been shown to promote endothelial cell activation (Verma *et al.*, 2003) and angiogenesis (Mu *et al.*, 2006) highlighting the important role this adipokine may play in vascular disorders. Resistin has also been shown to inhibit essential functions of polymorphonuclear leukocytes resulting altered immune response (Cohen *et al.*, 2008). Understanding how resistin molecules modulate the activity of components of this insulin-signalling pathway and so result in decreased insulin action on glucose metabolism might be a promising tool for identifying the aetiology of T2D.

1.7.3. Resistin: obesity and T2D

Serum resistin is elevated in diabetes and obesity (Kim *et al.*, 2001; Azuma *et al.*, 2003) hence it has been suggested that resistin may have a causative role in the insulin resistance and T2D (Steppan *et al.*, 2001). Functionally, administration of resistin resulted in impaired glucose tolerance and insulin sensitivity in wild-type mice, while immuno-neutralization of circulating resistin in diet-induced obese mice resulted in reduced blood glucose levels and significantly improved hyperglycaemia and insulin sensitivity (Steppan *et al.*, 2001). However, the physiological role of resistin in humans and the mechanism by which it neutralizes insulin action in rodents are still unclear.

A series of studies have been carried out since the initial discovery of resistin; some of which have provided data that support the original findings while others are conflicting. Insulin action is markedly impaired in individuals with visceral obesity (Carey *et al.*, 1996) and expression of resistin mRNA has been shown to be significantly higher in the visceral fat compared to subcutaneous fat depot (Gabriely *et al.*, 2002). Serum resistin levels and expression in WAT have previously been reported to be reduced in fasted mice and increased on re-feeding (Kim *et al.*, 2001; Steppan *et al.*, 2001) suggesting diet may also play a significant role.

The skeletal muscle has long been considered the major site of insulin-stimulated glucose uptake. Treatment of L6 rat skeletal muscle cells with recombinant resistin resulted in reduced insulin-stimulated glucose uptake and decreased insulin-stimulated GLUT-4 translocation (Palanivel *et al.*, 2006). The results also showed that resistin regulates the function of IRS-1 and Akt-1 by down-regulating production and tyrosine phosphorylation of IRS-1. In another study, prolonged exposure of rat skeletal muscles cells to resistin resulted in decreased GLUT-4 translocation and reduced glucose uptake in response to insulin (Fan Hong-qi *et al.*, 2007). These reports are in agreement with previous studies (Satoh *et al.*, 2004; Steppan *et al.*, 2001) demonstrating the possible role of resistin as a potential direct regulator of glucose homeostasis in rodents.

However in direct contrast, Way and co-workers showed that resistin gene expression was significantly decreased in adipose tissue of several different murine models of obesity including ob/ob, db/db, tub/tub, and KKAY mice compared with their lean counterparts (Way *et al.*, 2001). Surprisingly, resistin expression was also decreased in obese mice and increased in ob/ob mice and Zucker diabetic fatty (ZDF) rats in response to PPAR- γ agonists. This outcome raises serious questions concerning some of the previous reports since it has previously been hypothesised that one of the ways thiazolidinediones exert their effect is through downregulation of resistin expression. The precise mechanism however by which PPAR- γ activation by thiazolidinediones improves insulin sensitivity is still unclear.

Levy and colleagues reported that there was no difference in resistin expression in the epididymal fat pad of Fischer 344 rats, a model of age induced insulin resistance, compared with age-matched, insulin-sensitive Sprague Dawley rats (Levy *et al.*, 2002). Initial reports by Steppan *et al.*, in experimental animals indicated that resistin gene expression increases with adiposity and with insulin resistance (Steppan *et al.*, 2001). In this regard, resistin gene expression in the insulin-resistant, obesity-prone Fischer 344 rats should be higher than in Sprague Dawley rats, however this is not the case. This outcome clearly shows that resistin did not play a role in the insulin resistance characteristic of young Fischer 344 rats which raises further questions about the physiological role of resistin.

Degawa and co-workers demonstrated that resistin protein is present in human adipose tissue and blood, and that there is significantly more resistin in the serum of obese subjects compared to lean subjects (Degawa-Yamauchi *et al.*, 2003). This finding supports previous observations on resistin mRNA expression and suggests that resistin expression in adipose tissue is a combination from both adipocytes and other cells in the tissue. This outcome is also in agreement with a report which indicated that resistin protein was present in both adipocytes and stromal vascular cells of the adipose tissue (McTernan *et al.*, 2002b).

In as much as the study by Degawa-Yamauchi and colleagues used two different techniques to examine resistin protein in human; the parameters used in the study appear to be inconsistent and raises questions on the reliability of the study outcome.

For instance, the investigators compared lean and obese individuals of profoundly different ages and gender distribution. The lean group had a mean age of 33 ± 2 yr compared with 47 ± 1 yr in the obese group. Age-related changes in insulin resistance have previously been described with glucose tolerance shown to deteriorate with age (Boden *et al.*, 1993). It is possible that similar changes may influence resistin expression, an issue that was not properly addressed. From these reports, puzzling questions can be raised on the expression and exact regulation of adipose resistin, and thus on its physiological function.

The regulation of hepatic gluconeogenesis is an important process in the adjustment of the blood glucose level, and pathological changes in the glucose production of the liver are a central characteristic in T2D. Under normal circumstances, insulin inhibits hepatic glucose production when plasma glucose levels increase. Resistin has been shown to attenuate this effect of insulin by promoting increases in plasma glucose concentration (Rajala *et al.*, 2003). This is a key step of resistin involvement in the pathophysiology of T2D since the rate of hepatic gluconeogenesis is considerably increased in patients with T2D compared with control subjects, thereby contributing significantly to the fasting hyperglycaemia in diabetes (Accili, 2004). In another study, resistin was reported to induce insulin resistance, but did not affect glucose output in rat derived hepatocytes (Liu Feng *et al.*, 2008). Resistin was also shown to attenuate insulin-stimulated glycogen synthesis, phosphorylation of IRS, protein kinase B/Akt, GSK-3 as well as markedly inducing the gene and protein expression of SOCS-3, a known inhibitor of insulin signalling (Steppan *et al.*, 2005; Niederwanger *et al.*, 2007).

Metformin is a commonly used antidiabetic drug known to decrease hepatic glucose production. Therefore, it would be expected that metformin, an insulin-sensitizing drug would have a down-regulatory effect on resistin expression in adipose tissue as resistin has been shown to increase hepatic glucose output (Muse *et al.*, 2004; Rangwala *et al.*, 2004). In one study, metformin treatment was reported to improve hyperglycaemia and hyperinsulinemia in obese diabetic db/db mice; however results also unexpectedly showed that resistin protein expression in adipose tissue was increased by metformin treatment (Fujita *et al.*, 2002). This is in sharp contrast to the

proposed inflammatory role of resistin function as proposed by Steppan and co-workers.

Conversely, mice lacking the adipocyte hormone resistin exhibit low blood glucose levels after fasting, due to reduced hepatic glucose production, suggestive of a normal physiological role for resistin in glucose homeostasis (Banerjee *et al.*, 2004). Identification of resistin receptor and signalling pathways that mediate its action and analysis of the phenotypes resulting from deletion or over-expression of resistin in transgenic mice will help to further define the biological roles of this adipokine. The regulation of resistin thus still remains a subject of controversy, in particular, the question of its modulation in obesity emerging from contradictory results. Debate thus still exists regarding the role of resistin in the pathophysiology of insulin resistance. Further studies are needed to determine the mode of regulation and biological functions of resistin and whether it is an effector of insulin resistance in obesity.

1.8. Visfatin and beta cell function

1.8.1. Visfatin: Origin and structure

Visfatin also known as pre-B cell colony-enhancing factor (PBEF) or nicotinamide phosphoribosyltransferase (Nampt) is a novel adipokine that is highly expressed in visceral fat and up-regulated in obesity and T2D (Berndt *et al.*, 2005; Chen *et al.*, 2006). It was during a search for novel cytokine-like molecules secreted from human peripheral blood lymphocytes that visfatin was first identified as PBEF (Samal *et al.* 1994). The visfatin gene encodes for a 52 kDa secreted cytokine composed of 491 amino acids that acts as a growth factor for early stage B cells, even though it lacks a signal peptide (Samal *et al.* 1994).

However it was after visfatin was reported as a visceral fat-derived adipokine implicated in the development of obesity-associated insulin resistance and T2D that the molecule drew much attention (Fukuhara *et al.*, 2005). In this report, visfatin was shown to be up-regulated during adipocyte differentiation and detected in cell culture

media and plasma. The Fukuhara *et al.*, Science article has since been retracted on the basis that some preparations of visfatin did not bind the insulin receptor in their experiments. Subsequent studies have however revealed additional biological activities that suggest both intracellular as well as extracellular localization of visfatin (Rongvaux *et al.*, 2002; Revollo *et al.*, 2007b).

In addition to adipocytes, visfatin is expressed in many other tissues, where it has been linked to a variety of functions such as inflammatory responses and inhibition of apoptosis (Jia *et al.*, 2004). Most notable is the expression in skeletal muscle, liver, and immune cells hence it is likely that these tissues, whose function is altered in obesity, may be responsible for lower levels of circulating visfatin in obesity (Samal *et al.*, 1994). Previous reports have raised questions regarding the clinical relevance of visfatin, as it is ubiquitously expressed in different cell types (Jia *et al.*, 2004; Ognjanovic and Bryant-Greenwood, 2002; Curat *et al.*, 2006; Rongvaux *et al.*, 2002). However, recent studies support the view that visfatin is a true adipokine that is clearly expressed and secreted by human adipocytes (Haider *et al.*, 2006a; Kralisch *et al.*, 2005a and Pagano *et al.*, 2006)

The crystal structure of visfatin relating to its enzymatic function in nicotinamide mononucleotide (NMN) biosynthesis from nicotinamide has previously been determined (Kim *et al.*, 2006). The crystal structure of visfatin/nampt clearly demonstrates that the protein belongs to the dimeric class of type II phosphoribosyltransferases (Wang *et al.*, 2006; Khan *et al.*, 2006; Kim *et al.*, 2006)

1.8.2. Visfatin: proposed biological function

Visfatin is an adipokine that has been proposed to participate in glucose homeostasis due to its reported glucose-lowering effect. Initially visfatin was thought to have insulin mimicking functions, stimulating glucose utilization in peripheral tissues; however the precise mechanisms have not been fully elucidated. A previous report which as previously illustrated has since been retracted suggested that visfatin binds to insulin receptor at a distinct site from that of insulin (Fukuhara *et al.*, 2005) while other studies reported no effect (Revollo *et al.*, 2007b). This study sought to

investigate whether the reported insulin-like effects can be observed in clonal rodent pancreatic β -cells.

Research on visfatin is ongoing and various roles continue to emerge. Until now, visfatin has been shown to exert three distinct activities of vital importance to cellular function. Within the cell and possibly outside the cell, visfatin functions as an enzyme commonly referred to as intracellular nicotinamide phosphoribosyl transferase (iNAmpt) that catalyzes the rate-limiting step in mammalian nicotinamide adenine dinucleotide (NAD⁺) biosynthesis (Rongvaux *et al.*, 2002; Van der Veer *et al.*, 2005). Visfatin has been shown to induce the production of the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α in human monocytes (Moschen *et al.*, 2007). Therefore, visfatin appears to be a multifunctional protein acting as a hormone, cytokine, and/or enzyme.

As previously illustrated visfatin is abundantly expressed and secreted by adipocytes however the reported insulin mimetic activity of this adipokine remains controversial. The effects of visfatin are not restricted to glucose homeostasis. As illustrated in the previous paragraph, visfatin has been reported to lead to increased levels of cytokines, whereas insulin does not show cytokine-inducing effects, suggesting that induction of cytokines by visfatin may be mediated by engagement of unidentified receptor (Moschen *et al.*, 2007; Stephens and Vidal-Puig, 2006). It is also possible that there may be no visfatin receptor at all and visfatin solely be an enzyme. Further studies of visfatin's physiological role may lead to new insights into glucose homeostasis and new therapies for metabolic disorders such as diabetes.

NAD⁺ has been known for several decades as a classic coenzyme with a well-established role in cellular redox reactions. As illustrated previously, Nampt has both intra- and extracellular forms in mammals. Intracellular Nampt (iNAmpt) is an essential enzyme in the NAD biosynthetic pathway while the extracellular form of this protein has been reported to act as an adipokine.

Visfatin's enzymatic activity was originally reported in 1957 however it was until 2001 that the gene encoding Nampt was first identified in *Haemophilus ducreyi* (Preiss and Handler, 1957; Martin *et al.*, 2001). Recent progress in the field of NAD biochemistry has fuelled new interest in the NAD biosynthetic pathways with several

lines of evidence implicating this activity in a broad range of biological functions. Reports from recent findings have indicated that mammals predominantly use nicotinamide rather than nicotinic acid as a precursor for NAD⁺ biosynthesis. Nampt is the rate-limiting enzyme that catalyzes the transfer of a phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate to nicotinamide, forming nicotinamide mononucleotide (NMN) and pyrophosphate (Revollo *et al.*, 2007a; Revollo *et al.*, 2007b). NMN is then converted to NAD⁺ by nicotinamide mononucleotide adenylyltransferase ((NMNAT).

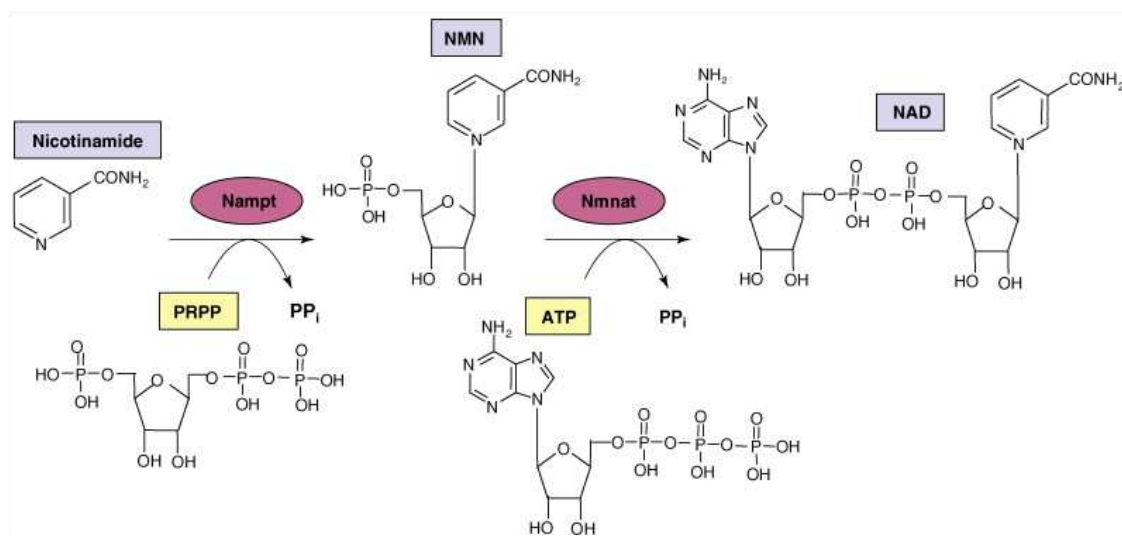


Figure 1.7 (Garten *et al.*, 2008): Visfatin actions in the NAD⁺ biosynthetic pathway. Nampt/Visfatin synthesize NMN from nicotinamide in a mammalian NAD⁺ biosynthetic pathway. The rate-limiting step in the biosynthetic pathway is the transfer of a phosphoribosyl residue from 5-phosphoribosyl-1-pyrophosphate (PRPP) to nicotinamide catalyzed by Nampt to produce NMN. The NMN produced is then converted to NAD by Nmnat.

Different cells utilize different strategies to make NAD⁺. In mammals, tryptophan, nicotinic acid and nicotinamide are three major precursors for NAD⁺ biosynthesis (Magni *et al.*, 1999; Rongvaux *et al.*, 2003). Tryptophan is an essential aromatic amino acid whose role as an a NAD⁺ precursor was first suggested by nutritional studies which showed that diets supplemented with tryptophan were able to cure

human pellagra (Krehl *et al.*, 1945). Pellagra is a disease characterized by a nicotinamide deficiency. Quinolinic acid (2, 3-pyridine dicarboxylic acid) reacts with 5-phosphoribosyl-1-pyrophosphate (PRPP) in the presence of magnesium ions to give rise to nicotinic acid mononucleotide (NaMN). The reaction is catalysed by quinolinic acid phosphoribosyltransferase (QPRTase) (Nishizuka *et al.*, 1963). NaMN is then converted to desamido-NAD (NaAD) by a nicotinic acid mononucleotide adenylyltransferase (NaMNAT). The NaAD is finally converted into NAD by a NAD synthase (NADS) (Preiss and Handler, 1958; Magni *et al.*, 1999).

1.8.3. Visfatin function and link to obesity and T2D

Visfatin is an endocrine, autocrine as well as paracrine protein with many functions including enhancement of cell proliferation, biosynthesis of nicotinamide mono- and dinucleotide and hypoglycaemic effect (Sommer *et al.*, 2008). Visfatin has also been shown to exert insulin mimetic and pro-inflammatory effects, also functioning as an intracellular enzyme to produce NAD (Rongvaux *et al.*, 2002; Moschen *et al.*, 2007). The observation that visfatin has insulin-mimetic functions has raised the hypothesis that a dysregulation of the activity of this molecule may contribute to the metabolic syndrome and diabetes. The multiple effects of visfatin has been proposed to be achieved by its role as an intra and extracellular NAD biosynthetic enzyme (Revollo *et al.*, 2007a; Revollo *et al.*, 2007b)

Visfatin expression and plasma concentrations are substantially increased with obesity in animals and humans (Berndt *et al.*, 2005). It has been shown that visceral fat is considerably reduced through weight loss after non-reversible surgical procedures e.g. gastric banding (Pontiroli *et al.*, 2002). However dietary approach as a solution to combating obesity is unsatisfactory since it is a complex, multi-factorial chronic disease involving environmental, genetic and metabolic components. Reduction of visceral fat would thus be linked to downregulation of visfatin levels. In one study visfatin concentration levels were shown to be increased in extremely obese individuals (BMI >40). However the elevated plasma visfatin and leptin concentrations were reportedly reduced after weight loss by gastric banding surgery (Haider *et al.*, 2006c). This suggests that visfatin may be involved in the beneficial

effect of weight loss and subsequent improvement of insulin resistance. Adiponectin concentration which is suppressed in obese state was shown to be increased. The changes reported in leptin and adiponectin are consistent with previous studies (Van Dielen *et al.*, 2002; Yang *et al.*, 2001).

In contrast to this, a separate study reported that massive weight loss after gastroplastic surgery in obese patients was accompanied by an increase in circulating concentrations of visfatin (Krzyzanowska *et al.*, 2006b). This finding is consistent with results of Pagano and co-workers who found reduced visfatin plasma concentrations in obese subjects compared with lean subjects (Pagano *et al.*, 2006).

After the retracted report by Fukuhara *et al.*, regarding insulin mimetic activity of visfatin, many research papers have been published on the topic, with controversial findings. In humans, plasma levels of visfatin were found to be elevated in subjects with visceral-fat accumulation and T2D and also in women with gestational diabetes (Chen *et al.*, 2006; Hammarstedt *et al.*, 2006; Krzyzanowska *et al.*, 2006a). It was consequently hypothesized that visfatin could play a role in the regulation of insulin sensitivity in humans and possibly link obesity to its complications.

In vitro studies showed that visfatin treatment increased glucose uptake in adipocytes and osteoblasts bearing resemblance to the actions of insulin with enhanced tyrosine phosphorylation of insulin receptor, IRS-1 IRS-2, and Akt (Moschen *et al.*, 2007; Xie *et al.*, 2007). These reports indicate that the regulation of glucose uptake, by visfatin in human adipocytes and osteoblasts involves insulin receptor phosphorylation which is the same signal-transduction pathway used by insulin.

The relationship between visfatin and obesity is however controversial. In one study, it was reported that plasma visfatin and mRNA expression in subcutaneous fat were positively correlated with adiposity (Berndt *et al.*, 2005) while another study reported a markedly higher visfatin / PBEF expression was present in visceral fat, compared with subcutaneous fat (Fukuhara *et al.*, 2005). In contrast, Pagano and co-workers showed that, in human obesity, plasma visfatin and its mRNA in subcutaneous adipose tissue were significantly lower in obese subjects, compared with normal-weight controls however higher visfatin mRNA was found in visceral adipose tissue

of obese subjects, compared with lean controls; suggesting a divergent regulation of visfatin in different fat depots (Pagano *et al.*, 2006).

The findings obtained by Fukuhara *et al.* may be different due to the limited observations as only two subjects groups were considered. Subsequent studies have shown a high variability of visfatin / PBEF expression in adipose tissue which is a possible explanation of this discrepancy in the reports from the different groups. In a previous study, enzymatic function of visfatin was shown to require dimerization hence it may circulate as a dimer in human serum (Kim *et al.*, 2006; Revollo *et al.*, 2007a). This may have implications on the immunological detection as well as biological actions of visfatin. Differences in the qualitative and quantitative detection of visfatin by immunoassays need to be considered in clinical association studies of visfatin, obesity and diabetes due to possible differences in the specificity of the immunoassays applied. This may help explain the conflicting observations that have previously been reported.

Subsequent clinical studies have confirmed the association between visfatin and diabetes with certain reports showing that circulating visfatin is increased with progressive β -cell deterioration (Chen *et al.*, 2006; Dogru *et al.*, 2007; Lopez-Bermejo *et al.*, 2006; Varma *et al.*, 2007). These studies also showed that visfatin is highly expressed in subcutaneous fat of lean and more insulin sensitive subjects however this observation is attenuated in subjects with high intramyocellular lipids, low insulin sensitivity and high levels of inflammatory markers. The link between visfatin and beta-cell function is further emphasised by reports indicating regulatory effect of glucose and insulin on visfatin concentrations in humans (Haider *et al.*, 2006a). Findings from this study showed that circulating visfatin concentrations are increased by hyperglycaemia; however this effect was suppressed by exogenous hyperinsulinaemia or somatostatin infusion. Glucose signalling for visfatin release was also reported to involve the PI3-kinase/AKT pathway.

It has been suggested that visfatin is also regulated by hormones known to alter insulin sensitivity and pro-inflammatory adipose-secreted factors. In one study, visfatin mRNA expression in 3T3-L1 adipocytes was significantly down-regulated by growth hormone (GH), tumour necrosis factor-alpha (TNF- α) and β -adrenergic

agonist isoproterenol (Kralisch *et al.*, 2005b). GH has long been known to potently antagonize insulin action on insulin sensitive tissues such as muscle, fat and liver (Frank, 2001) while several studies have shown that TNF- α potently induces insulin resistance with the serum levels of TNF- α increased in human and murine obesity (Peraldi *et al.*, 1996). These reports suggest a direct regulation of visfatin mRNA expression by insulin resistance-inducing hormones that might constitute an important element in the pathogenesis of obesity related insulin resistance. This further supports the view that visfatin might be an interesting novel candidate linking core components of obesity and insulin resistance.

The increase in visfatin expression is not uniform in all models of obesity. Although visfatin has been reported as having insulin-like qualities, there is no data to indicate that serum concentrations change acutely or in any way after eating especially glucose rich diet. The evidence of a direct link between visfatin genotype and human T2D is thus still weak and more molecular, physiological and clinical studies are needed to determine the role of visfatin in the aetiology and pathogenesis of T2D. Precise understanding of physiological and molecular actions of visfatin will lead to the discovery of effective therapeutic intervention. Novel manipulations which promote visfatin signalling and function may be promising strategies to attenuate the inflammatory pathways in the β -cells and ultimately reduce the progression of obesity-related insulin resistance.

As the list of adipokines continues to grow, it has become apparent that factors that control the production of adipokines vary according to the species under study thus future work will require elaborate approaches in animal models and especially humans to elucidate the biology of adipokines and their impact on diseases.

1.9. Aims of this study

The molecular mechanisms underlying the link between obesity and T2D have been elusive. It is therefore important to characterize the mechanisms of insulin resistance associated with obesity in order to develop effective therapeutic approaches to T2D. An accumulating body of evidence suggests that inflammation may play a crucial intermediary role in pathogenesis, thereby linking diabetes with a number of commonly coexisting conditions thought to originate through inflammatory mechanisms. In this regard, substantial amount of experimental evidence suggest that resistin and visfatin, two adipokines that are highly expressed in obese and diabetics compared to normal and lean individuals are associated with hyperglycaemia, insulin resistance, and overt T2D.

Cross-sectional investigations have provided conflicting reports on the role resistin and visfatin may play in inflammation and aetiology of T2D. Additional experimentation is thus required to shed more light on resistin and visfatin physiology and pathophysiological role.

The aim of this study was therefore to investigate the effect of the adipokines visfatin and resistin on beta-cell function and regulation of key genes involved in glucose homeostasis. This involved assessing the ability of recombinant resistin and visfatin to directly alter insulin secretion in clonal pancreatic beta-cells and to investigate the effect of resistin and visfatin on insulin receptor expression and activation, MAPK signalling and beta-cell viability.

Chapter 2

Materials and Methods

2.1. Materials

For equipments, kits, reagents and materials, please refer to Appendix 1

2.1.1. Cell lines

The rat clonal pancreatic BRIN-BD11 cells were a gift from Professor Peter Flatt, University of Ulster, Coleraine, Northern Ireland, UK.

The clonal mouse pancreatic β TC-6 cells were purchased from the American Type Culture Collection (ATCC) collection (LGC Promochem, UK).

2.1.2. Antibodies

Actin	Santa Cruz Biotechnology Inc.
Goat anti rabbit IgG HRP	Autogen Bioclear (UK) LTD
Insulin receptor antibody	Abcam (UK) LTD
Insulin receptor (phosphor Y972) antibody	Abcam (UK) LTD
Phospho MAPK (ERK1/2)	Cell signalling technology Inc
MAPK (ERK1/2)	Cell signalling technology Inc
Visfatin antibody	Abcam (UK) LTD

2.1.3. Buffers

2.1.3.1. Preparation of 5X Sample loading buffer

250 mM TrisHCl pH6.8	12.5ml
10% SDS	10g
30% Glycerol	30ml
5% β -mercapitaethanol	5ml
0.02% bromophenol blue	52ml

2.1.3.2. Protein extraction buffer

20mM Tris
150mM NaCL
1mM EDTA
1% Triton X

2.1.3.3. Running buffer (1X)

Tris-Glycine-SDS PAGE Buffer (10X)	100ml
Distilled water	900ml

2.1.3.4. Transfer buffer (1X)

Tris-Glycine Electro-Blotting Buffer (10X)	100ml
Methanol	200ml
Distilled water	700ml

2.1.3.5. Blocking solution

BSA (Bovine Serum Albumin)	5g
Tris buffered saline-Tween 20	100ml

2.1.3.6. Tris buffered saline (TBS) 10X

Tris Base	24.22g
NaCl	80g
Water	986ml
HCL (Absolute – add in the hood)	14ml
pH	7.6

2.1.3.7. Tris buffered saline with tween 20 (TBS-tween)

10X Tris buffered saline	100ml
Distilled water	900ml
Tween 20	500 μ l

2.1.3.8. Cell lysis buffer

(20 mmol/l Tris,
150 mmol/l NaCl,
1 mmol/l EDTA,
1% Triton X,
Protease inhibitor cocktail
Phosphatase inhibitor

2.2. Cell culture

2.2.1. General tissue culture for Brin-BD11 and β TC-6 cells

The insulin-secreting rat β -cell line BRIN-BD11 cells and mouse β TC-6 cells were cultured in RPMI 1640 medium supplemented with 11 mM glucose, 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were seeded at 3×10^6 cells per flask and maintained in 175cm³ vented tissue culture flask in humidified incubator at 37°C in 5% CO₂ and 95% air atmosphere and routinely passaged upon attaining 80% confluence (determined by microscopic inspection). All cells used in experiments were between passages 25 to 45.

2.3. Cell Treatment

2.3.1. Cell Viability Assay and Cell Death Assay

Cell viability was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit. The cells were plated out at a density of 2×10^4 cells per well in a 96-well plate and grown overnight to attach. The cells were serum starved and then treated with various doses of resistin, visfatin and NMN for 24, 48 and 72 hours. For 48 and 72 hour experiments, treatment media was supplemented with 1% serum since the β -cells are particularly sensitive to oxidative stress resulting from withdrawal of survival factors in serum. Upon completion of treatment, treatment media was aspirated and replaced with 100 μ l of fresh serum free media. 20 μ l of MTS reagent was added into each well of the 96-well assay plate and incubated for further 2 hours. The absorbance at 510 nm was recorded using an ELISA plate reader. The percentage of the absorbance of treated cells was calculated against untreated cells.

To determine cell death, β TC-6 cells were seeded at a concentration of 2×10^4 cells per well, in a 96-well cell culture plates containing RPMI 1640 medium supplemented

with 10% foetal calf serum, 2 mM L-glutamine, penicillin (400 IU/ml) and streptomycin sulphate (200 µg/ml) overnight to attach. The cells were then treated with different concentrations of resistin for 48 hours in serum depleted media to ensure serum depletion induced cell death. Upon completion of treatment, treatment media was aspirated and replaced with 100µl of fresh serum free media. 20 µl of MTS reagent was added into each well of the 96-well assay plate and incubated for further 2 hours. Absorbance was read at 510.

2.3.2. Cell Count

Cells were seeded at an initial density of 1×10^6 cells per flask in a 25cm³ vented tissue culture flask and grown overnight to attach. The cells were then treated with recombinant resistin (20ng/ml and 40ng/ml) in RPMI 1640 serum free media and cultured in a humidified incubator at 37°C in 5% CO₂ and 95% air atmosphere for 24 hours. After completion of treatment, the cells were trypsinized and centrifuged at 500g for 5 minutes. Cell pellet was re-suspended in fresh culture media and cell count conducted using a Coulter counter.

2.4. Real Time RT-PCR studies

2.4.1. Primer Design

All primers were designed using the reference sequence in the NCBI Nucleotide sequence database. Specificity of all primer sets were confirmed using melt-curve analysis and sequencing where appropriate.

Primer	Strand	Primer Sequence
Mouse Actin	Sense	GCTGTATTCCCCTCCATCGTG
	Anti-sense	CGTCCCAGTTGGTAACAATGCC
rat GAPDH	Sense	GGAGTCTACTGGCGTCTTCA
	Anti-sense	ATGAGCCCTTCCACGAT
Mouse Insulin receptor	Sense	AATGGCAACATCACACACTACC
	Anti-sense	CAGCCCTTTGAGACAATAATCC
Mouse GcgR	Sense	TTGGCGATGACCTCAGTGTG
	Anti-sense	AACCAGCAATAGTTGGGTATGATG
Rat resistin	Sense	AGAAGGCACAACCGTCACT
	Anti-sense	CCGCTGTCCAGTCTATGCT
Mouse visfatin	Sense	GCCTCCTGGATTTTCTCTTTG
	Antisense	AACAATACCCACCCAACACAA

Table 2.1 All the primers were supplied by VH Bio Limited, UK except for rat GAPDH and rat visfatin which was supplied by PrimerDesign Ltd, Southampton, UK.

2.4.2. Total RNA extraction and quantification

Beta-cells were treated with resistin or visfatin for 24 hours in serum free media supplemented with 11mM glucose. After treatment, the cells were collected, washed in ice cold PBS and centrifuged at 300g for 5 minutes. Pelletted cells were re-suspended in 175µl of SV RNA cell lysis buffer and passed through a 21-gauge needle to shred high molecular weight DNA. 350µl of SV RNA Dilution Buffer was added to the 175µl cell lysate and mixed by inversion. The solution was then placed in a heat block at 70°C for 3 minutes and thereafter centrifuged at 14,000g for 10 minutes at room temperature before being transferred to a fresh eppendorf.

200µl of 95% ethanol was added to the lysate, and thoroughly mixed using a pipette. The solution was then transferred to a silica membrane spin column assembly and centrifuged at 14,000g for 1 min. The spin basket was removed from the assembly and the run through solution discarded. The spin basket was then replaced and 600µl of SV RNA Wash Solution added to the spin column and centrifuged at 14,000g for 1 min. The collection tube was emptied and DNase I treatment performed.

600µl of SV RNA Wash Solution was added to the spin column and centrifuged at 14,000g for 1 min. The collection tube was emptied and 250µl SV RNA Wash Solution added and centrifuge at 14,000g for further 2 minutes. The cap from the spin tube was removed by twisting and the spin basket transferred to 1.5 ml elution tube. 95µl of nuclease free water was directly added to the membrane, ensuring complete cover of the surface. This was centrifuge at 14,000g for 1 minute. The spin basket was removed and discarded. 5µl of RNase inhibitor was added to the collected RNA. The collected RNA quantified and stored at -80°C.

Quantification of total RNA was performed using Genequant technology by measuring absorbance of at 260/280nm. Sample absorbance, concentrations and 260/280 ratios were recorded. Only samples with a ratio > 1.7 were used, any ratio below this mark was discarded.

2.4.3 mRNA Reverse Transcription

1µg of total RNA in each sample was reverse transcribed using the cDNA Synthesis Kit in a 50µl reaction volume containing nuclease free water, AMV RT buffer, Oligo(dt)₁₅ primer, dNTP mix and avian myeloblastoma virus (AMV) reverse transcriptase for 1 hour at 40°C. The resulting cDNA was stored at -20°C.

2.4.4. SYBR[®] Green Real time RT-PCR studies for gene expression analysis

The real-time RT-PCR reactions were performed using the iCycler Real-Time PCR Detection System. Reaction volumes used were 25µl containing 5µl of diluted cDNA (1 in 2 dilution), 1µl of each pair of primers, 5.5µl nuclease free water and 12.5µl of SYBR Green Mastermix. Two housekeeping genes (Actin and GAPDH) were amplified in separate reaction to normalize results. Denorm software was used to analyse the data hence the reason for selecting Actin and GAPDH as housekeeping genes. Thermal cycling conditions included:

95° C for 1 minute (initial denaturing)

95°C for 30 seconds

58°C for 30 seconds

95°C for 30 seconds

72°C for 10 minutes (final extension)

Each sample reaction was run in triplicate and expression quantified as the number of cycles (C_T) after which fluorescence exceeds the background threshold minus the C_T for the housekeeping control (ΔC_T). The RT-PCR statistical analysis was performed using student T test for two group comparisons and analysis of variance (ANOVA) for multiple comparisons. The relative expression levels were calculated by the formula $2^{-\Delta\Delta C_T}$, comparing treatment samples and untreated control samples.

2.4.5. Analysis of gene expression profiles using RT² Profiler™ PCR Array

The expression profiles of selected target genes specific to diabetes was investigated using Mouse Diabetes RT² Profiler™ PCR Array. The kit contains primers for 84 genes involved in beta cell function and T2D; plus wells for negative controls and four housekeeping genes which were used to normalise the data. Total RNA of the treated samples (resistin and visfatin treated) and untreated control was extracted and quantified spectrophotometrically as described in section 2.4.2. cDNA synthesis and preparation to the array were done in triplicate as described in section 2.4.3. Reactions were all performed using the iCycler Real-Time PCR Detection System in 96-well reaction plates. Thermal cycling conditions included:

95° C for 1 minute (initial denaturing)

95°C for 30 seconds

58°C for 30 seconds

95°C for 30 seconds

72°C for 10 minutes (final extension)

Results were analysed using a spreadsheet analysis template provided by the supplier (SABioscience Corp). For each set of triplicates, the mean value for each gene was determined and used to calculate the fold-changes (treatment versus untreated control). Relative gene expressions were calculated by using the $2^{-\Delta\Delta C_t}$ method, in which C_t indicates the fractional cycle number where the fluorescent signal reaches detection threshold.

2.5. Western Blot and protein work

2.5.1. Protein extraction (whole cell extracts) for expression studies

β -cells were treated in serum-free media with the desired concentration of resistin or visfatin for 24 hours. Following experimental treatments, media was aspirated and collected. Cells in the flasks were washed once with ice-cold PBS and collected. Total cellular protein was extracted from untreated and treated cells for 1 h at 4 °C in a detergent based cell lysis buffer (for preparation, refer to section 2.1.3.8). Samples were then spun at 13000g for 10 minutes at 4° C and supernatant collected for protein quantification assay and Western blot analysis. The collected protein was quantified using a proprietary protein quantification kit (DC protein assay; section 2.5.3) and 5X sample loading buffer was added (for preparation; refer to section 2.1.3.1) after quantification. The mixture was immediately boiled at 95°C for 5 minutes to reduce and denature the protein. Samples were stored at -20°C until use.

2.5.2. Cell Lysis/Protein Extraction for signalling studies

Cells were cultured in six-well plates overnight to attach prior to treatment. After attaining confluence the cells were pre-incubated in serum free media for four hours after which the various treatment compounds were administered directly into the media. Upon completion of treatment, the cells were washed in ice-cold PBS and lysed in ice-cold cell lysis buffer (for preparation; refer to section 2.1.3.8). The cells were scraped off the plates and transferred to an appropriate eppendorf tube. The cell suspension (cells and lysis buffer) was collected and centrifuged at 13000g for 10 minutes. The supernatant was then collected for protein quantification assay and western blot or ELISA analysis.

2.5.3. DC Assay Protein Quantification

Protein content in cell lysates was determined using the DC Protein Assay Kit (Bio-Rad). The concentration of the unknown protein samples was determined by comparing the absorbance at 690 nm to the standard curve prepared using BSA (Bovine Serum Albumin) protein standards. All samples were quantified in triplicate and averages used in quantification.

2.5.4. SDS-PAGE and Western Blot Analysis

For Western blot analysis equal amounts of solubilised proteins (40 μ g) were resolved by SDS-PAGE using running buffer (for preparation, refer to section 2.1.3.3) at 200V for 1 hour. The percentage resolving gel used was determined based on the size of protein analysed (8 to 12%). The proteins were then transferred to nitrocellulose membranes in transfer buffer at 100V for 1 hour (for preparations, refer to section 2.1.3.4). All membranes were stained with Ponceau-S protein staining solution to assess transfer efficiency. The membranes were then washed in distilled water to remove the Ponceau-S stain and air-dried overnight.

To reduce non-specific antibody binding, the nitrocellulose membranes were blocked with 5% bovine serum albumin (BSA) dissolved in TBS-tween (TBS-T) for 1 hour at room temperature. The membranes were then incubated with primary antibody (of protein under investigation) dissolved at the required concentrations in 1% BSA overnight at 4°C. After incubation, the membranes were subjected to two 5 minute washes in TBS-T. Bound antibodies were detected by incubation with HRP labelled secondary antibody for 1 h at room temperature. The membranes were then washed five times for 5 min each, in TBS-tween and exposed to Enhanced Chemiluminescence (ECL+) detection solution for 5 minutes. The membranes were viewed using Storm® Phosphorimager using blue laser to measure fluorescence at 600mV.

2.5.5. MAPK Activation Assay

Activation of the MAPKs ERK1/2 and p38 was analysed using a proprietary phospho-specific ELISA kit (RayBio® Cell-Based ELISA Sampler Kit). 30,000 cells were seeded into each well in a 96 well plate and incubated for overnight at 37°C, 5% CO². The cells were then serum starved for 4 hours and treated with 200ng/ml visfatin, 100µM NMN and 100nM Phorbol 12-myristate 13-acetate (PMA). The detection reagents were added to cells grown and attached to a 96-well plate. The plate was incubated overnight at 4°C, which gives the best detection of target protein. After incubation, plate contents were discarded and washed 4 times. 100µl of detection antibody was added to each well and sealed with tape and the plate incubated for 1 hour at 37°C. The plate was again washed 4 times. 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and sealed with tape and the plate incubated for further 10 minutes at 37°C or 30 minutes at 25°C. 100µl of stop Solution was then added to each well with gentle shaking and absorbance read at 450 nm

2.5.6. Insulin Receptor Activation Assay

Activation of the insulin receptor was analysed using a proprietary phospho-specific ELISA kit (RayBio® Cell-Based ELISA Sampler Kit). 30,000 cells were seeded into each well in a 96 well plate and incubate for overnight at 37°C, 5% CO². The cells were then serum starved for 4 hours and treated with various concentrations of visfatin (50ng/ml, 200ng/ml and 500ng/ml) and 10nM insulin as positive control. The detection reagents were added to cells grown and attached to a 96-well plate. The plate was incubated overnight at 4°C, which gives the best detection of target protein. After incubation, plate contents were discarded and washed 4 times. 100µl of detection antibody was added to each well and sealed with tape and the plate incubated for 1 hour at 37°C. The plate was again washed 4 times. 100µl of TMB substrate was added to each well and sealed with tape and the plate incubated for further 10 minutes at 37°C or 30 minutes at 25°C. 100µl of stop Solution was then added to each well with gentle shaking and absorbance read at 450 nm

2.6. Determination of insulin secretion

To determine the effect of resistin on insulin secretion, glucose responsive clonal rodent pancreatic beta-cell line BRIN-BD11 was used. The cells were plated out at a density of 2×10^4 cells per well in a 96-well plate and incubated overnight in RPMI-1640 tissue culture medium supplemented with 10% foetal calf serum, 100 iu/ml penicillin and 100 μ g streptomycin to attach. The cells were subsequently treated with 0, 20 and 40ng/ml resistin for 24-hours in serum free RPMI. After treatment, the cells were immersed in serum free RPMI media containing 2.25mM glucose for 1 hour and then stimulated with 22mM glucose for another 1 hour. The media from this last treatment was collected and assayed for insulin content using a proprietary Rat insulin ELISA (Merckodia, Sweden).

For visfatin and NMN, glucose responsive clonal rodent pancreatic beta-cell line β TC-6 cells were pre-incubated at 37°C for 1 hour in 2.25mM glucose. After pre-incubation, the medium was removed, and cells were treated with visfatin in 2.25mM glucose for 1 hour. For high glucose study, the cells were pre-incubated in 2.25mM glucose and then treated with visfatin in 22mM glucose for 1 hour. After incubation, the treatment medium was collected and centrifuged at 13,000g for 5 minutes. The supernatant was collected and assayed for insulin content using a proprietary Mouse insulin ELISA (Merckodia, Sweden). The effect of NMN on insulin secretion was determined using the same procedure as visfatin.

2.7. Statistical analysis

Statistical analysis was performed using the GraphPad® Prism software Version 4.0 (GraphPad Software, San Diego) and Microsoft Excel 2003 (Microsoft Corporation, WA), based on the mean values of independently reproduced experiments. Data were presented as mean±SEM of treated samples compared to the untreated control. Significant differences between treatment and control values were determined by the 2-tailed student t-test and 1-way ANOVA with Tukey's post-test.

Chapter 3

Regulation of beta-cell line viability by visfatin and resistin

3.1. Introduction

Pancreatic β -cells have a dense capillary network, which is essential for the necessary provision of nutrients to allow for accurate glucose sensing, and to disperse hormones to the systemic circulation (Virtanen *et al.*, 2008). In response to glucose sensing, sufficient β -cells are required to maintain normal glucose concentrations, as demonstrated by the development of diabetes when β -cell mass is decreased surgically or by β -cell toxins (Goodner *et al.*, 1989, Kjems *et al.*, 2001).

The ability to preserve or enhance β -cell mass and function would thus have important therapeutic implications. The relationship between insulin resistance, deficient β -cell mass, and impaired insulin secretion is still poorly understood however. This is partly because it is still not possible to assess β -cell mass in vivo in humans due to difficulty in obtaining pancreatic tissue, since the tissues are only available from a deceased organ donor (Bonner-Weir S, 2000).

Regulation of β -cell mass is largely a balance between β -cell replication and apoptosis (Butler *et al.*, 2003). Developing new ways to replenish the deficit in β -cell mass in diabetes is vital to new and successful therapies. Ongoing research is aimed at regeneration of insulin producing cells by triggering the body to grow its own new β -cells either by neogenesis or replication of existing ones or by islet transplantation (Lipsett and Finegood, 2003; Shapiro *et al.*, 2006).

Hypertrophic adipocytes have been shown to produce excess secretory factors which alter β -cell function (Guerre-Millo, 2004). Recently, visfatin has been reported to exert beneficial functions on glucose and lipid metabolism, as well as insulin sensitivity (Sun *et al.*, 2009) while resistin has been shown to exhibit inflammatory properties (Steppan *et al.*, 2001). Viability of pancreatic β -cells and isolated islets is one of the main obstacles limiting β -cell adaptation in times of stress. The purpose of this study was to investigate the effects of resistin and visfatin on cell viability of clonal pancreatic β -cells.

3.2. Materials and methods:

Details of materials and methods used in the experiments for this chapter can be found in chapter 2

3.3. Results

3.3.1. 1. The effect of resistin on β -cell cell viability over 24 hours

Figure 3.1.1 shows the result of 24-hour treatment of β TC-6 cells with resistin. Incubation with resistin significantly increased β -cell viability at 5, 10 and 20 ng/ml but not at concentrations higher than this. The increase was maximal at 10 ng/ml (45% increase compared to control; $p < 0.05$).

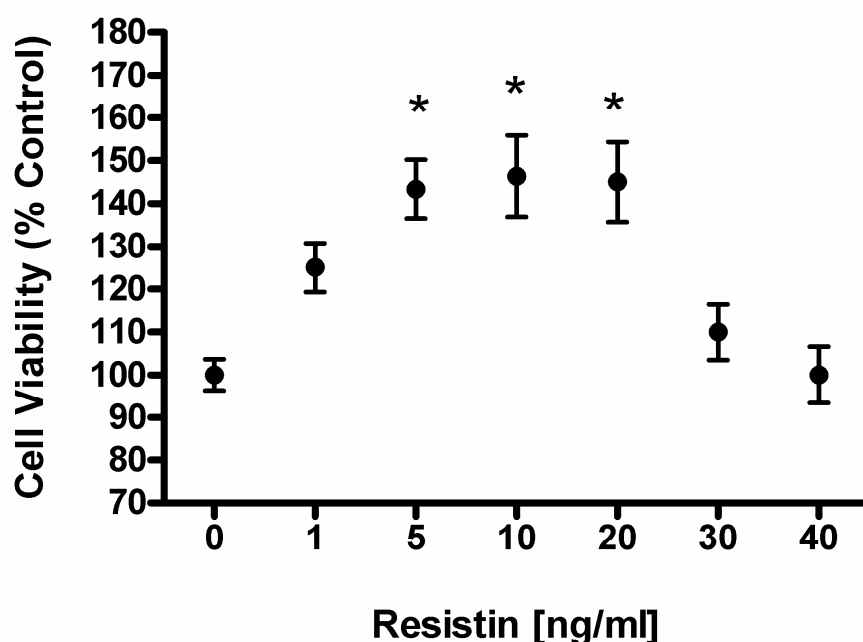


Figure 3.1.1 Resistin increases β TC-6 cell viability at physiological concentrations. Cell viability was determined using modified MTS assay. The results show a marked difference with the low concentration reported in lean non-diabetics (20ng/ml) causing an increase in cell viability while high concentration associated with obesity and diabetes (40ng/ml) not eliciting any increase compared to control. The values represent means and standard error of the mean (SEM). * $p \leq 0.05$ using ANOVA and Tukey's multiple comparison test for the comparison between untreated and resistin treated cells. (n = 5).

3.3.1.2. The effect of resistin on β -cell cell number over 24 hours

To assess whether the increases in cell viability seen in Figure 3.1.1 were due to proliferative changes in the β TC-6 cells, a total cell count was carried out. This technique showed that the number of cells significantly increased after 24-hour incubation with 20ng/ml resistin compared to untreated cells ($p < 0.05$). This increase might initially appear modest at only about 20% compared to untreated cells, but as the treatment period was only 24-hours this is still an interesting finding. This suggests that resistin promotion of cell viability is at least in part through increased cell proliferation. However since this increase in cell number is not as drastic as the marked increase in viability illustrated in Figure 3.1.1, it is possible that there could be other mechanisms by which resistin promote cell viability.

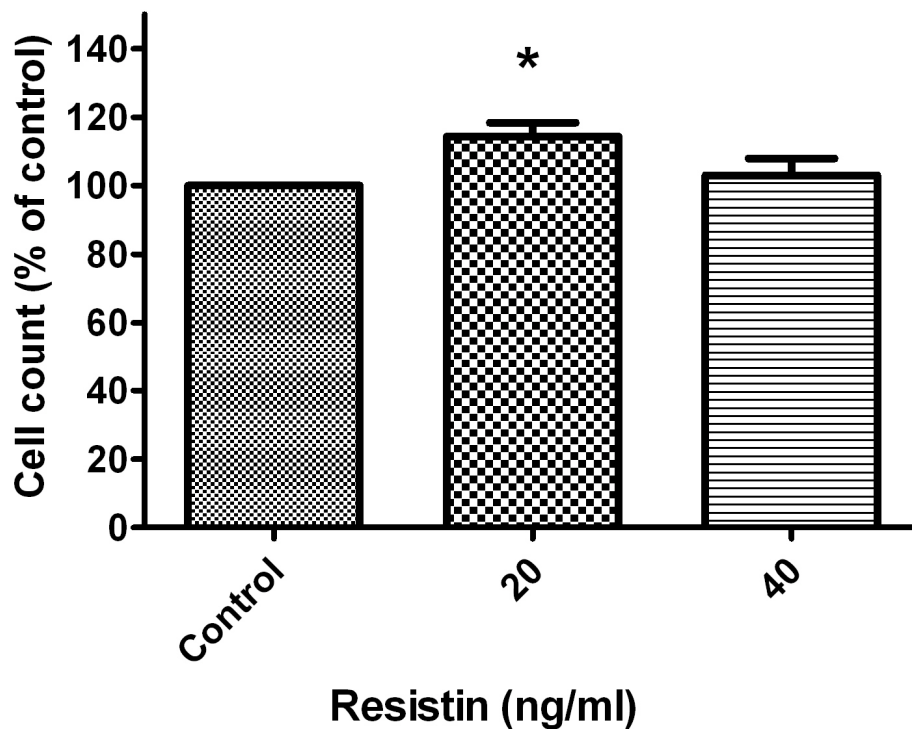


Figure 3.1.2 Resistin increases β TC-6 cell total cell number. Total cell count determined using a Coulter counter. The experiment was repeated three times. * $p \leq 0.05$ using ANOVA for the comparison between untreated and resistin treated cells ($n = 3$).

3.3.1.3. The effect of resistin on serum-depletion induced β -cell death

To determine whether resistin can protect β -cells against serum depletion induced cell death, mouse β TC-6 cells were cultured with or without resistin for 48 hours. Sub-confluent β TC-6 cells were treated with a range of resistin concentrations (0-40ng/ml) in serum deprived media. As expected, serum withdrawal over 48 hours triggered cell death of the β TC-6 cells however resistin did not exert any protective effects from cell death induced by serum depletion at any of the concentrations tested.

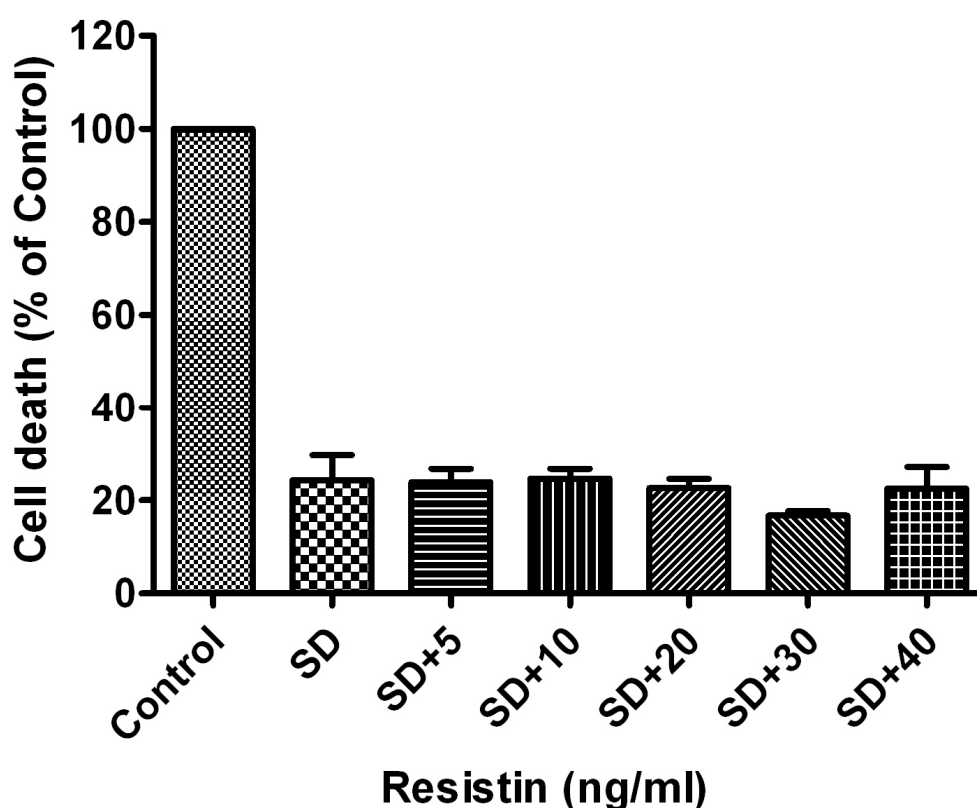


Figure 3.1.3 Resistin does not protect β TC-6 cells from serum-depletion induced cell death. β TC-6 cells were cultured in varying concentration of resistin (5 to 40ng/ml) for 48 hours. After incubation, viability of the cells was measured using modified MTS assay. The results show that resistin did not have any protective effects from serum depletion induced cell death (n = 5). SD=serum depletion without resistin; control= complete media; SD+5, 10, 20, 30 and 40=doses of resistin

3.3.1.4. The effect of resistin on β -cell cell viability over 48 hours

The previous results showed that resistin promotes β -cell viability by limited increase in proliferation but without attenuating cell death. To further explore the long term effects of resistin, viability of the β -cells over 48 and 72 hours was determined. As illustrated in Figure 3.1.4., the effect of resistin on cell viability declined after 48 hours with a slight non-significant increase at 1 and 5ng/ml in cell viability and a non-significant downward trend in cell viability with increasing resistin concentrations compared to basal.

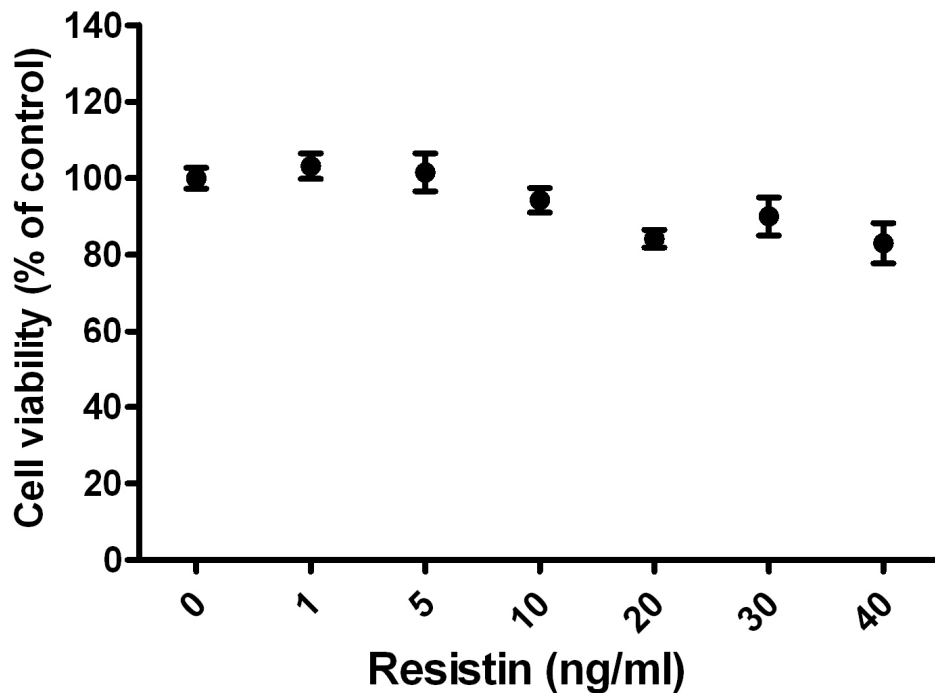


Figure 3.1.4 Resistin treatment has no significant effect on cell viability over 48 hours. The cells were treated with resistin for 48 hours in media supplemented with 1% serum and cell viability determined using modified MTS assay. The β -cells are particularly sensitive to oxidative stress resulting from withdrawal of survival factors in serum, for this reason treatment medium for the β -cells was supplemented with 1% serum for long term study (48 and 72 hours) (n = 5).

3.3.1.5. The effect of resistin on β -cell cell viability over 72 hours

Further treatment with resistin over 72 hour period resulted in non-significant increase in cell viability of β TC-6 cells at 1, 5 and 10ng/ml and a non-significant downward trend in at 30 and 40ng/ml. This result shows that prolonged exposure to resistin has no significant effect in promoting survival of the mouse β TC-6 cells.

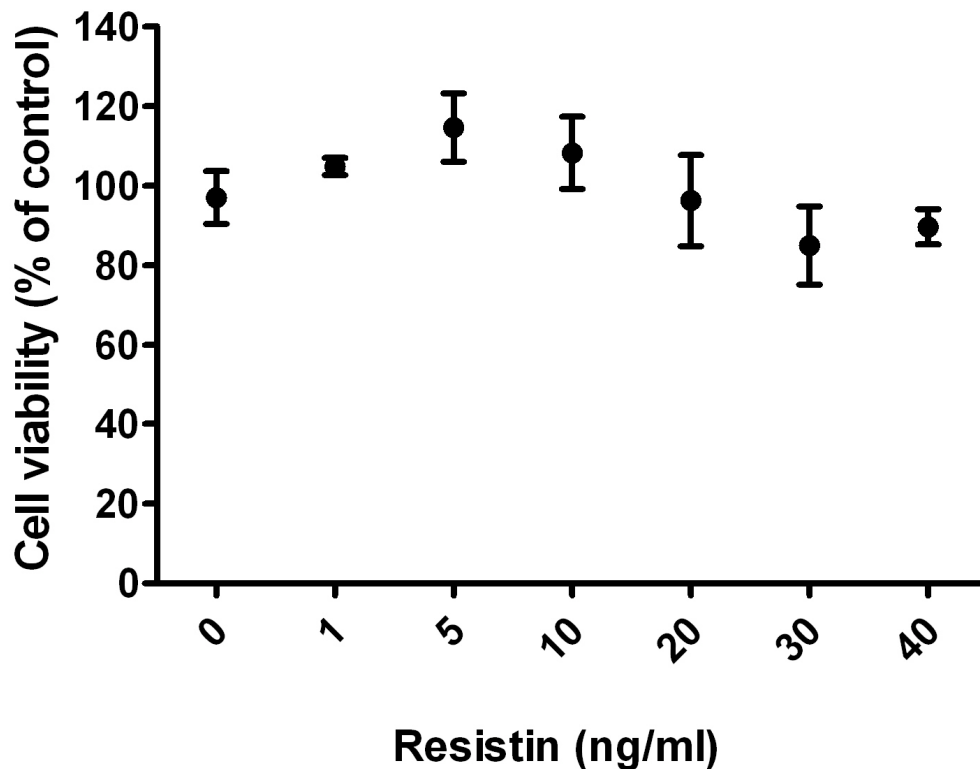


Figure 3.1.5 Resistin treatment results in a non-significant increase in cell viability after 72 hours. β TC-6 cells (1×10^4 cells per well) were plated in a 96 well plate. The cells were treated with resistin (0-40ng/ml) for 72 hours in medium supplemented with 1% serum and cell viability determined using modified MTS assay. Results are expressed as percent relative to control cells in serum-free medium (n = 5).

3.3.1.6. The effect of 24-hour exposure to low visfatin concentration on β cell viability

The effect of visfatin at low circulating concentrations on β TC-6 cells was tested. Exposure of the cells to visfatin at low circulating concentrations for 24 hours did not induce any significant effect in cell viability. The results show that visfatin treatment at levels similar to those found in circulation neither promotes nor attenuates cell viability of the β -cells over 24 hours.

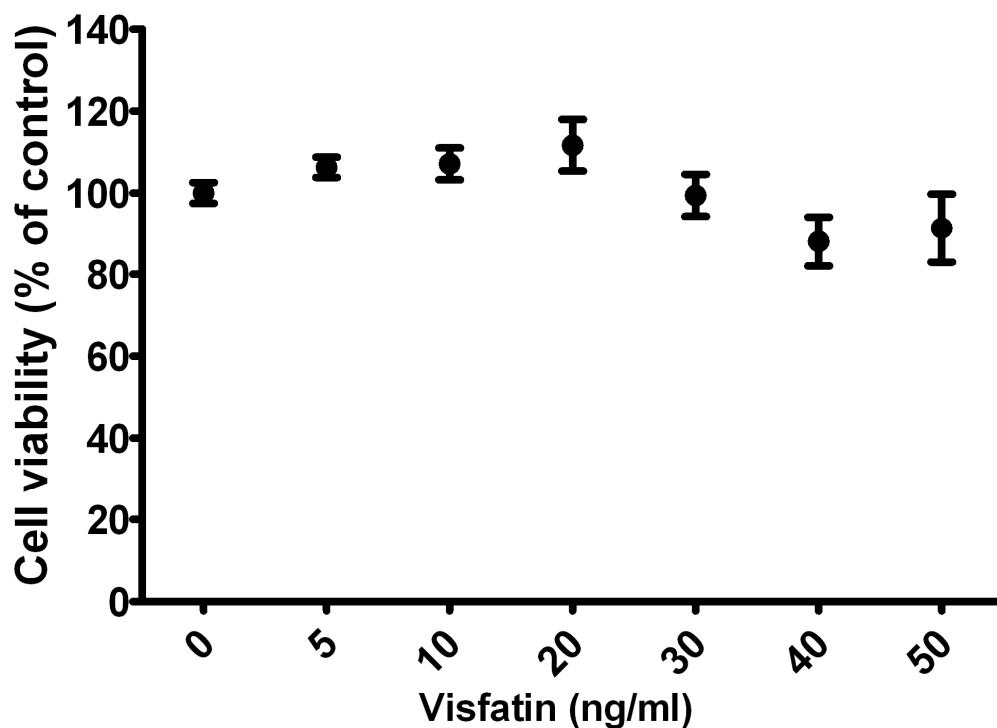


Figure 3.1.6 Visfatin has no effect on β TC-6 cell viability over 24 hours. β TC-6 cells (2×10^4 cells per well) were seeded out in 96 well and treated with visfatin for 24 hours. Cell viability was determined using modified MTS assay. Results are expressed as percent relative to control cells in serum-free medium (n= 5).

3.3.1.7. The effect of 48-hour exposure to low visfatin concentrations on β -cell viability

Treatment of β TC-6 cells with visfatin for 48 hours at concentrations from 0 to 50ng/ml resulted in no significant effect on cell viability of β TC-6 cells.

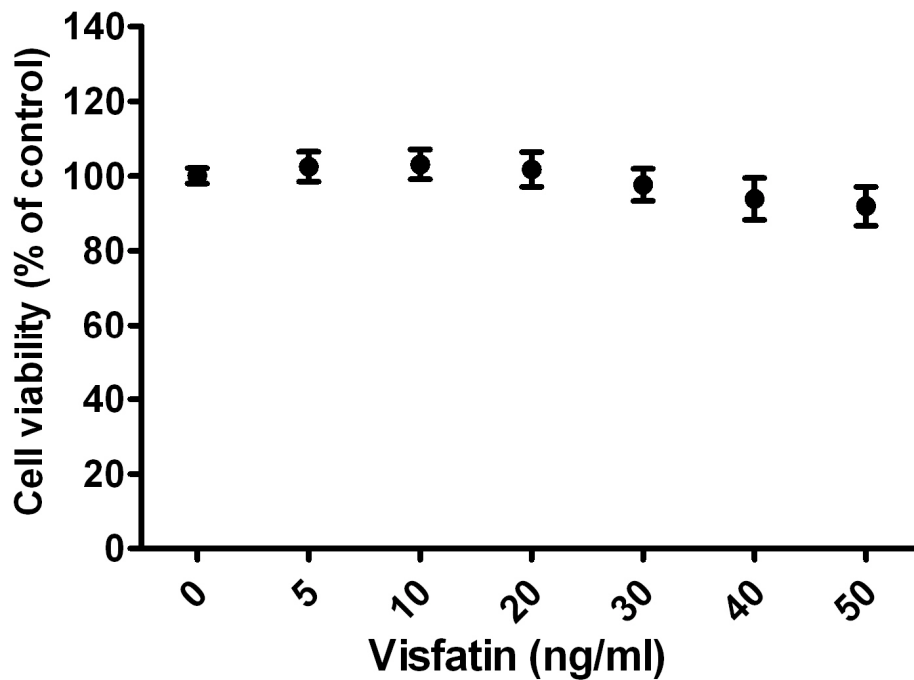


Figure 3.1.7 Low visfatin concentration does not promote cell viability over 48 hours. β TC-6 cells (1×10^4 cells per well) were plated in a 96 well plate and treated with visfatin for 48 hours in media supplemented with 1% serum. Results are expressed as percent relative to control cells in serum-free medium (n=5)

3.3.1.8. The effect of 72-hour exposure to low visfatin concentrations on β -cell viability

Subsequent treatment with visfatin over 72 hour period resulted did not have a significant effect on cell viability of β TC-6 cells. This result shows that prolonged exposure of the cells to visfatin does not promote survival of the mouse β TC-6 cells

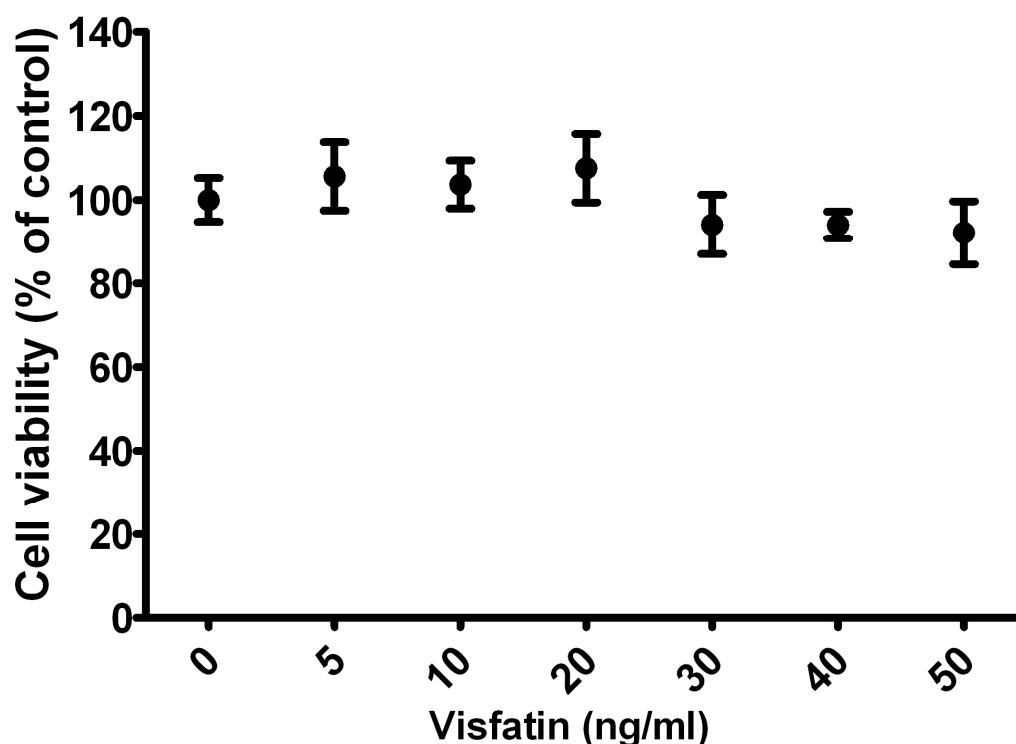


Figure 3.1.8 Effects of visfatin on cell viability of β TC-6 cells over 72 hours. β TC-6 cells (1×10^4 cells per well) were plated in a 96 well plate. The cells were treated with visfatin for 72 hours in media supplemented with 1% serum and cell viability determined using modified MTS assay. Results are expressed as percent relative to control cells in serum-free medium (n=5).

3.3.1.9. The effect of 24-hour exposure to high visfatin concentrations on β -cell viability

Circulating visfatin concentration has been shown range from 0.4ng/ml in healthy children (Haider *et al.*, 2006b) to 90ng/ml in gestational diabetes (Krzyzanowska *et al.*, 2006a) with other studies proposing a possible higher concentration as there is only one ELISA test kit for visfatin that is presently commercially available (Phoenix Peptides, Karlsruhe, Germany). A higher concentration of visfatin compared to low circulating concentration has also been proposed due to the dramatic elevation of visfatin levels in the visceral adipose tissue of obese mice (Sethi and Vidal-Puig, 2005). Exposure of β cells to high concentrations of visfatin for 24 h resulted in a gradual decrease in cell viability with a significant decrease observed at 500ng/ml.

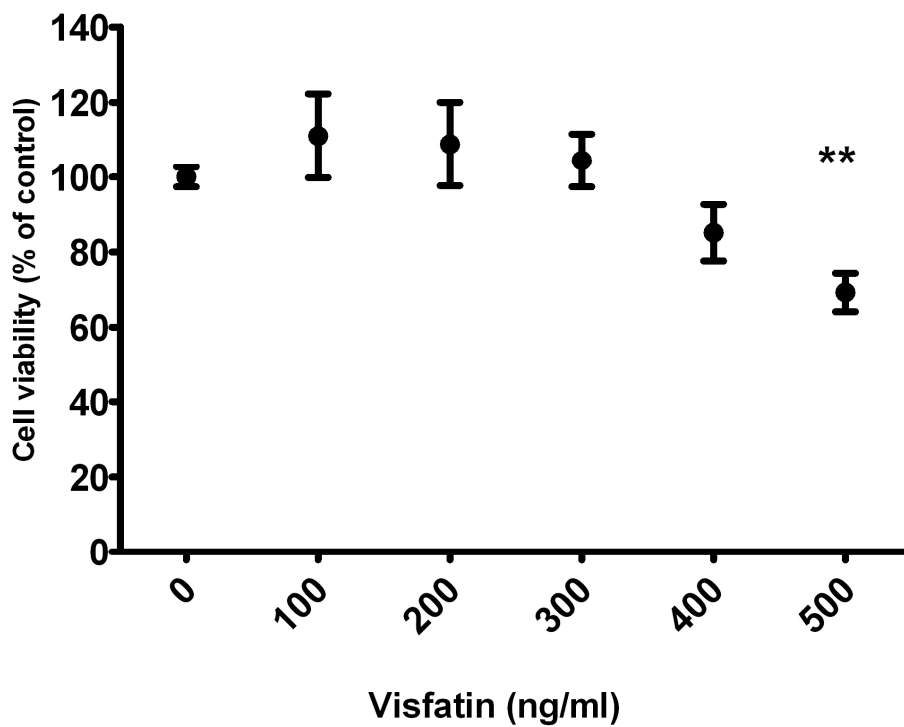


Figure 3.1.9 Visfatin decreases β TC-6 cell viability at high concentrations. β TC-6 cells were plated in a 96 well plate and cell viability determined using modified MTS assay. Results are expressed as percent relative to control cells in serum-free medium (n=5). The values represent means and standard error of the mean (SEM). ** denotes significant (P < 0.01)

3.3.2.0. The effect of 48-hour exposure to high visfatin concentrations on β -cell viability

To further investigate the long term effect of high visfatin concentrations, the mouse β cells were exposed for 48 or 72 hours to varying high concentrations of visfatin in treatment media supplemented by 1% serum. A relatively marked effect was observed when the β -cells were exposed to these elevated levels over longer time episodes. Unlike 24h, both 400 and 500ng/ml treatments significantly reduced viability of the β cells.

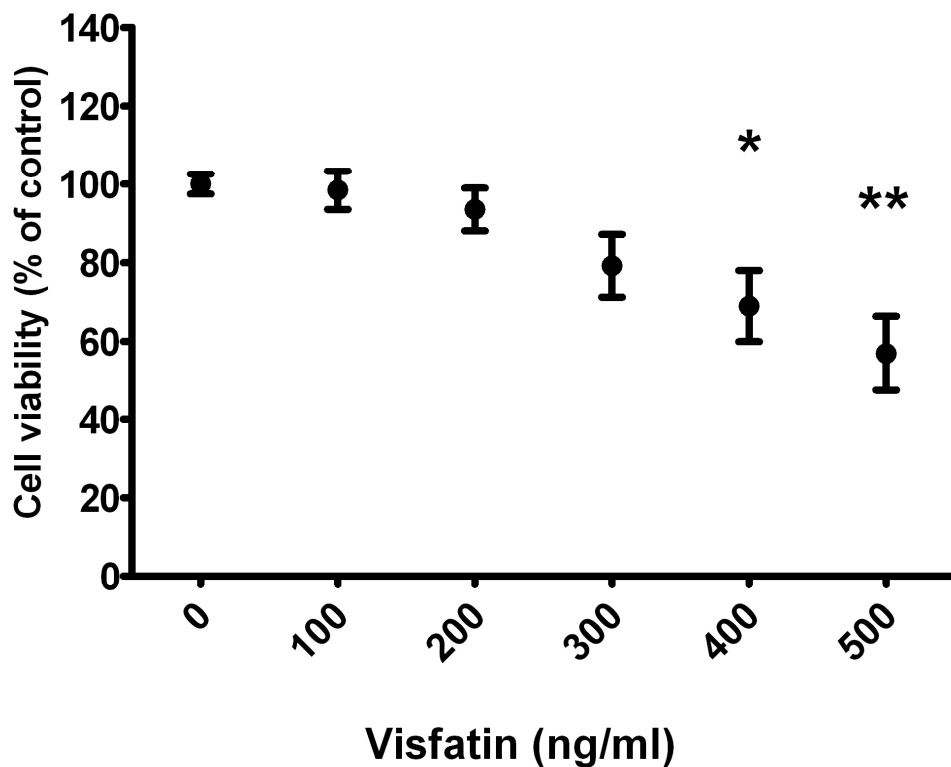


Figure 3.2.0 Visfatin decreases β TC-6 cell viability at high concentrations over 48 hours. β TC-6 cells were treated with visfatin for 48 hours in medium supplemented with 1% serum, and cell viability determined. There was a significant decrease in cell viability at concentrations of 400 and 500ng/ml. The values represent means and standard error of the mean (SEM). * $p \leq 0.05$ and ** $p \leq 0.01$ using ANOVA and Tukey's multiple comparison test for the comparison between untreated and visfatin treated cells. (n=5).

3.3.2.1. The effect of 72-hour exposure to high visfatin concentrations on β -cell viability

The exposure of the β TC-6 cells in long-term culture to elevated levels of visfatin for 72 hours resulted in significant reduction in cell viability. There was no effect on cell viability between 100 to 300ng/ml however a significant decline was evident at 400 and 500ng/ml treatment compared to untreated control.

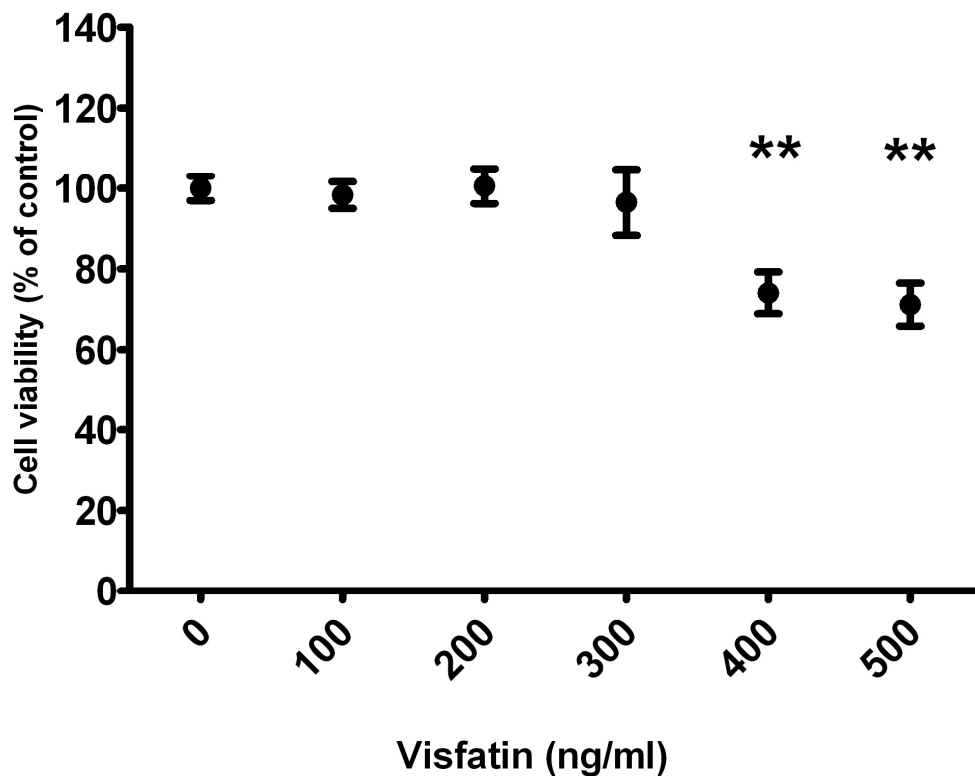


Figure 3.2.1 Visfatin decreases β TC-6 cell viability at high concentrations over 72 hours. β TC-6 cells (2×10^4 cells per well) were plated in 96 well plate. The cells were treated with visfatin for 72 hours in medium supplemented with 1% serum, and cell viability determined using modified MTS assay. There was a significant decrease in cell viability at concentrations of 400 and 500ng/ml. ** $p \leq 0.01$ using ANOVA and Tukey's multiple comparison test for the comparison between untreated and visfatin treated cells. (n=5).

3.3.2.2. The effect of 24-hour exposure to NMN on β -cell viability

To further address the mechanism by which elevated levels of visfatin regulate β -cell viability, the role of NMN (a product from the visfatin enzymatic reaction) on viability of the β cells was assessed. The cells were cultured for 24 hours in serum free media with various treatment concentrations of NMN. As for 48 and 72 hours, treatment media supplemented with 1% serum in the absence or presence of NMN was used. The results show that NMN did not influence the viability of the β TC-6 cells and parallel experiments performed over 48 and 72 hours demonstrated that longer term exposure to NMN by itself has no influence β cell viability.

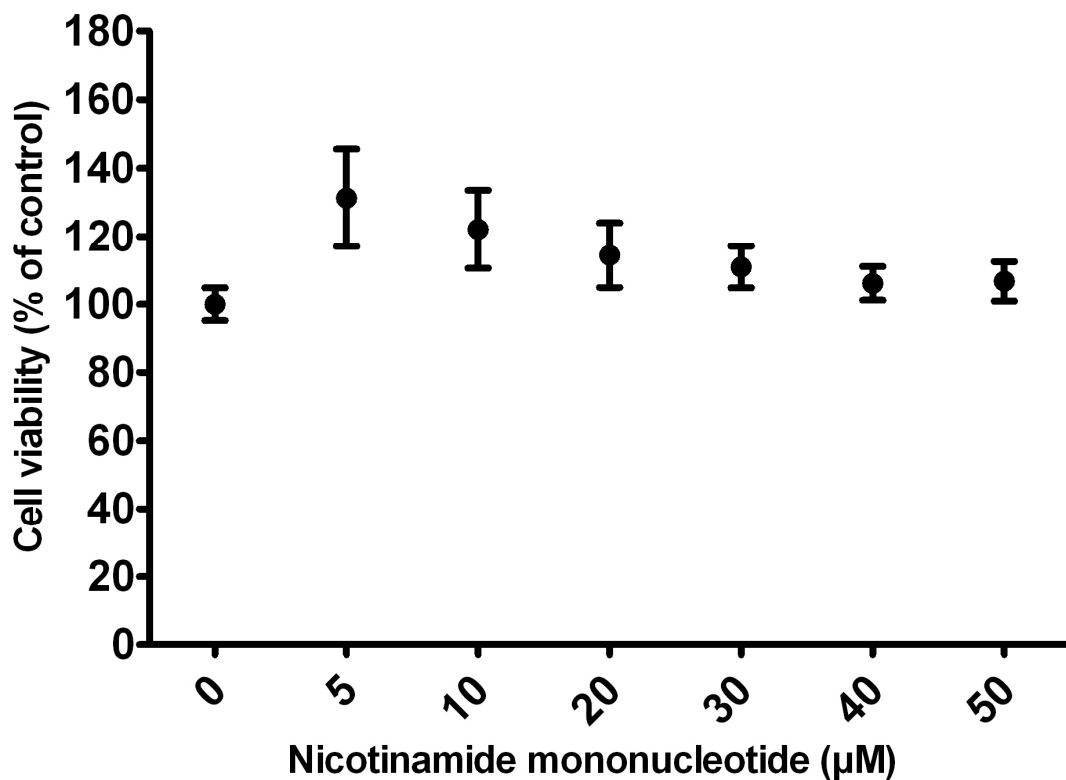


Figure 3.2.2 NMN did not have any effect on beta cell viability over 24 hours. β TC-6 cells (2×10^4 cells per well) were plated in a 96 well plate. The cells were treated with NMN for 24 hours, and cell viability determined using modified MTS assay as previously illustrated (n=5).

3.3.2.3. The effect of 48-hour exposure to NMN on β -cell viability

To further evaluate the long term effects of NMN on β cell viability, the cells were subjected to treatment over 48 hour period. Exposure of β TC-6 cells to NMN for 48 hours resulted in a small but non-significant decrease viability of the beta cells at highest concentration.

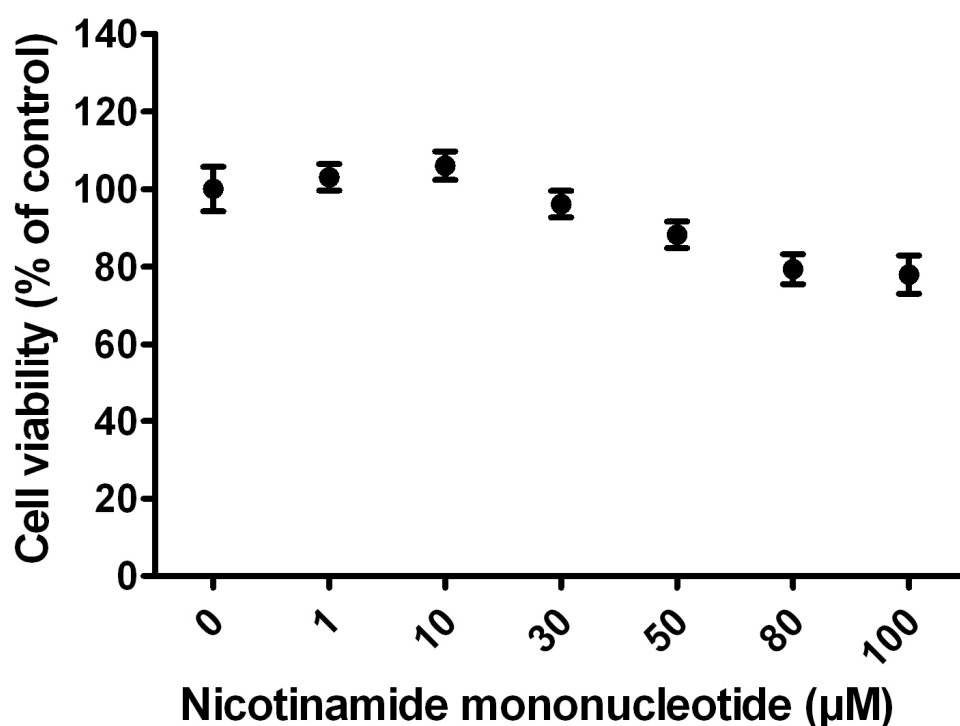


Figure 3.2.3 Exposure to NMN results in a non-significant decrease β cell viability over 48 hours. β TC-6 cells (2×10^4 cells per well) were plated in a 96 well plate. The cells were treated with NMN for 48 hours in media supplemented with 1% serum, and cell viability determined using modified MTS assay as previously described (n=5).

3.3.2.4. The effect of 72-hour exposure to NMN on β -cell viability

72 hour treatment of NMN treatment resulted in a small but non-significant decrease viability of the beta cells at 100nM.

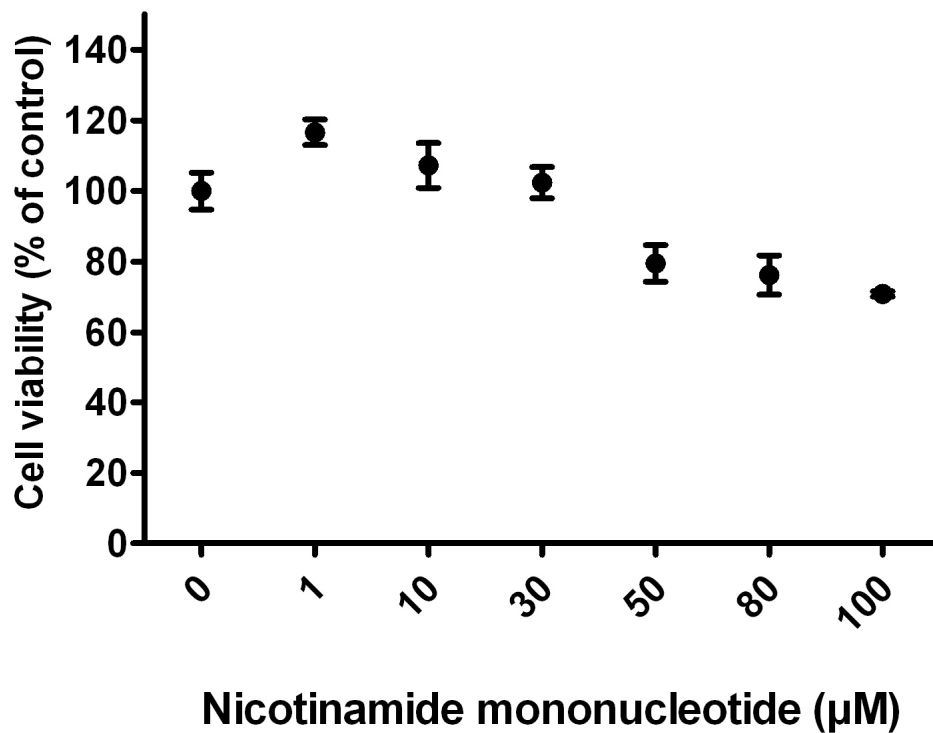


Figure 3.2.4 Exposure to NMN results in a non-significant decrease β cell viability over 72 hours. β TC-6 cells (2×10^4 cells per well) were plated in 96 well plate. The cells were treated with NMN for 72 hours in media supplemented with 1% serum, and cell viability determined using modified MTS assay as previously described. (n=5).

3.4. Discussion

The capacity of β -cell mass to increase in the face of insulin resistance is very important to β -cell function because it prevents or delays the onset of T2D (Del Prato *et al.*, 2004). Proliferation of pancreatic β -cells is a major event in the development of β -cell mass, thus there is considerable interest in understanding the mechanisms that stimulate pancreatic islet cell growth and differentiation. Little is known about the capacity, mechanisms, or timing of growth in β -cell mass in humans and animals. In as much as numerous advances have been made, major gaps in understanding the developmental biology of the β -cell remain to be elucidated.

Varieties of hormones, growth factors, metabolites and cytokines have been implicated in the regulation of pancreatic β -cell viability (Saldeen, 2000). To evaluate the direct effects of resistin on β -cell viability, serum-starved cells were treated for 24 hours with increasing concentrations (0 to 40ng/ml) of resistin. For the duration of 24 hour treatment, as has been reported in a publication based on this study (Brown *et al.*, 2007), resistin promotes cell viability in clonal mouse pancreatic β TC-6 cells. This observation is supported by two studies in a different cell type which showed that resistin promotes cell activation, proliferation and migration in endothelial cells (Verma *et al.*, 2003; Mu *et al.*, 2006).

The maximal stimulatory effects of resistin of up to 45% on β TC-6 cell viability was achieved at concentration of 20 ng/ml ($P < 0.01$) as compared to the untreated control, which is comparable with the clinical plasma levels of resistin in lean non-diabetic subjects (Fujinami *et al.*, 2004; Yannakoulia *et al.*, 2003). A higher dose of 40 ng/ml which is the reported concentration in obesity and diabetic patients (Fehmann *et al.*, 2002; Zhang *et al.*, 2003) did not promote viability as compared to the untreated control ($P > 0.05$). These data in Figure 3.1.1 strongly suggest that resistin is involved in β -cell survival and confirm the effect of resistin on continued existence of pancreatic β -cells. Based on these observations, it is possible that at the lower and more 'physiological' levels, resistin may act as a beta-cell growth factor maintaining beta-cell mass from stress induced by excessive accumulation of fat in adipose tissue. However at the much higher 'pathological' concentrations, the observed proliferative

effect diminishes, potentially suggesting that the protective effective could be defective in times of elevated serum resistin concentrations.

Studies in rodents have shown that there is a compensatory increase in β -cell mass in response to insulin resistance or obesity (Bonner-Weir *et al.*, 2000). Thus the stimulatory effect of resistin illustrated above highlights the potential role of this adipokine in β -cell survival. Clinically, this is a vital novel finding since protection of pancreatic β -cells from stress and inflammatory induced cell death is key to developing successful therapeutic interventions.

The mode of action by which resistin exerts its effect on β -cell viability is still unknown. The possibility that increase in viability required induction of proliferation and / or decrease in cell death was considered. Thus in the next series of experiments, the effect of resistin on cell death and total cell number was determined. The results show an increase in cell number after 24 hour treatment with 20ng/ml resistin compared to untreated control and no effect resulting from 40ng/ml treatment. The increase resulting from 20ng/ml resistin treatment was by a small margin of only 20% compared to control. This is not sufficient to sustain the cell survival in comparison with the trend observed in Figure 3.1.1 between concentrations of 5 to 20ng/ml. This implies that resistin may be promoting viability of the β -cells by increase in cell number and a different mechanism.

β -cell death was induced by the removal of survival factors that accompanies serum withdrawal. Serum starvation is a widespread working model for inducing cell death and apoptosis in different cell types (Granata *et al.*, 2004). As expected, culture of β TC-6 cells in medium devoid of serum led to a marked cell death over 48 h (Figure 3.1.3). However, the extent of this response was not reduced when the cells were also exposed to resistin at various concentration ranges of 1 to 40ng/ml during the period of serum withdrawal. Resistin did not exhibit any protective effects confirming that reduction in β -cell death was not required for the observed response in viability (Figure 3.1.1) and therefore doesn't protect from apoptosis at least in this model of cell death.

It is vital to note that resistin expression is up-regulated in the obese insulin resistant state in the adipose tissue of rodents and humans (Steppan and Lazar, 2004; McTernan *et al.*, 2002b), suggesting that this adipokine may provide a physiological adaptation by improving survival of the β -cells as they cope with increased insulin demand. Irrespective of the mechanisms involved, this study together with concurrent studies involving other cell types (Verma *et al.*, 2003; Mu *et al.*, 2006) has revealed that resistin has the unique capacity to promote viability via different pathways in pancreatic β -cells even though it is regarded as an inflammatory molecule.

The effect of resistin on cell viability of the β -cells over the long term was also examined. Resistin did not have any effect on β -cell viability over 48 and 72 hours. The time course study reveals that resistin prolonged survival of the β -cells in serum deprived media during the 24 hour episode however the effect diminished over the 48 and 72 hour periods. The observed effect over 24 hours is physiologically important because if cellular stress is not delayed or eliminated or cells fails to recover from the stressful insult, apoptosis ensues (Donath *et al.*, 1999).

Visfatin has only recently been discovered as an adipokine, and as such, its physiological and/or patho-physiological role in the β -cells is only beginning to emerge. In this study, the effects of a range of concentrations of visfatin on the viability of mouse β TC-6 cells were evaluated. Two concentration ranges were used: a set of concentrations mirroring that seen in circulation (0 to 50ng/ml) and a higher range of concentrations (100 to 500ng/ml) representing what might be seen in localised tissue where visfatin is secreted. The low visfatin concentrations did not elicit any effect on the viability of the β -cells (Figure 3.1.6, 3.1.7. and 3.1.8.). This suggests that low circulating concentrations of visfatin are not physiologically vital in β -cell survival.

Treatment with the high visfatin concentrations resulted in a significant decrease in viability of the β -cells. The modified MTS assays revealed that cell viability was significantly impaired in β TC-6 cells after 24h, 48h and 72h treatments (Figure 3.1.9, 3.2.0. and 3.2.1.). The elevated visfatin concentrations may in part mimic the situation in type 2 diabetes where β -cells are already insulted by various stressful conditions such as exposure to high cytokine concentration and oxidative stress (Robertson *et al.*,

2004; Akerfeldt *et al.*, 2008). In one previous study, circulating visfatin was shown to be increased with progressive beta-cell deterioration (López-Bermejo *et al.*, 2006). The observations outlined in this study (Figure 3.1.9, 3.2.0. and 3.2.1) might be directly correlated to these previous reports and help to illustrate the negative regulatory influence of high visfatin concentrations on the β -cells in obese and T2D states. The precise mechanisms involved in this cytotoxic action of visfatin remain to be defined, but are probably multifactorial.

One major highlight is the decline in cell survival as a result of the high visfatin concentration range used and time episode employed. From the results illustrated in Figure 3.1.9, 3.2.0 and 3.2.1, treatment with elevated levels of visfatin is more potent in decreasing cell viability over longer period of time. There was no effect with 400ng/ml visfatin over 24 hours; however a significant decrease ($p < 0.05$) in viability of the β -cells was observed with this concentration (400ng/ml) after 48 and 72 hours. Based on these observations, it is conceivable that prolonged exposure of β -cells to elevated concentrations of visfatin as observed in the obese state may lead to reduced survival and subsequent β -cell dysfunction.

Visfatin has recently been shown to exhibit robust NAD⁺ biosynthetic activity with NMN acting as a key intermediate (Revollo *et al.*, 2004). To investigate whether the observed effects of visfatin was via the production of NMN, the effect of NMN on cell viability was investigated. As illustrated in Figure 3.2.2, 3.2.3 and 3.2.4 NMN did not have any effect on cell viability of the β cells both in the short term (24 hours) and long term (48 and 72 hours) suggesting that visfatin may elicit this effect through a different and yet to be identified pathway. This might be a cell specific effect as NMN has been shown to upregulate cell viability in prostate cancer cells (personal communication Dr J Brown, University of Warwick). Since visfatin receptor is yet to be identified its mode of action remains elusive. Reversing the deleterious effects of elevated visfatin concentrations and understanding possible mechanism of action could thus prove a vital tool in improving β -cell function.

Chapter 4

Gene expression profiling of cultured clonal pancreatic β -cells

4.1. Introduction

The expression of functional and regulatory genes by pancreatic β -cells is vital to maintaining glucose homeostasis and ensuring secretion of sufficient insulin in response to glucose to meet the metabolic demand. Dysregulation of any of the β -cell specific genes could result in alterations in β -cell differentiation, survival and insulin secretion, all of which are potentially implicated in the onset of T2D (Kutlu *et al.*, 2009).

The adipose tissue is now known to be a central player in the pathogenesis of insulin resistance associated with obesity (Kershaw and Flier, 2004). Circulating adipokines regulate the function of pancreatic β -cells at multiple levels: Adiponectin has previously been shown to rescue the β -cells from cytokine and fatty acid-induced apoptosis (Rakatzi *et al.*, 2004) and play a key role in mediating insulin resistance in the β -cells (Retnakaran *et al.*, 2005) while leptin has been shown to suppress insulin secretion by the activation of ATP-sensitive K⁺ channels in pancreatic beta-cells (Kieffer *et al.*, 1997). Chronic exposure to elevated levels of adipokines is thus likely to modulate β -cell function over the longer term and eventually bring about adaptive changes in gene expression. Regulation of β -cell and adipocyte specific gene expression is one of the most important targets for the prevention of obesity and amelioration of insulin sensitivity (Shepherd *et al.*, 2009).

The aim of this study was to assess the effect of the adipokines resistin and visfatin on the expression profile of genes involved in obesity, β -cell function and onset of T2D. To do this, mouse β TC-6 cells were exposed for 24 h to resistin or visfatin. The expression profile of the related genes was analyzed using Mouse Diabetes RT² Profiler™ PCR Array kit.

4.2. Materials and methods:

Details of materials and methods used in the experiments for this chapter can be found in chapter 2

4.3. Results

4.3.1. Effects of resistin on gene expression profile in mouse β TC-6 cells

RT-real time PCR array analysis of transcripts expressed in β TC-6 cells exposed to resistin resulted in altered patterns of gene expression. A total of 84 gene transcripts were analysed. Upon analysis of the regulated genes, 14 genes were identified which were significantly up-regulated while 5 genes were significantly downregulated. Table 4.1 shows details of the genes that were significantly altered after resistin treatment. A list of all the gene transcripts analysed but which were not significantly altered by resistin treatment can be found in Appendix 2.1.

	Description	Fold Change	p value
Ace 1	Angiotensin-converting enzyme	-1.382	0.00153
CD28	CD28 antigen	1.447	0.00295
Ctla4	Cytotoxic T-lymphocyte-associated protein 4	3.102	0.0287
Foxc2	Forkhead box C2	2.297	0.0345
G6pc	Glucose-6-phosphatase, catalytic	1.350	0.00101
G6pd2	Glucose-6-phosphate dehydrogenase 2	3.102	0.00706
Gcg	Glucagon	-1.047	0.0392
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	3.102	0.0310
HNF4a	Hepatic nuclear factor 4	1.047	0.0457
Il6	Interleukin 6	1.447	0.0261
Inpp1l	Inositol polyphosphate phosphatase-like 1	2.194	0.0381
Ins1	Insulin I	1.023	0.0443

Mapk14	Mitogen-activated protein kinase 14	2.297	0.0118
Mapk8	Mitogen activated protein kinase 8	2.297	0.0329
Pax4	Paired box gene 4	-1.414	0.0528
Pfkfb3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	17.549	0.00962
Pik3cd	Phosphatidylinositol 3-kinase catalytic delta	31.269	0.0260
Retn	Resistin	-1.259	0.000656
Slc2a4	Solute carrier family 2 (facilitated glucose transporter),member 4	2.701	0.0352
Stx4a	syntaxin 4A (placental)	17.549	0.00582
Tcf2	Transcription factor 2	32	0.000214
Vamp3	Vesicle-associated membrane protein 3	-6.349	0.0269
Vegfa	Vascular endothelial growth factor A	-4.702	0.0103

Table 4.1 RT-PCR array data for β -cells treated with resistin. Gene Expression Analysis of resistin treated β TC-6 cells using Mouse Diabetes RT² Profiler™ PCR Array. Analysis of differential expression was performed using the spreadsheet provided by the array company. Significant differences between the treatment and the control samples were analyzed with a 2-tailed student t-test ($p < 0.05$, $n = 3$). Values represented are average fold change for significantly regulated gene compared with non-treatment control ($p < 0.05$). CD28, Ctla4, Foxc2, G6pc, G6pd2, Gpd1, HNF4a, Il6, Inpp11, Ins1, Mapk14, Mapk8, Pfkfb3, Pik3cd, Slc2a4, Stx4a, Tcf2 genes were significantly upregulated by resistin treatment whilst Ace 1, Gcg, Pax4, Retn, Vamp3, Vegfa genes were significantly downregulated compared to control cells.

4.3.2. Schematic representation of resistin regulation of insulin sensitivity based on the results from PCR array study

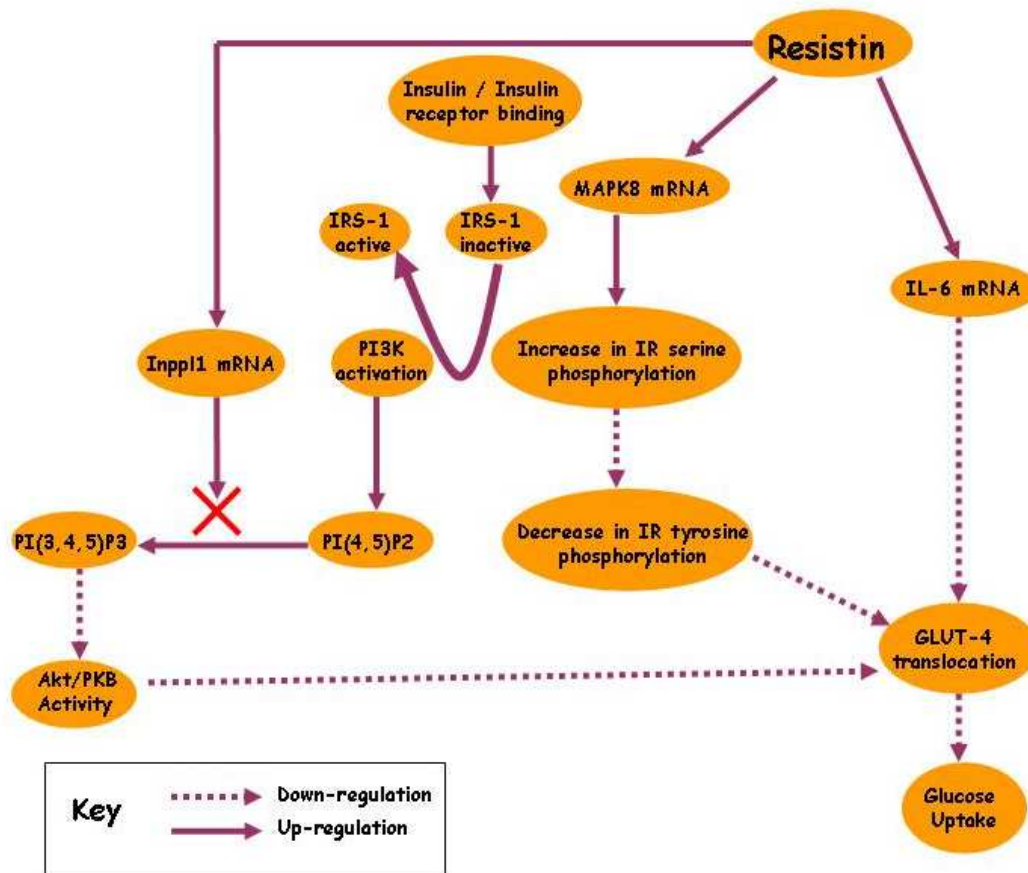


Figure 4.1 Effect of resistin on mRNA expression of Mapk8, Inpp1 and IL-6 and potential downstream effects in insulin signalling pathways. The phosphorylated insulin receptor binds to and phosphorylates IRS proteins, which in turn phosphorylates various downstream signaling proteins. Of critical importance is PI3K which makes PI(3,4,5)P3 from PI(4,5)P2 upon stimulation. Increase in Inpp1 mRNA by resistin could potentially result in decreased PI(3,4,5)P3 synthesis and inhibit the actions of downstream molecules e.g. Akt/PKB and subsequent decrease in glucose uptake. Mapk8 has been shown to increase IR serine phosphorylation. Up-regulation of Mapk8 by resistin could thus promote this effect and subsequently reduce IR tyrosine phosphorylation which in turn decreases Glut-4 translocation. The same effect is observed with IL-6 has also been shown to inhibit Glut-4 translocation. Key: IR=insulin receptor, IRS=insulin receptor substrate, PI3K=Phosphatidylinositol-3 kinase, PIP3= phosphatidylinositol triphosphate

4.3.3. Effects of visfatin on gene expression profile in mouse β TC-6 cells

RT-real time PCR array analysis of 84 genes was performed to reveal genes regulated by visfatin. It is evident that visfatin treatment significantly alters the expression patterns of genes involved in inflammation, including proinflammatory cytokines and genes associated with obesity, insulin secretion, insulin resistance and the early onset of T2D. Comparison of differentially expressed genes between the visfatin treated cell samples and untreated controls indicated that a total of 12 genes were significantly altered ($p < 0.05$). The details are illustrated in Table 4.2. A list of all the gene transcripts analysed but which were not significantly altered by visfatin treatment can be found in Appendix 2.2.

	Description	Fold Change	p value
Ace 1	Angiotensin-converting enzyme	-3.732	0.0157
Akt2	Protein kinase B, beta	24.251	0.000668
CD28	CD28 antigen	97.006	0.00251
CeaCam	Carcinoembryonic antigen-related cell adhesion molecule pseudogene 1	-2.828	0.0178
DPPIV	Dipeptidyl-peptidase 4	1.0968	0.0751
HNF4a	Hepatic nuclear factor 4	16	0.0118
Ins1	Insulin I	8.979	0.0369
Mapk8	Mitogen activated protein kinase 8	-1.122	0.0386
Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	40.317	0.0189
PPARa	Peroxisome proliferator activated receptor alpha	-1.259	0.0434
Stx4a	Syntaxin 4A (placental)	17.549	0.0352
Tcf2	Transcription factor 2	32	0.00313
UCP2	Uncoupling protein 2, mitochondrial	-1.289	0.00479

Table 4.2 RT-PCR array data for β cells treated with visfatin. Gene Expression Analysis of visfatin treated mouse β TC-6 cells using Mouse Diabetes RT² Profiler™ PCR Array. The Superarray system compared the relative levels of mRNA expressed in β TC-6 cells with or without visfatin treatment. Analysis of differential expression was performed using the spreadsheet provided by the array company. Significant differences between the treatment and the control samples were analyzed with a 2-tailed student t-test ($p < 0.05$, $n = 3$). Values represented are average fold change for each gene compared with non-treatment control. Akt2, CD28, DPPIV, HNF4a, Ins1, Nfkb1, Stx4a, Tcf2 mRNA was significantly upregulated upon visfatin treatment whilst Ace 1, CeaCam, Mapk8, PPARa, UCP2 genes were significantly downregulated.

4.3.4 Schematic representation of visfatin regulation of insulin secretion based on the results from PCR array experiments

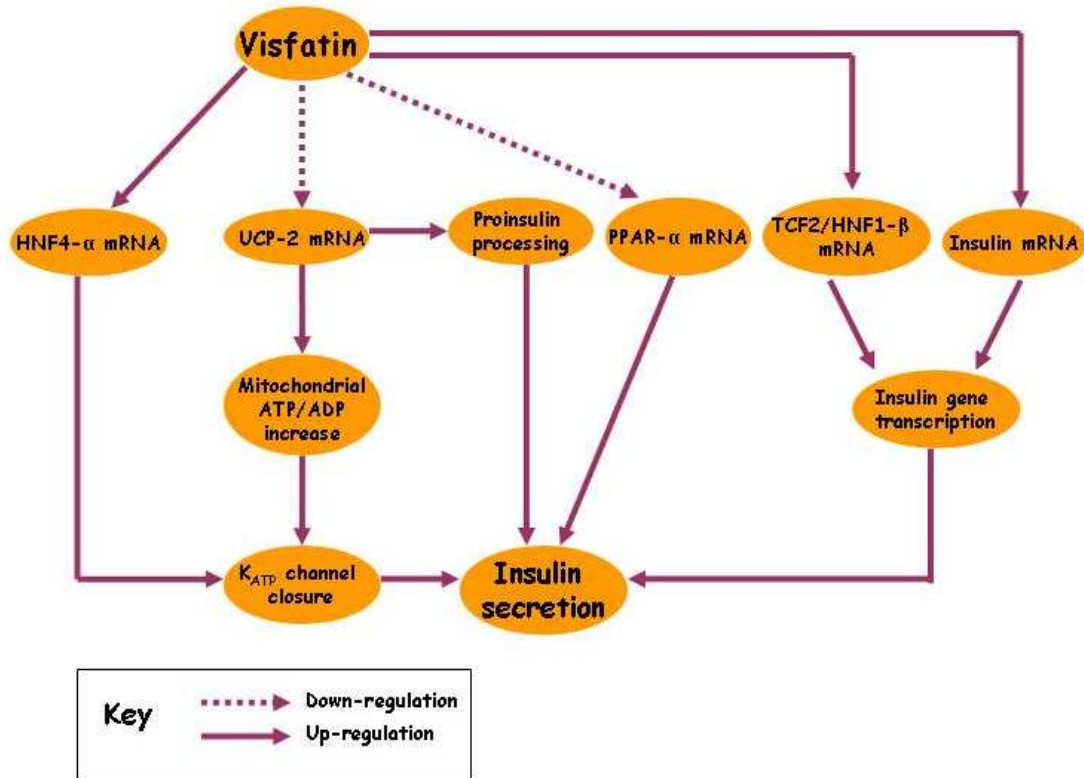


Figure 4.2 The role of visfatin in the regulation of insulin secretion. The potential pathways involved in visfatin induced insulin secretion are schematically depicted. Loss of HNF4- α function has previously been shown to result in reduced insulin secretion via K_{ATP} channel activity. Upregulation of HNF4- α mRNA by visfatin highlights a possible pathway of visfatin induced insulin secretion. UCP2 proteins have been shown to impair mitochondrial ATP generation and alter K_{ATP} channel activity with subsequent reduced insulin secretion. Down-regulation of UCP-2 mRNA by visafatin illustrates a potential pathway by which visfatin promotes insulin secretion. A similar effect is observed with decreased PPAR- α mRNA. Insulin gene transcription is a vital regulatory process in insulin biosynthesis, a process which precedes insulin secretion. TCF2 / HNF1- β is known to be involved in transcriptional regulation of the insulin gene and the observed increase in TCF2 and insulin mRNA by visfatin illustrates possible involvement of visfatin in this process.

4.3.5 Schematic representation of visfatin regulation of insulin sensitivity based on the results from PCR array experiments

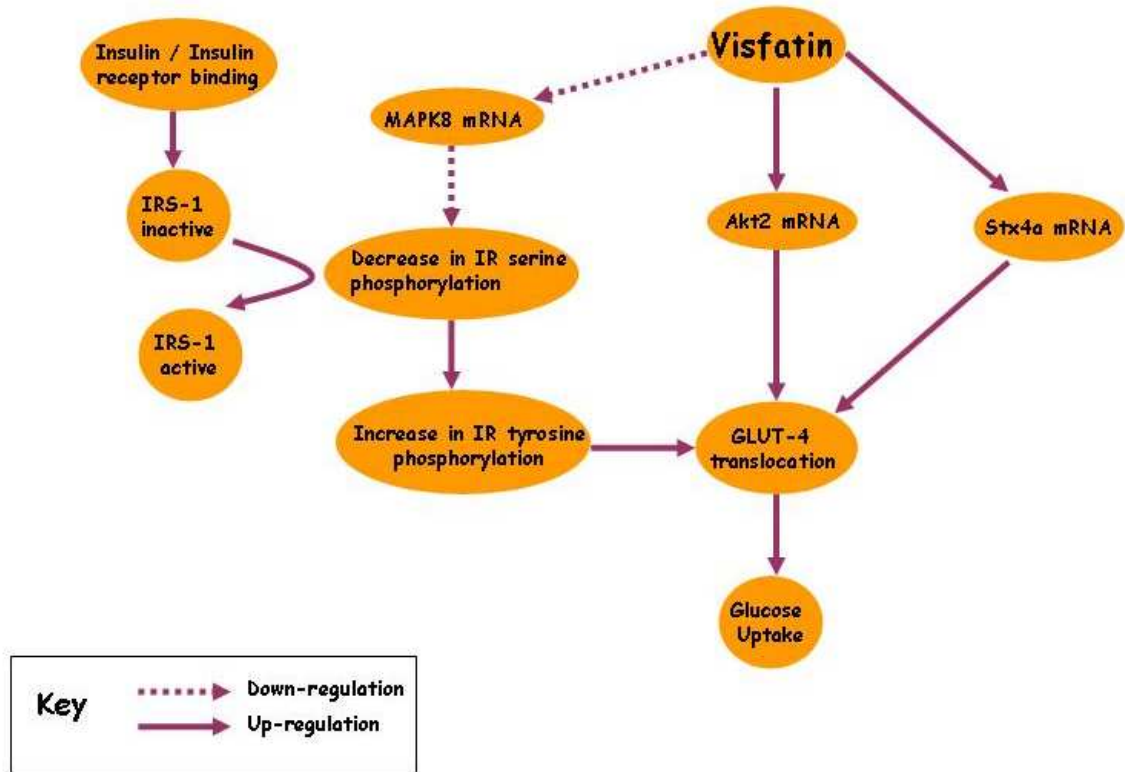


Figure 4.3 Schematic illustrations of genes regulated by visfatin and potential effects on signaling pathways of insulin and glucose uptake.

Akt2/PKB, a downstream molecule of PI3-kinase has been implicated in insulin-signaling pathway and known to regulate GLUT-4 trafficking. Akt2 mRNA upregulation by visfatin illustrates a possible pathway by which visfatin regulates glucose uptake. Mapk8 activation has been shown to be involved in serine phosphorylation of IR and subsequently reduced tyrosine phosphorylation of IR and glucose uptake. The observed reduced transcription activity of this molecule by visfatin highlights a possible pathway by which visfatin improves insulin sensitivity. Syntaxin 4 (Stx4a) knockout mice have previously been shown to have reduced insulin-stimulated glucose uptake with parallel reduction in GLUT-4 translocation. This highlights the role of this protein in glucose homeostasis and the observed upregulation by visfatin demonstrate a potential pathway in visfatin regulation of GLUT-4 vesicle trafficking and glucose uptake.

4.4. Discussion

In this study, the gene expression profile of clonal mouse pancreatic β -cells treated with resistin and visfatin was examined using real time PCR array. It is evident that treatment of β TC-6 cells with both visfatin and resistin leads to up-regulation or down-regulation of a number of genes involved in signal transduction and inflammation, including pro-inflammatory cytokines and genes associated with secreted factors and transcription activity. The current results provide novel observations that may help to define the underlying molecular pathways by which the adipokines resistin and visfatin may contribute to obesity-related changes in β -cell function and alterations that may result in insulin resistance and onset of T2D.

Elevated serum resistin in human diabetes and rodent diabetic models is often associated with inflammation (McTernan *et al.*, 2002a). The pro-inflammatory nature of resistin is more evident from the ability of the protein to stimulate the production of inflammatory cytokines including TNF- α and IL-12 (Silswal *et al.*, 2005). The present study shows that treatment of β -cells with 40ng/ml resistin resulted in up-regulation of iL-6 mRNA expression by 1.447 fold ($p=0.0261$), an observation which implicates this adipokine in potential inflammatory response in the β -cells. Interestingly, the concentration eliciting this response is associated with obesity, T2D and inflammatory response (Fehmann *et al.*, 2002; Zhang *et al.*, 2003). This observation is clinically significant as elevated levels of C-reactive protein (CRP) and IL-6 have previously been shown to predict the development of type 2 diabetes (Pradhan *et al.*, 2001). Interleukin-6 also induces insulin resistance in 3T3-L1 adipocytes (Rotter *et al.*, 2003). Conversely, IL-6 has also been shown to protect pancreatic β -cells from inflammatory cytokines-induced cell death (Choi *et al.*, 2004). It is thus possible that the promotion of cell viability of beta cells by resistin as observed in Figure 3.1.1 could be mediated by upregulation of IL-6 mRNA. This highlights a possible dual function of IL-6 in resistin activity.

The signalling pathways involved in resistin-induced insulin resistance are still poorly understood. The present study demonstrates that resistin treatment significantly upregulated Mitogen-activated protein kinase 8 (Mapk8) gene also known as c-Jun N-

terminal kinase 1 (JNK-1) by 2.297 fold ($p=0.0329$) compared to control. Mapk8 is known to affect insulin signalling and has been implicated in the progression of obesity and T2D in humans (Hirosumi *et al.*, 2002). In one published study, knockdown of Mapk8 / JNK1 gene expression by siRNA significantly increased GLUT-4 translocation in human embryonic kidney cells (Liu *et al.*, 2009) hence mRNA upregulation would be expected to elicit opposite effect. Mapk8 has also been previously reported to mediate fatty acid-induced cellular insulin resistance (Solinas *et al.*, 2006) which further illustrates the molecule's wider role in responding to activation by environmental stress and pro-inflammatory cytokines. The involvement of Mapk8 as a negative regulator of the insulin signalling and its up-regulation by resistin in the β -cells highlights a possible pathway by which resistin induces insulin resistance.

Among the metabolic enzyme-related genes, it is noteworthy that resistin increased the expression of mRNAs for glucose-6-phosphate dehydrogenase (G6pd2) by 3.102 fold ($p=0.00706$) and Glucose-6-phosphatase (G6pc) by 1.350 fold ($p=0.00101$). G6pc is a key gluconeogenic enzyme which catalyzes the hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate (Nordlie *et al.*, 1993). Overexpression of the glucose-6-phosphatase has previously been shown to attenuate glucose sensitivity of insulin secretion in clonal mouse pancreatic beta cells (Iizuka *et al.*, 2000) suggesting that resistin could have long term effects on β -cell function through this observed effect on G6pc. Up-regulation of G6pc mRNA by resistin as demonstrated in this study could potentially induce enzymatic activity of glucose-6-phosphatase and disrupt glucose stimulated insulin secretion. This association highlights a possible pathway by which resistin may disrupt beta cell secretory function (Nakata *et al.*, 2007).

Glucose-6-phosphate dehydrogenase is one of the major NADPH-producing enzymes which catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate (Pandolfi *et al.*, 1995). Overexpression of G6pd has previously been shown to result in obesity induced lipid metabolism disorders and insulin resistance (Park *et al.*, 2005). Metabolic diseases including T2D are closely associated with a failure of lipid homeostasis, however molecular mechanisms underlying lipid metabolism disorders have not been clearly understood (Kahn BB and Flier JS., 2000). G6pd expression

levels in the β -cells may thus be a promising indicator of obesity related insulin resistance and defects in insulin secretion arising from fatty acid toxicity. Resistin has previously been shown to induce insulin resistance in pancreatic islets and also impair glucose-induced insulin release (Nakata *et al.*, 2007). The presently observed up-regulation of G6pd mRNA by resistin in the β -cells may represent a pathological pathway by which resistin mediates insulin resistance the beta cells and defects in insulin release.

The inositol polyphosphate phosphatase-like 1 (Inpp1) gene encodes for the Lipid Phosphatase SHIP2 which negatively regulates insulin signalling by disrupting PI3-kinase pathway (Ishihara *et al.*, 1999; Clement *et al.*, 2001; Marion *et al.*, 2002). PI3-kinase functions as a lipid kinase producing $PI(3,4,5)P_3$ from $PI(4,5)P_2$ upon stimulation by a variety of ligands (Hiles *et al.*, 1992; Stephens *et al.*, 1993). $PI(3,4,5)P_3$ acts as a lipid second messenger often after receptor stimulation to activate downstream molecules, including Akt which results in translocation of GLUT-4 to the plasma membrane (Stephens *et al.*, 1993; Rameh and Cantley, 1999; Saltiel and Kahn, 2001). Lipid Phosphatase SHIP2 has previously been shown to hydrolyze $PI(3,4,5)P_3$ to produce $PI(3,4)P_2$, in effect disrupting the PI3 kinase pathway and negatively regulating insulin signalling (Ishihara *et al.*, 1999; Sleeman *et al.*, 2005).

Resistin up-regulation of Inpp1 mRNA was by 2.194 ($p=0.0381$) and is thus significant in unravelling the mode of action and negative regulation of insulin signalling in the beta and skeletal muscle cells. Previous studies have shown that prolonged exposure of resistin to skeletal muscle cells results in decreased GLUT4 translocation and glucose uptake in response to insulin (Palanivel *et al.*, 2005; Fan *et al.*, 2007). In the beta-cells, Lipid Phosphatase SHIP2 has been demonstrated to be a negative regulator of the PI3K signal transduction pathway; with enhanced Akt, glycogen synthase kinase 3 (GSK3) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation in INS1E cells observed following SHIP2 inhibition (Grempler *et al.*, 2007). The inhibitory role by resistin to the effects of insulin in the beta cells and other target tissues involves disruption of PI3 kinase activity and it is interesting to speculate that Inpp1 may be an important gene in regulating resistin activity on insulin's metabolic actions, as many of them require the downstream products of PI3 kinase.

6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (Pfkfb3) is a potent activator of phosphofructokinase-1 (PFK-1), which is a rate-limiting enzyme of glycolysis (Pilkis *et al.*, 1995; Okar *et al.*, 2001). In the present study resistin significantly up-regulated Pfkfb3 mRNA by 17 fold. This observation highlights the involvement of resistin in regulation of glucose metabolism. Changes in the level and the activity of this enzyme would markedly affect both the glycolytic and the glyconeogenic fluxes. PFKFB3 gene silencing has previously been shown to decrease glycolysis in HeLa cells (Calvo *et al.*, 2006) hence it is possible that up-regulation of Pfkfb3 mRNA by resistin as illustrated in this study could induce glycolysis in the beta-cells. Studies involving other cell types e.g. treatment of L6 rat skeletal muscle cells with recombinant resistin resulted in reduced insulin-stimulated oxidation of glucose via the Krebs cycle although lactate production was unaltered (Palanivel *et al.*, 2006). Oxidative glucose disposal in the target tissues is the main pathway utilised by cells to generate ATP and increased non-oxidative glucose disposal by glycolysis and reduced Krebs cycle activity by resistin could potentially result in glucose intolerance. The effect of resistin on Pfkfb3 mRNA expression illustrated in the PCR array data is a starting point and further functional experiments are needed to elucidate potential interactions in the beta-cells.

The most highly up-regulated mRNA by resistin treatment in this study was the transcription factor 2 (Tcf2) gene, more commonly known as hepatocyte nuclear factor 1 β (HNF1 β) which was increased by 32-fold compared to control. Tcf2 belongs to a network of transcription factors expressed in liver, kidney, gut, and pancreatic β cells (Coffinier *et al.*, 1999; Reber *et al.*, 2001). It also plays a role in the specific regulation of gene expression in these tissues. Mutations of HNF-1 β (TCF2) produce maturity-onset diabetes of the young, type 5 (MODY5) (Horikawa *et al.*, 1997). Affected patients are characterized by an insulin secretion defect. Selective Deletion of the Hnf1 β in the beta cells has previously been shown to result in impaired glucose tolerance, dysregulated islet gene expression, and reduced glucose-stimulated insulin secretion (Wang *et al.*, 2004). The finding that Tcf2 mRNA expression was increased suggests a role for resistin in mediating transcription responses during pancreas development. This outcome is also consistent with the observed upregulation of HNF4- α and Ins1 mRNA by resistin since the insulin gene has previously been shown

to be a target gene of hepatocyte nuclear factor-1alpha (HNF-1alpha) and HNF-1beta (Okita *et al.*, 1999).

As mentioned in the previous paragraph, the PCR array data presented Table 4.1 also demonstrates that resistin induced an increase in *Ins1* mRNA by 1.023 fold ($p=0.0443$). Insulin gene is the key regulatory hormone involved in glucose homeostasis. The regulation of insulin gene expression in response to increases in blood glucose levels is crucial for maintaining normal glucose homeostasis. The observed increase in *Ins1* mRNA expression highlights a possible mechanism by which adipose tissue secreted proteins may regulate insulin secretion. Due to increased energy demands in obesity, the increased insulin gene transcription by resistin highlights a possible pathway by which the adipose tissue adapts by causing increased insulin secretion and subsequent glucose uptake to meet energy needs.

Resistin also increased the expression level of mRNA for, *Mapk14*, *Pik3cd*, *Slc2a4*, *Stx4a* and significantly decreased *Gcg*, *Vamp3* and *Vegfa* mRNA. *Slc2a4* encodes a protein that functions as a facilitative glucose transporter in a similar fashion to insulin responsive GLUT-4. *Stx4a* mediates fusion and the translocation of *SLC2A4* and GLUT-4 from intracellular vesicles to the plasma membrane. *Pik3cd* is a downstream effector of tyrosine-phosphorylated insulin receptor substrate proteins. It is unclear; the roles of these molecules with regard to resistin function in the beta-cells. One possibility is an indirect effect by resistin on other transcription factors or regulatory signalling molecules which results in their modulation. These observations highlight a more complex function of resistin and further studies are needed to elucidate and define its potential role.

Genetic susceptibility to T2D involves many genes, most of which are still unknown. Significant alterations in genes controlling transcription were observed with *Pax4* mRNA downregulated while *Foxc2* mRNA was strongly upregulated. *Pax4* plays an important role in the differentiation and development of pancreatic islet β -cells (Sosa-Pineda *et al.*, 1997). Resistin also upregulated the mRNA for cytotoxic T-lymphocyte-associated protein 4 (*Ctla4*) and *CD28*. These genes are more commonly associated with type 1 diabetes and immune responses and their role in obesity induced insulin resistance and T2D is still unclear. Most of the significantly altered genes however are

novel targets of resistin and belong to functional classes that can account for most of the biological and metabolic effects of this adipokine in the β -cells.

In addition to resistin, the effect of visfatin on mRNA of the β -cell genes was investigated. The results from cells treated with visfatin demonstrate altered expression of genes involved in insulin signalling pathway, including the down-regulation of Mapk8 and the up-regulation of Akt2 gene. Akt2 also known as Protein kinase B plays a pivotal role in insulin signalling. Evidence in animal models suggests that Akt2-deficient mice exhibit insulin resistance and mild diabetes (Cho *et al.*, 2001). Akt2 activation in response insulin and IGF-1 has been shown to influence growth, protein synthesis and glucose metabolism (Brazil and Hemmings, 2001). The results in Table 4.2 show that visfatin increased the transcription of Akt2 gene in the β -cells by 24-fold. The high fold increase suggests that visfatin has a high potency in regulating the gene expression level of Akt2 and may have a therapeutic advantage in improving insulin sensitivity.

Mapk8 has previously been shown to increase the phosphorylation of insulin receptor substrate-1 (IRS-1) at serine residues which in effect inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 (Aguirre *et al.*, 200). In addition, TNF alpha and saturated fatty acids which induce insulin resistance are mediated by Mapk8 activity (Solinas *et al.*, 2006). Improvement in insulin sensitivity and tyrosine phosphorylation by visfatin in rodents (Sun *et al.*, 2009) could be due to its downregulation of Mapk8 mRNA expression. The increased tyrosine signalling capacity of insulin receptors by visfatin observed in Figure 6.1.4 in chapter 6 could also be mediated by the observed visfatin downregulation of Mapk8 mRNA expression.

Insulin is a key hormone in glucose homeostasis and regulation of insulin gene expression is essential for maintaining this process. Insulin gene transcription in the β -cells is induced by increases in blood glucose levels (Efrat *et al.*, 1991). The induction of insulin gene is a key step in restoring normal blood glucose levels during hyperglycaemia and culminates in insulin secretory response which lowers blood glucose. The 8-fold increase in insulin gene transcription by visfatin highlights the involvement of this adipokine in the process. It is possible that increased insulin gene expression in response to visfatin treatment may result in improved responsiveness of

the insulin promoter to acute glucose stimulation or chronic hyperglycaemia illustrating a possible pathway by which visfatin improves insulin sensitivity as illustrated in a recent report (Sun *et al.*, 2009).

Glucose uptake in the beta cells, skeletal muscle and adipose tissue is mediated by insulin-stimulated translocation of GLUT-4 glucose transporters from an intracellular vesicular pool to the plasma membrane (Ishihara *et al.*, 1993; Kanai *et al.*, 1993). Insulin-stimulated translocation of GLUT4 has previously been shown to involve SNARE-complex proteins syntaxin 4 (Stx4a) and SNAP23 (Cheatham *et al.*, 1996). Inhibition of syntaxin 4 has been shown to repress insulin-stimulated GLUT4 translocation (Volchuk *et al.*, 1996) suggesting that this protein plays an essential role in insulin-stimulated glucose transport. The high fold increase (17.5 fold) of Stx4a gene by visfatin strongly implicates visfatin in this process. Visfatin has previously been shown to regulate of glucose uptake in human osteoblasts (Xie *et al.*, 2007). Eventhough this effect (glucose uptake) is observed in the osteoblasts and not β -cells; it is possible that this insulin-like effect by visfatin is mediated by induction of Stx4a gene.

Uncoupling Protein-2 (UCP-2) is an inner mitochondrial membrane transporter which disrupts the electrochemical gradient across the inner mitochondrial membrane, producing heat instead of ATP from respiration (Li *et al.*, 2000; Brown *et al.*, 2002). Overexpression of UCP-2 has previously been shown to suppress glucose-stimulated insulin secretion in normal islets (Chan *et al.*, 1999). In a similar fashion, UCP2-deficient mice had higher islet ATP levels and increased glucose-stimulated insulin secretion (Zhang *et al.*, 2001). Visfatin treatment resulted in decreased UCP-2 mRNA expression in the β -cells. In one study, Silent Information Regulator T-1 (Sirt1) was shown to positively regulate insulin secretion by repressing UCP2 in pancreatic beta cells, conversely Sirt1 knockout mice displayed high UCP2 expression (Bordone *et al.*, 2006). Sirt1 is a NAD-dependent deacetylase (Imai *et al.*, 2000) and thus closely linked to visfatin which catalyses the rate-limiting step in the NAD⁺ biosynthetic pathway (Revollo *et al.*, 2004). In this regard, the observed visfatin induced insulin secretion illustrated in Figure 6.2.2 of chapter 6 could partly be mediated by visfatin's ability to suppress UCP-2 mRNA expression in the β -cells.

In the same way, the significant downregulation of PPAR- α gene by visfatin could also be linked to visfatin action on insulin secretion. In one study, PPAR- α was shown to suppress insulin secretion and induce UCP-2 expression in insulinoma cells (Tordjman *et al.*, 2002). The suppression of glucose-stimulated insulin secretion in this study could be due to the ability of PPAR- α to induce fatty acid oxidation and stimulate lipid uptake (Schoonjans *et al.*, 1996; Frohnert *et al.*, 1999) which may result in lipotoxicity. Taken together, the reduced transcription activity of both the UCP-2 gene and PPAR- α by visfatin suggest the involvement of both genes in mediating visfatin induced insulin secretion as observed in Figure 6.2.2 in chapter 6 of this study.

Hepatocyte nuclear factor 4 alpha (HNF-4 α) is a member of the nuclear receptor superfamily known to regulate a large number of genes in hepatocytes and pancreatic beta-cells (Wang *et al.*, 2000). The dysfunction of this transcription factor has been associated with diabetes mellitus. Mutations in the human HNF4- α gene leads to maturity onset diabetes of the young subtype 1 (MODY1) (Yamagata *et al.*, 1996). HNF4- α is critical to β -cell function and adequate insulin secretion as illustrated by HNF4- α knockout mice, which exhibit impaired glucose tolerance and defective insulin secretion (Miura *et al.*, 2006). The data outlined in Table 4.2 indicate that visfatin up-regulated HNF4 alpha mRNA expression by 16-fold. The increase observed in these important transcription factors, HNF4- α and Tcf2 illustrate a possible pathway by which visfatin causes an increase in insulin secretion (Figure 6.2.2) as they are both thought to be involved in insulin gene transcription (Bernardo *et al.*, 2008)

Another transcription factor upregulated by visfatin is NF kappa B (NF- κ B) which plays a major role in inflammatory responses. Visfatin has recently been shown to enhance the expression of adhesion molecules in endothelial cells through NF- κ B activation (Kim *et al.*, 2008). In another study involving endothelial cells, NF- κ B signalling was shown to mediate visfatin-induced activation of gelatinases factors that are essential in the pathogenesis of vascular inflammation (Adya *et al.*, 2008a). In the beta-cells, the involvement of NF- κ B in the IL-1 β induced nitric oxide synthase expression and signaling mechanism has previously been demonstrated (Kwon *et al.*, 1995). Additional studies have shown that cytokines IL-1 β , interferon-gamma (IFN- γ)

and TNF- α activate NF- κ B and induce the production nitric oxide (NO) in human pancreatic islets (Flodström *et al.*, 1996). NO is a key mediator in inflammation. The increase in NF κ B mRNA by visfatin as illustrated in Table 4.2 highlights a potential role for this transcription factor in mediating visfatin function. This is critical to future studies since serum visfatin has previously been reported to increase with progressive β -cell deterioration (López-Bermejo *et al.*, 2006) suggesting a possible involvement of visfatin in inflammatory processes

In conclusion, cDNA RT-PCR array technology represents a powerful technique for defining adipokine-regulated genes. The data outlined in this study suggests several candidate genes which may be involved in resistin induced β -cell dysfunction and visfatin mediated insulin secretion and induction of β -cell sensitivity. They will help to better identify the mechanisms involved in obesity induced insulin resistance and T2D, a disease characterized by defective regulation of gene expression in response to insulin.

Chapter 5

Resistin regulation of insulin secretion and insulin receptor expression

5.1. Introduction

β -cells are responsible for maintaining a narrow range of blood glucose levels. This is achieved by efficient insulin secretion in response to increases in circulating glucose levels and insulin action in the target tissues (Ashcroft *et al.*, 1994). Reduced β -cell function has been shown to precede impaired glucose tolerance (IGT) (Festa *et al.*, 2008). A better understanding of the pathophysiology of glucose intolerance is thus vital in preventing the development of T2D or at least delaying the onset. Key to this are the signalling cascades by which insulin mediates its effects in the tissues involved in glucose utilisation.

Insulin signalling pathways have previously been reported to be present in the pancreatic β -cells and their activation following binding of insulin to its receptor has been demonstrated (Kulkarni *et al.*, 2002). Disruptions of these pathways have profound effects on β -cell function; with knockout of insulin receptor in a β -cell specific manner resulting in hyperglycaemia and defects β -cell growth (Otani *et al.*, 2004). The presence of insulin resistance and defects in pancreatic β -cell function are thus key characteristics of type 2 diabetes (Lillioja *et al.*, 1993). Adipocyte secreted factors including resistin have been reported as key players in the pathogenesis of insulin resistance associated with obesity (Steppan *et al.*, 2001).

Elevated levels of resistin have been proposed to cause insulin resistance and therefore may serve as a link between obesity and type 2 diabetes (Steppan *et al.*, 2001). However, in humans, the physiological role of resistin is far from clear and its role in obesity and insulin resistance and / or diabetes is controversial. Studies on resistin gene expression have been inconsistent with previous studies rodent and animal models reporting conflicting results (Savage *et al.*, 2001; Nagaev *et al.*, 2001). The aim of this study was to first characterise the expression of resistin in rodent clonal pancreatic β -cells and then establish its role in insulin receptor expression and glucose stimulated insulin secretion.

5.2. Materials and methods:

Details of materials and methods used in the experiments for this chapter can be found in chapter 2

5.3. Results

5.3.1. Resistin mRNA is expressed in clonal rat pancreatic β cells

Real-time RT-PCR was performed using cDNA derived from clonal rat BRIN-BD11 cell line to investigate resistin expression in the beta cells. Analysis revealed that resistin is expressed in this cell line (Ct values of between 24 and 26 being observed). This result suggest that resistin previously described as a adipose tissue specific protein is also found in pancreatic β -cells.

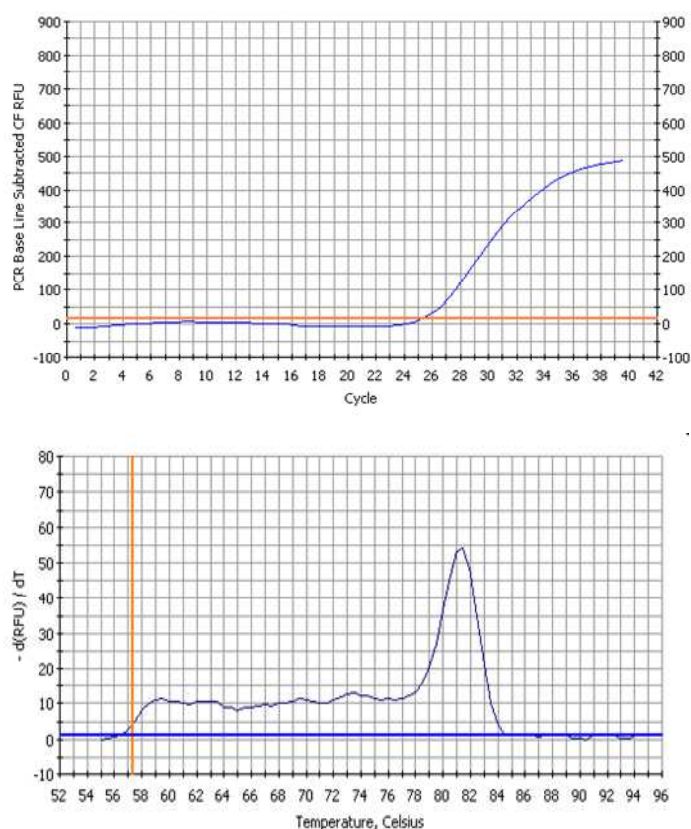


Figure 5.1 Resistin is expressed in clonal rat pancreatic BRIN-BD11 cells. Resistin mRNA expression suggests that resistin is another ‘adipose tissue-specific’ protein found in pancreatic β -cells. In this experiment, $1\mu\text{g}$ of mRNA from clonal rat BRIN-BD11 cells was reverse transcribed to cDNA and used in a $25\mu\text{l}$ RT-PCR reaction. To exclude the possibility of genomic DNA contamination, the mRNA was DNased before the reverse transcription (RT) reaction. (n=3).

5.3.2. Low concentrations of resistin have no effect on insulin receptor gene expression

To determine whether resistin at concentrations reported in lean non-diabetics modulates insulin receptor gene expression, mouse β TC-6 cells were treated with 20ng/ml resistin for 24h. Real-time RT-PCR analysis revealed that resistin treatment did not have any effect on the levels of insulin receptor (IR) mRNA as compared to untreated controls ($P>0.05$).

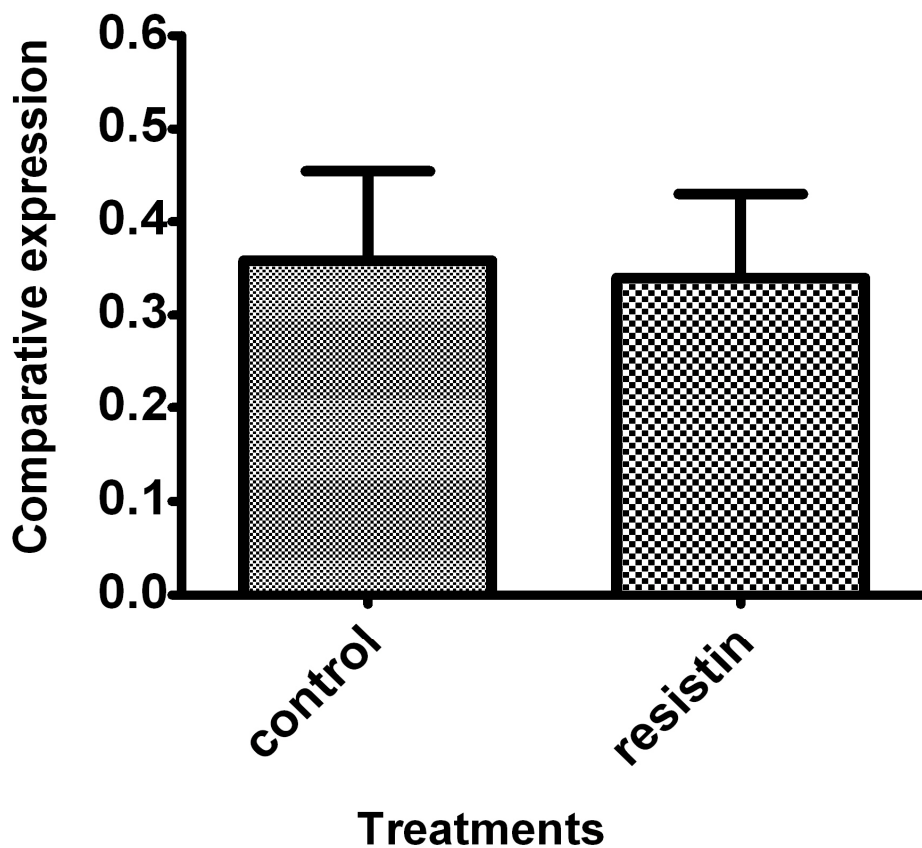


Figure 5.2 Resistin did not have any effect on Insulin receptor gene expression after treatment with 20ng/ml. Total RNA was extracted from treated and non-treated cells, reverse transcribed and subjected to quantitative real-time RT-PCR. Insulin receptor gene expression was normalized to β -actin and GAPDH gene expression. Resistin did not have any effect on IR mRNA expression at the concentration (20ng/ml) used. Results are the means \pm S.E.M. comparing resistin-treated with untreated control cells. (n=4).

5.3.3. Low concentrations of resistin have no effect on insulin receptor protein expression

Western blot analysis of insulin receptor protein expression in mouse β TC-6 cells showed that resistin does not alter insulin receptor protein expression at concentrations reported in lean non-diabetics suggesting absence of pathological action at this concentration.

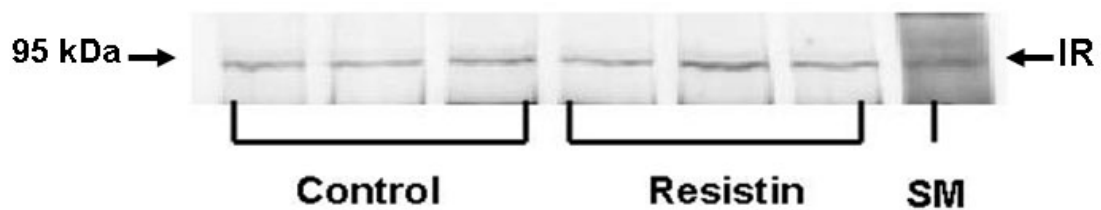


Figure 5.3 Resistin did not have any effect on insulin receptor protein expression. Proteins from lysed cells were separated on 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti insulin receptor antibody. Resistin did not have any effect on insulin receptor (IR) protein expression at the concentration (20ng/ml) used. Mouse skeletal muscle cell lysate (SM) was used as a positive control. (n=3).

5.3.4. Insulin receptor mRNA levels in β TC-6 cells are decreased by high resistin concentrations

To further investigate the effect of resistin at concentrations reported in obese type 2 diabetics, β TC-6 cells were treated with 40ng/ml resistin for 24h. Resistin treatment dramatically reduced the levels of insulin receptor mRNA expression by 70% as compared to untreated controls.

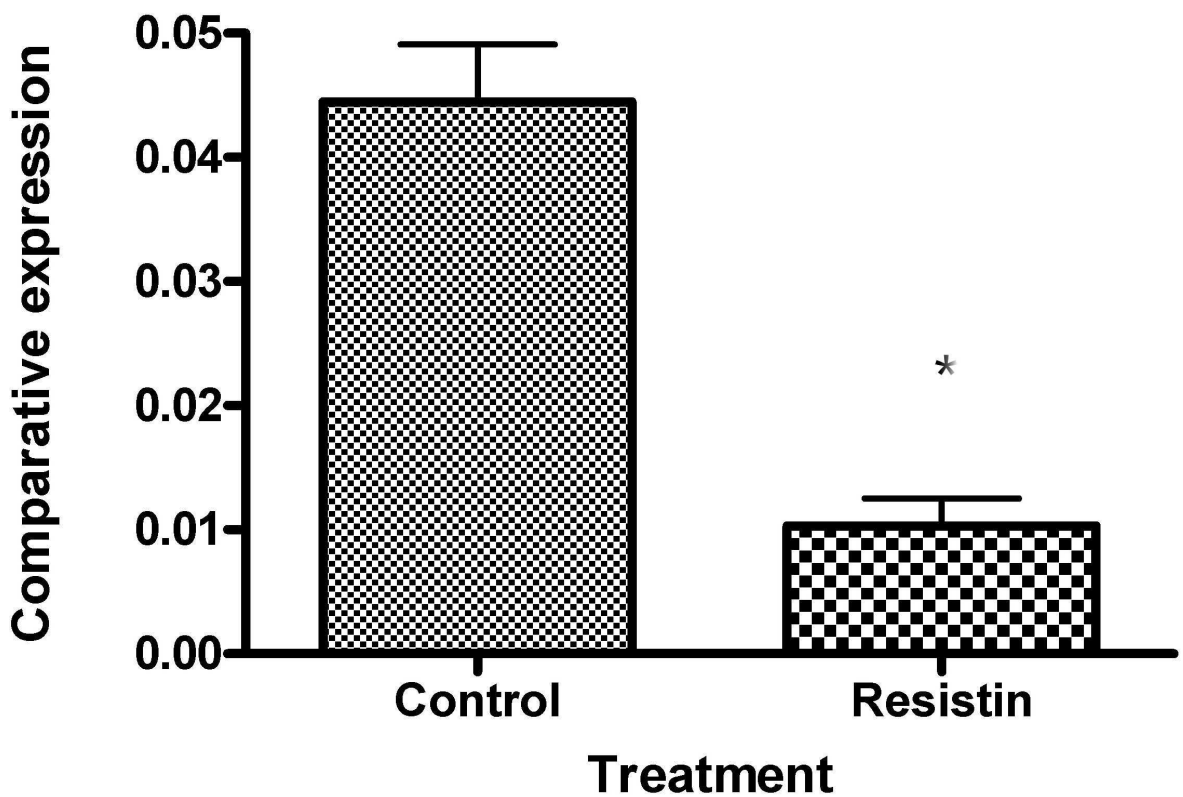


Figure 5.4 Resistin downregulates Insulin receptor gene expression. β TC-6 cells treatment with 40ng/ml resistin for 24 hours resulted in reduced IR mRNA expression compared to untreated control. Total RNA was extracted, reverse transcribed and subjected to quantitative real-time RT-PCR to determine insulin receptor mRNA levels and normalized to β -actin and GAPDH expression means. ($p < 0.05$). $n = 4$.

5.3.5. Insulin receptor protein levels are decreased by high resistin concentrations

The changes noted at the mRNA level were also reflected at the protein level in the clonal pancreatic mouse β TC-6 cells. Resistin significantly decreased insulin receptor protein expression in resistin treated samples compared to non-treated controls with a decrease in detected band intensity of 60% being recorded.

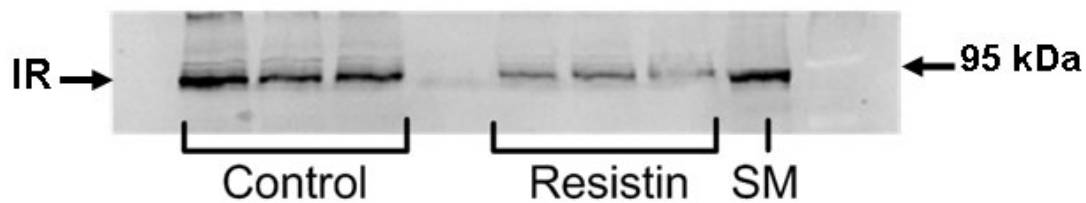


Figure 5.5 Resistin downregulates Insulin receptor protein expression.

Western blot analysis of protein extracts shows reduced IR expression in resistin treated samples compared to non-treated controls. Total protein was extracted from the cell lysate followed by separation using 8% SDS-PAGE and immunoblot analysis. Mouse skeletal muscle cell lysate (SM) was used as a positive control. Key: IR = insulin receptor (n=3).

5.3.6. Resistin has no effect on insulin secretion in BRIN-BD11 cells

The glucose-responsive clonal rodent pancreatic BRIN-BD11 cell line was used for the insulin secretion study. Resistin at concentrations of 20 and 40ng/ml did not exert any significant effect on high glucose (22 mmol/l) stimulated insulin secretion over 1 h from the clonal rat BRIN-BD11 cell line.

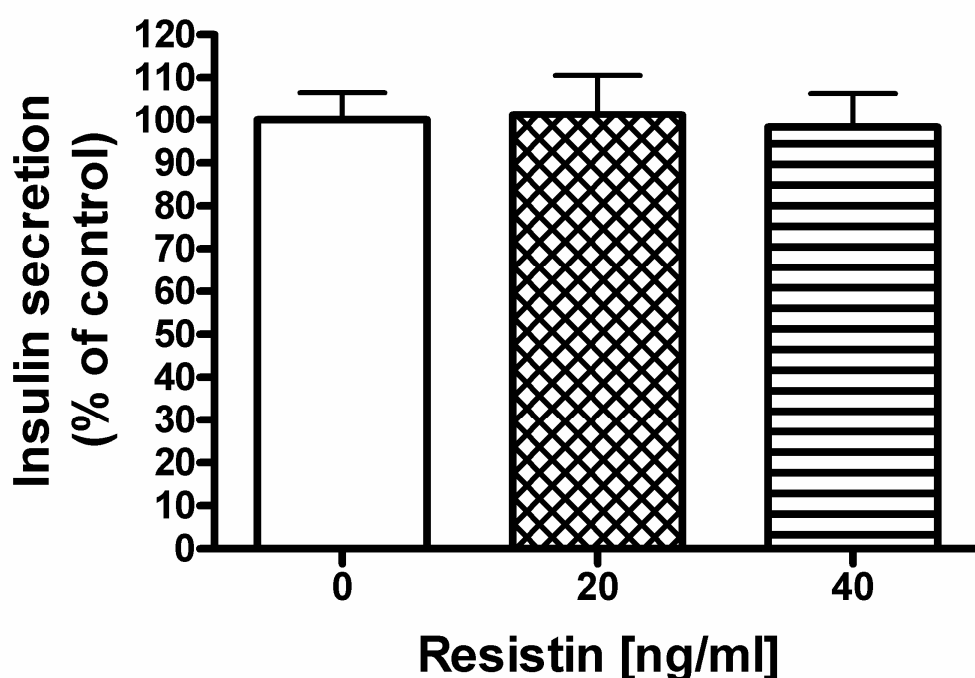


Figure 5.6 Resistin did not have any effect on glucose stimulated insulin secretion. BRIN-BD11 cells were treated with 0, 20 and 40ng/ml resistin for 24 hours in serum free RPMI medium. After treatment, the old medium was aspirated and replaced by serum free media containing 2.2 mM glucose for 1 hour and then stimulated with 22 mM glucose for another 1 hour. The medium from this last treatment was then collected and assayed for insulin content using a proprietary Rat insulin ELISA. Resistin treatment did not have any effect on GSIS both at physiological and pathological concentrations (n = 4).

5.4. Discussion

Resistin, a circulating protein derived from adipocytes in rodents has been implicated in obesity-associated diabetes (Steppan CM and Lazar MA, 2004). Despite numerous studies since its discovery, the role of resistin in insulin sensitivity and obesity remains controversial. Previous reports have demonstrated that resistin mRNA is expressed in mouse brain and pituitary (Morash *et al.*, 2002) however studies of resistin gene expression in humans have given conflicting results (Nagaev *et al.*, 2001; Engeli *et al.*, 2002)

In order to gain insight into the physiological role of resistin, analysis of resistin gene expression in rat BRIN-BD11 cells was performed using real-time RT-PCR. The results outlined above demonstrate that resistin is expressed in rat β -cells with Ct values of 24 and 26 being observed. This data confirms that resistin, previously thought to be an adipose specific protein both in rodents and humans (Steppan *et al.*, 2001; McTernan *et al.*, 2002b) is also found in the β -cells and raises the question of its potential role in β -cell function.

In one previous study resistin deficiency was shown to improve glucose tolerance and insulin sensitivity in severely obese mice (Qi *et al.*, 2006). Original studies by Steppan and co-workers showed that administration of resistin to mice resulted in glucose intolerance, whereas immuno-neutralization of resistin improved insulin action (Steppan *et al.*, 2001). These reports illustrate the role of resistin in the pathophysiology of T2D and the expression of resistin in the β -cells as illustrated in this study may have potential pathological role on β -cell function e.g. mediator of inflammation. Further studies are thus needed to identify factors that regulate resistin expression in the β -cells.

Insulin mediates its action in glucose homeostasis by first binding to the insulin receptor (IR) localized on the cell surface (Gammeltoft, 1984). The regulation of insulin receptor expression and activity is thus important in directing insulin to specific target tissues involved in glucose utilisation (Desbuquois *et al.*, 1993). β -cell specific disruption of the insulin receptor has previously been shown to result in

impaired glucose tolerance further illustrating the important role of IR expression in β cell function (Kulkarni *et al.*, 1999). In another study, reduction of IR expression by small interfering RNA in MIN6 insulinoma cell lines resulted in alterations in expression of numerous genes with diverse functions and disruption of the insulin signalling pathway (Ohsugi *et al.*, 2005).

In order to clarify the role of resistin in regulation of insulin receptor at concentrations reported in lean non-diabetics and obese diabetic individuals, clonal mouse β TC-6 cells were treated with 20ng/ml resistin, which is representative of serum levels measurable in non-diabetic subjects (Fujinami *et al.*, 2004; Yannakoulia *et al.*, 2003) and 40ng/ml which is the reported concentration range in obese T2D (Fehmann *et al.*, 2002; Azuma *et al.*, 2003; Zhang *et al.*, 2003). Resistin did not have any effect on either gene or protein expression of insulin receptor at the lower concentration of 20ng/ml.

However at 40ng/ml, as has been reported in a publication based on this study (Brown *et al.*, 2007), 24-hour treatment of β TC-6 cells with elevated resistin concentration (40ng/ml) resulted in a significant decrease in insulin receptor mRNA and protein expression compared to control. This effect observed on insulin receptor has profound importance. Resistin has previously been shown to induce insulin resistance in pancreatic β -cells (Nakata *et al.*, 2007) and rat derived hepatocytes (Liu *et al.*, 2008). It is thus possible that this induction of β -cell insulin resistance by resistin could involve reduction of insulin receptor expression as reported in this study.

The expression of resistin in the β -cell which is the site of insulin production raises the question of the potential role resistin may play in insulin secretion. The secretory response to glucose by the β -cells is modulated by a variety of hormones and enzymes (Zawalich *et al.*, 1995; Harris *et al.*, 1996). More recently adipose tissue secreted factors have been implicated in modulating insulin secretion. Tumour necrosis factor - alpha (TNF- α) and leptin, both adipose tissue-derived hormones have previously been reported to suppress glucose-stimulated insulin secretion (GSIS) in pancreatic β -cells (Zhang *et al.*, 1995; Tsiotra *et al.*, 2001). However the contribution of resistin to the regulation of insulin release from pancreatic β -cells has not been fully explored.

In this regard the direct actions of resistin on GSIS were investigated. Clonal mouse β TC-6 cells were treated with resistin both at 20 and 40ng/ml. As illustrated in Figure 5.1.7, resistin did not have any effect on GSIS. This is in contrast to a previous study which found that resistin decreased GSIS in isolated islet tissue (Nakata *et al.*, 2007). The difference could partly be due to the islet tissue used by Nakata *et al.*, which is well established as being more glucose-responsive than the clonal pancreatic β -cell line used in this study. Further studies using primary β -cells or islets are thus required to elucidate the underlying mechanisms of resistin and its pathophysiological role in insulin secretion.

Identification of resistin mRNA in the β -cells and the regulatory effect on IR expression at pathological concentrations raises the possibility that this adipose tissue derived hormone may play a pivotal role in obesity induced insulin resistance. Taken together these results demonstrate that resistin elicits its pathological effects only at elevated concentrations reported in obese individuals providing a potential link between increased adiposity and T2D.

Chapter 6

Visfatin regulation of insulin secretion and insulin receptor signalling in pancreatic beta cells

6.1. Introduction

Visfatin has recently been identified as an adipokine that is abundantly expressed and preferentially produced in visceral adipose tissue (Berndt *et al.*, 2005; Kovacicova *et al.*, 2008). Initial (and subsequently retracted on the basis that some preparations of visfatin did not bind the insulin receptor in their experiments) reports indicated that visfatin mimics the effects of insulin by binding to the insulin receptor at a site distinct from that of insulin, however other studies were unable to reproduce some of these results (Revollo *et al.*, 2007b). The physiological role of visfatin in glucose homeostasis is thus controversial and remains to be elucidated. More importantly, the expression of visfatin has been shown to be regulated by cytokines interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6); all of which are known to promote insulin resistance (Ognjanovic *et al.*, 2001; Kralisch *et al.*, 2005a; Tan *et al.*, 2009).

Insight into the biological functions of visfatin continues to grow with ongoing research. Recently Revollo *et al.*, have shown that visfatin is a biosynthetic enzyme which mediates its functions in the β -cells via NAD⁺ biosynthetic pathway (Revollo *et al.*, 2007b). Visfatin has also been previously reported to regulate NAD⁺-dependent protein deacetylation in human vascular smooth muscle cells, maturation and survival (Van der Veer *et al.*, 2005).

In the present study, therefore, the effect of visfatin on insulin receptor activation and secretory function of the β -cell was examined. These two components together play a critical role in the regulation of glucose homeostasis in the pancreatic β -cells and insulin target organs. In addition the effects of visfatin and nicotinamide mononucleotide, the product of visfatin's enzymatic actions, on intracellular signalling pathways such as extracellular signal-regulated kinase (ERK 1/2), MAPK p38 were investigated.

6.2. Materials and methods:

Details of materials and methods used in the experiments for this chapter can be found in chapter 2

6.3. Results

6.3.1.1. Visfatin mRNA expression in the pancreatic β -cells

Analysis of mRNA from clonal pancreatic mouse β TC-6 cells showed that visfatin is expressed at a relatively high level with Ct values of between 21 and 23 generally being observed. Visfatin has previously been shown to be predominantly expressed in the visceral adipose tissue in both humans and mice and the high expression level observed highlights a potential role, the peptide may play in the pancreatic β -cells.

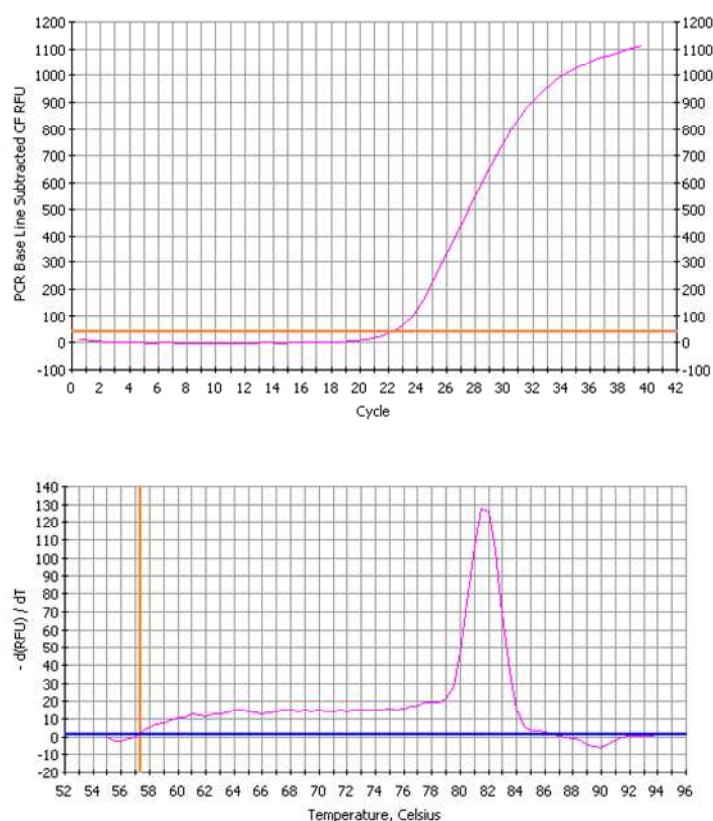


Figure 6.1.1 Visfatin mRNA expression analysis by real-time quantitative RT-PCR. The high visfatin mRNA expression suggests that visfatin is yet another adipose tissue-specific protein found in pancreatic β -cells. For this experiment, total RNA was extracted from the mouse β TC-6 cells samples and quantified. 1 μ g of total mRNA was DNAsed to exclude the possibility of genomic DNA contamination and reverse transcribed (RT) and amplified in a 25 μ l real time RT-PCR reaction. (n=3).

6.3.1.2. Visfatin protein expression in the beta cells

To determine whether visfatin is also expressed at the protein level, cell lysates from the beta cells were analyzed using western blot. As illustrated in figure 6.1.2, the observations noted at the mRNA level were also reflected at the protein level in the beta-cells. Finding visfatin at the source of insulin production raises the possibility that this adipokine may play a role in insulin secretion, consistent with the notion of visfatin being involved in glucose homeostasis (Revollo *et al.*, 2007b).

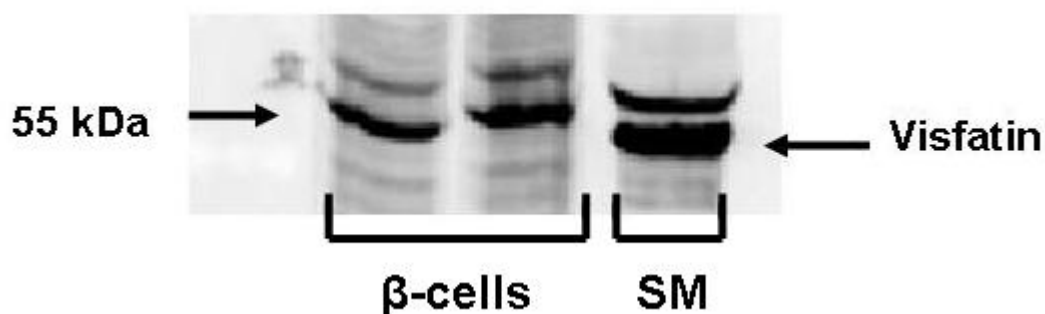


Figure 6.1.2 Visfatin Protein is expressed in the beta cells. Western blot analysis of protein extracts from clonal mouse β TC-6 cells, separated on 12% SDS-PAGE and immunoblotted with anti visfatin antibody. As illustrated in the figure, visfatin protein is highly expressed in the β -cells. The antibody against visfatin recognized bands with apparent molecular masses of 55 kDa which corresponds to molecular mass of visfatin. The two lanes for β -cells are from the same lysate sample. Mouse skeletal muscle cell lysate (SM) was used as a positive control. (n=3).

6.3.1.3. Dose-dependent visfatin regulation of insulin receptor phosphorylation

To study the effect of visfatin on insulin receptor phosphorylation, mouse β TC-6 cells were stimulated with visfatin and insulin (positive control) or left untreated and analysed by ELISA.

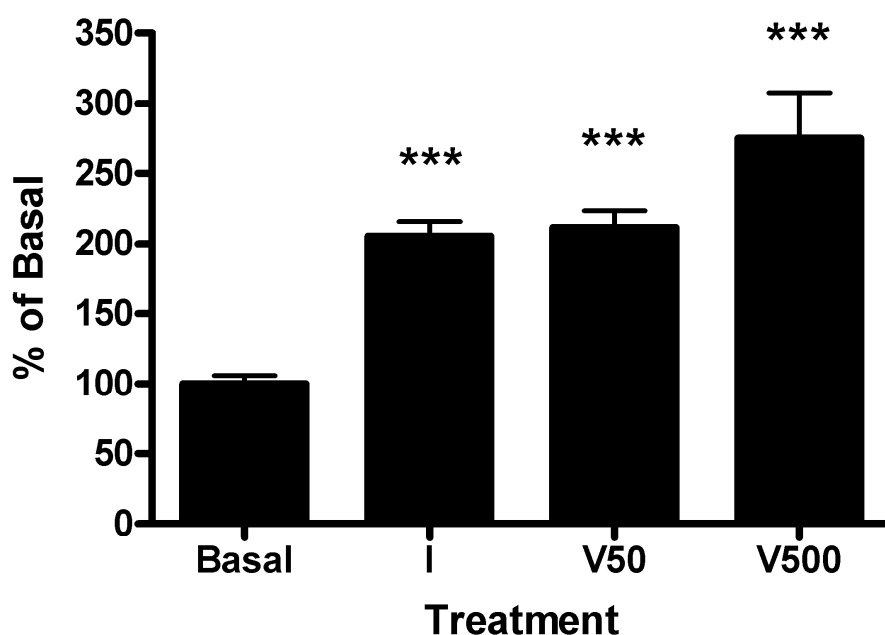


Figure 6.1.3 Visfatin stimulates insulin receptor phosphorylation in clonal pancreatic mouse β TC-6 cells. Visfatin (50 and 500ng/ml) caused significant 104 % ($p < 0.001$) and 178 % ($p < 0.001$) increases in insulin receptor phosphorylation respectively. The results show that insulin receptor was significantly activated by visfatin after 15 minutes stimulation ($p < 0.001$). Phosphorylated IR was determined by ELISA using specific antibody against tyrosine phosphorylated IR. Total IR was also determined using anti-total IR based ELISA to normalise data. V50 = 50ng/ml visfatin, V500 = 500ng/ml visfatin and I = 10nM insulin (positive control) ($n=4$).

6.3.1.4. Time-dependent visfatin regulation of insulin receptor phosphorylation

To investigate the time course effect of visfatin on insulin receptor activation, 200ng/ml of visfatin was used to stimulate the cells at 2, 5, 15, 30 and 60 minutes and the cells lysed in lysis buffer supplemented with phosphatase inhibitors and protease inhibitors. Visfatin induced insulin receptor phosphorylation after 15 minutes.

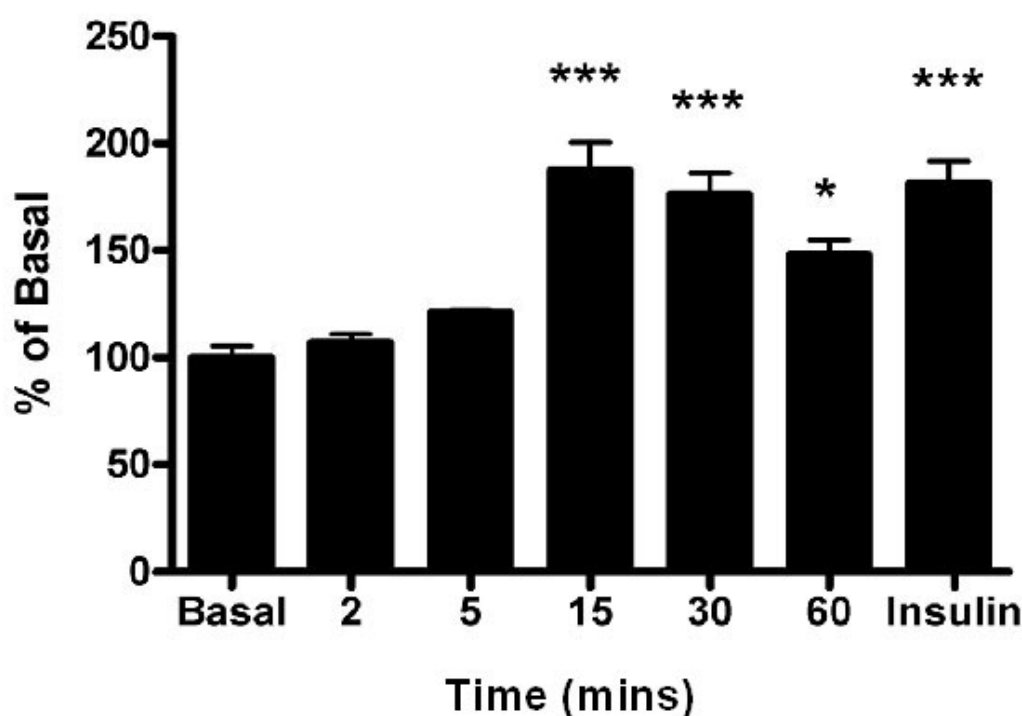


Figure 6.1.4 Visfatin activation of insulin receptor in mouse β TC-6 cells (time course). There was no effect of visfatin on tyrosine phosphorylation of IR after 2 and 5 minutes. However, visfatin significantly increased insulin receptor phosphorylation at 15, 30 and 60 minutes. The maximal IR tyrosine phosphorylation was observed after 15 minutes ($P < 0.001$) and remained significantly higher than basal for 60 minutes ($p < 0.05$). Cells were stimulated with insulin (positive control) for 15 minutes. Insulin receptor phosphorylation was determined by specific enzyme-linked immunosorbent assay (ELISA) kit. Values are mean \pm SEM, $n=4$.

6.3.1.5. The effect of FK866 on visfatin induced insulin receptor phosphorylation

To test whether NMN, a visfatin reaction product is involved in visfatin–IR activation in the β -cells, the cells were treated with visfatin and FK866 (a potent visfatin inhibitor) for 15 minutes. Co-incubation of visfatin and FK866 significantly suppressed the activation of IR induced by visfatin compared to the cells stimulated with visfatin alone ($p<0.05$). These data indicate that IR activation by visfatin in the β cells is mediated by NMN.

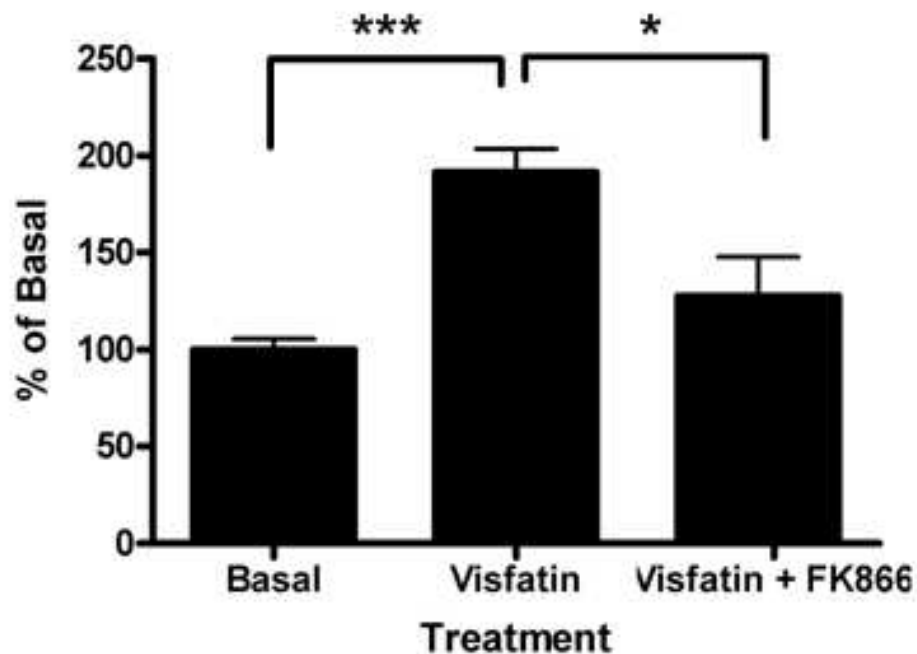


Figure 6.1.5 FK866 induced reduction in visfatin activation of the insulin receptor. β TC-6 cells were incubated with visfatin alone or co-incubated with 200ng/ml visfatin and 10nM FK866 for 15 minutes and lysed in cell lysis buffer supplemented with phosphatase inhibitors and protease inhibitors. The effect of FK866 on visfatin induced IR phosphorylation was determined by specific enzyme-linked immunosorbent assay (ELISA) kit. FK866 significantly reversed the effects of visfatin induced insulin receptor activation. $n=4$

6.3.1.6. Time-dependent activation of insulin receptor by NMN

Because the stimulatory effect of visfatin on insulin receptor activation was reversed by FK866 (Figure 6.1.5.), it was vital to examine the effect of NMN (visfatin reaction product) whose production is inhibited by FK866 in the NAD⁺ biosynthetic pathway.

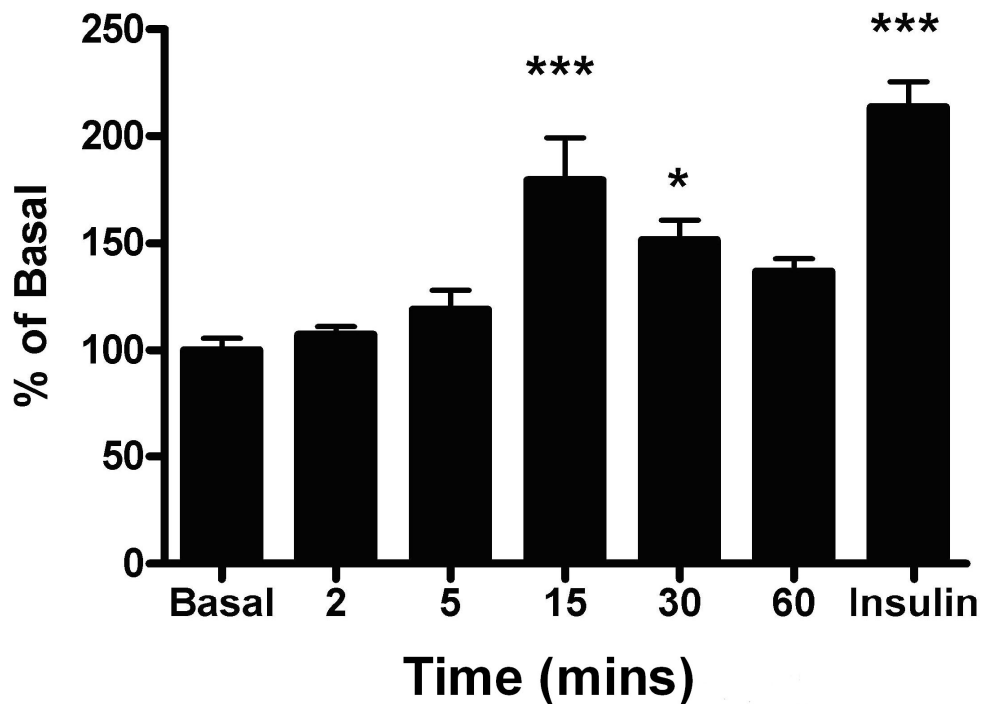


Figure 6.1.6 NMN time-response curves for insulin receptor activation in mouse β TC-6 cells. NMN induced an increase in IR phosphorylation in a similar trend to visfatin with the maximal effect attained after 15 minutes ($P < 0.001$). Insulin receptor phosphorylation was determined by specific enzyme-linked immunosorbent assay (ELISA) kit. Cells were stimulated with insulin (positive control) for 15 minutes ($n=4$).

6.3.1.7. Visfatin and extracellular signal-regulated kinase (ERK 1/2) activation

Visfatin has previously been shown to cause activation of the ERK1/2 in endothelial cells (Adya *et al.*, 2008b). The effect of visfatin on ERK1/2 activation in the β -cells was thus investigated. The cells were exposed to 200ng/ml visfatin at varying times (0, 2, 5, 15, 30 and 60 minutes) and phosphorylated, active ERK 1/2 was detected using specific enzyme-linked immunosorbent assay (ELISA) kit. PMA was used as a positive control.

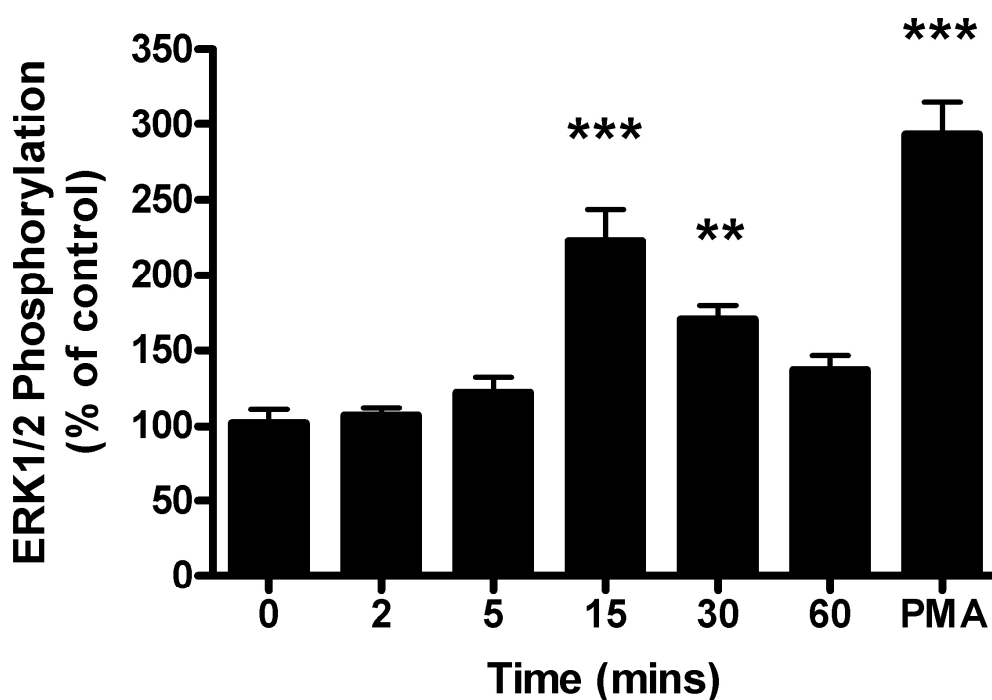


Figure 6.1.7 Time-dependent activation of ERK1/2 by visfatin. As shown in figure 6.1.7, treatment of cells with 200ng/ml visfatin caused a significant 122% increase ERK1/2 phosphorylation after 15 minutes ($P<0.001$). The level of phosphorylated active ERK1/2 was subsequently decreased, and had reached nearly the basal level by 60 minutes. Cells were stimulated with PMA (positive control) for 15 minutes. $n=4$

6.3.1.8. Time-course response of NMN and ERK 1/2 activation

To further elucidate the role of nicotinamide mononucleotide (NMN) which is a visfatin reaction product in the NAD biosynthetic pathway, the cells were stimulated with NMN for 15 minutes and activity of ERK 1/2 analyzed.

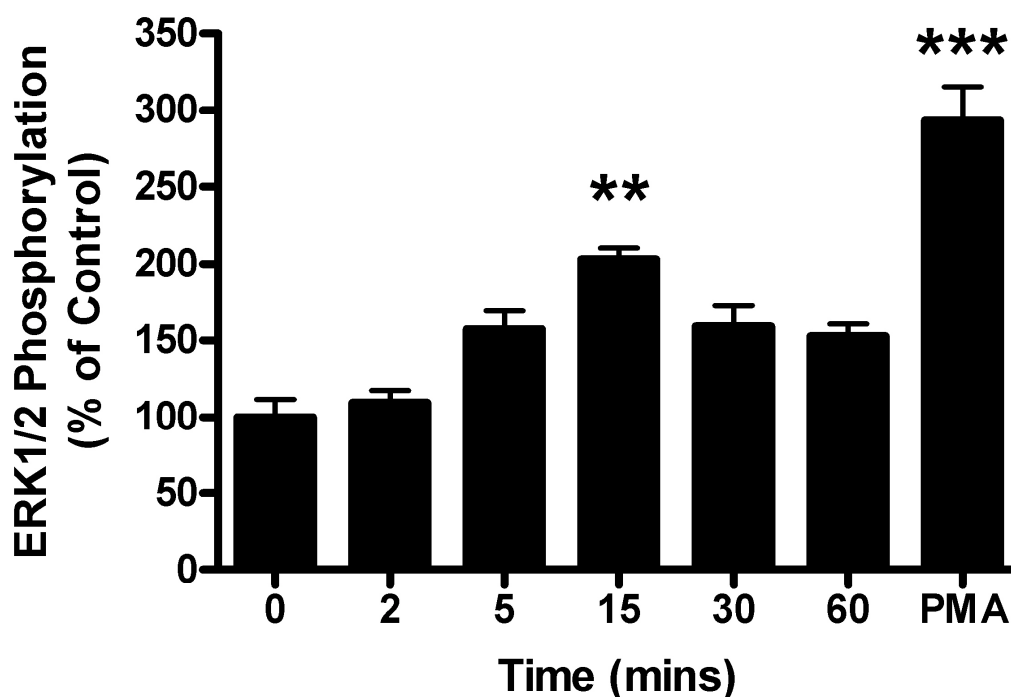


Figure 6.1.8 Time response curves of ERK 1/2 activation by NMN. The cells were stimulated with 100 μM NMN at varying times (0, 2, 5, 15, 30 and 60 minutes). NMN stimulation resulted in ERK 1/2 phosphorylation with the maximal effect (105% increase compared to basal) attained after 15 minutes ($p < 0.01$). This transient increase in ERK 1/2 activation is consistent with the time-course increase in visfatin and ERK 1/2 activation as illustrated in Figure 6.1.7. Cells were stimulated with PMA (positive control) for 15 minutes. Phosphorylated ERK 1/2 was detected using specific enzyme-linked immunosorbent assay (ELISA) kit. PMA was used as a positive control. $n=4$

6.3.1.9. Effect of FK866 on visfatin induced ERK 1/2 activation

Visfatin has previously been shown to act as a nicotinamide phosphoribosyltransferase (Nampt) and mediates its function via biosynthesis of nicotinamide mononucleotide. To test this enzymatic activity of visfatin in ERK 1/2 activation, the cells were co-incubated for 15 minutes with visfatin and FK866 (a specific Nampt inhibitor) and ERK 1/2 activation determined using specific enzyme-linked immunosorbent assay (ELISA) kit. PMA was used as a positive control.

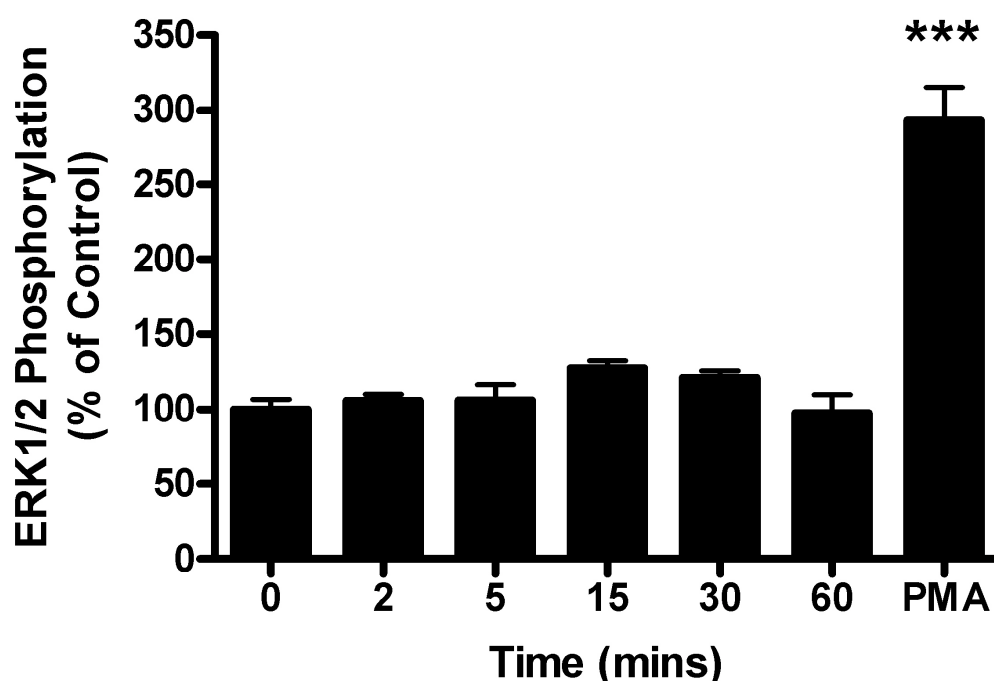


Figure 6.1.9 FK866 significantly reduces visfatin induced ERK 1/2 activation. The cells were co-incubated with 200ng/ml visfatin and FK866 (10nM) for 15 minutes. The visfatin-induced increase in phosphorylated active ERK 1/2 as observed in Figure 6.1.7 was markedly inhibited by FK866 which has been shown to act as a highly selective inhibitor of visfatin and thereby inhibiting synthesis of visfatin reaction product (NMN) in the NAD biosynthetic pathway. Cells were stimulated with PMA (positive control) for 15 minutes. n=4

6.3.2.0. Comparison of visfatin and (visfatin + FK866) on ERK 1/2 activation

Co-incubation of visfatin with 10 nM FK866 significantly inhibited visfatin induced ERK 1/2 activation ($p < 0.01$). The level of phosphorylated ERK 1/2 was subsequently decreased to nearly the basal level in the (visfatin + FK866) samples compared to the samples treated with visfatin alone.

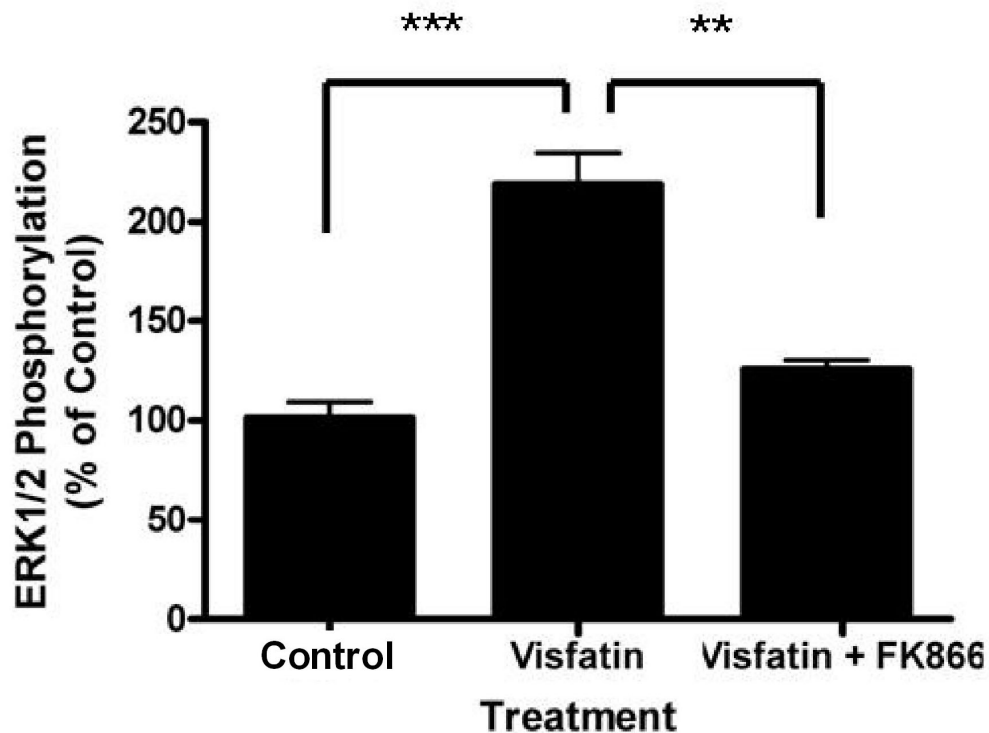


Figure 6.2.0 Comparative analysis of visfatin treated samples and the effect of visfatin inhibitor (FK866) on ERK 1/2 activation. Visfatin treatment caused a significant increase in ERK1/2 phosphorylation compared to untreated control ($p < 0.001$) however FK866 significantly reduced the effect of visfatin on ERK1/2 activation compared to visfatin treatment alone ($p < 0.01$)

6.3.2.1. Visfatin and MAPK p38 activation

Visfatin has previously been implicated in inflammatory response ((López-Bermejo *et al.*, 2006). p38 mitogen-activated protein kinases (MAPK) are a class of MAPK's which are responsive to stress stimuli and activated by a variety of cellular and external stress signals including inflammatory cytokines (Kumar *et al.*, 2003). To examine whether visfatin's role in inflammatory response is mediated by MAPK signalling pathway, β TC-6 cells were stimulated with 200ng/ml visfatin at varying time points.

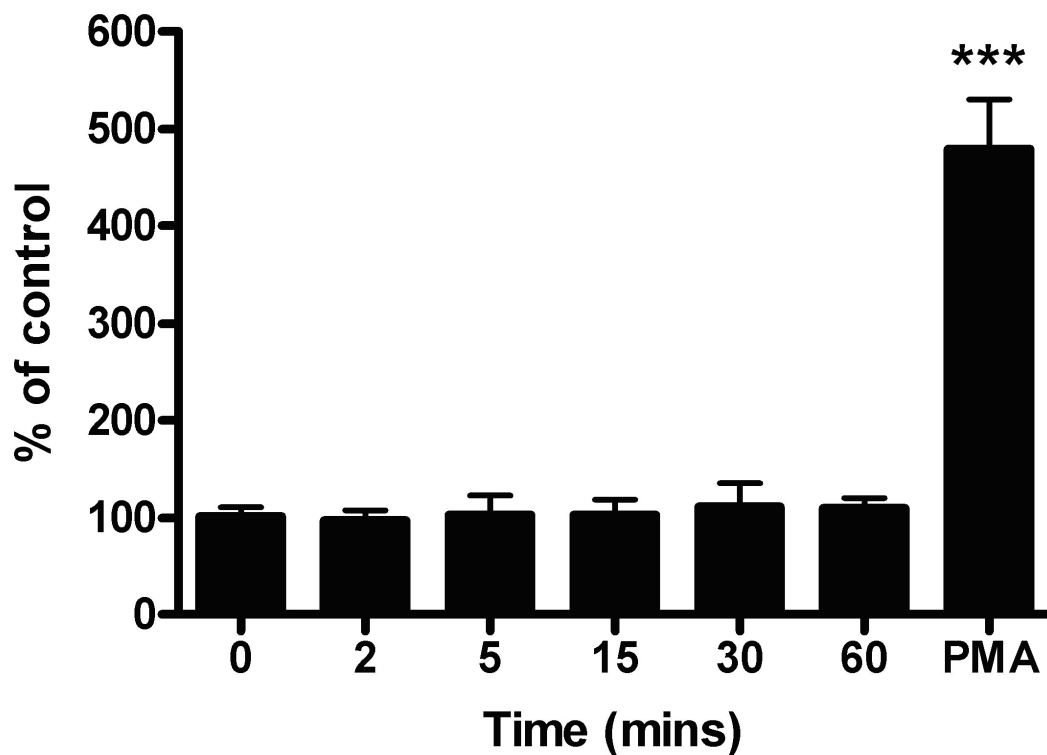


Figure 6.2.1 Time response curves of MAPK p38 activation by visfatin. No significant effect of 200ng/ml visfatin was seen on p38 activation compared to basal. Phosphorylated p38 was detected using specific enzyme-linked immunosorbent assay (ELISA) kit. Cells were stimulated with PMA (positive control) for 15 minutes n=4.

6.3.2.2. Visfatin regulation of on insulin secretion (low glucose).

The effect of visfatin on the insulin secretory response of β -cells at a sub-stimulatory glucose concentration (2.25mM) was examined. The β -cells were pre-incubated in 2.25mM glucose and varying concentrations of visfatin (0 – 200ng/ml), visfatin 200ng/ml + FK866 inhibitor (200 + F) and visfatin 200ng/ml + UO126 inhibitor (200 + U) for 1 hour and insulin content assayed using proprietary Mouse insulin ELISA kit.

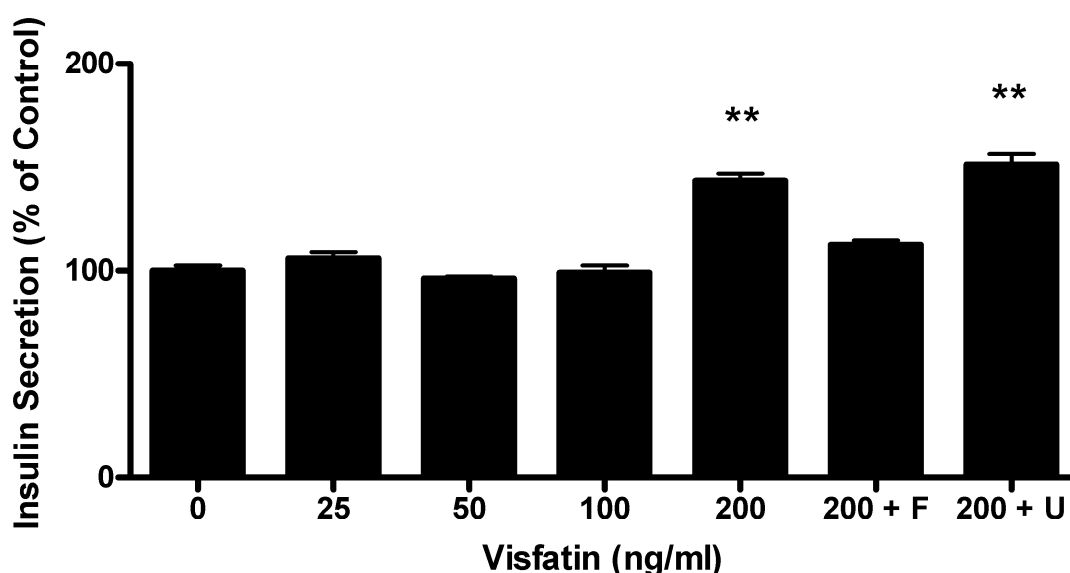


Figure 6.2.2 Effect of visfatin on insulin release from clonal pancreatic mouse β TC-6 cells at low glucose. The exposure of the mouse β TC-6 cells to 25, 50 and 100ng/ml visfatin did not elicit any secretory response however 200ng/ml visfatin evoked significant increase in insulin secretion compared to untreated cells ($p < 0.01$). The stimulatory effect of 200ng/ml visfatin on insulin secretion was significantly blocked by co-incubation with FK866 (visfatin inhibitor). Co-incubation with UO126 (ERK 1/2 inhibitor) did not significantly alter the effects of 200ng/ml visfatin on β -cell insulin secretion compared to visfatin alone. Incubation period was 1 hour. $n = 6$.

6.3.2.3. Visfatin regulation of insulin secretion (high glucose)

The effect of visfatin on insulin secretion in elevated glucose conditions (22mM) was investigated. In contrast to low glucose conditions, visfatin did not stimulate insulin secretion from β TC-6 cells at elevated glucose concentrations.

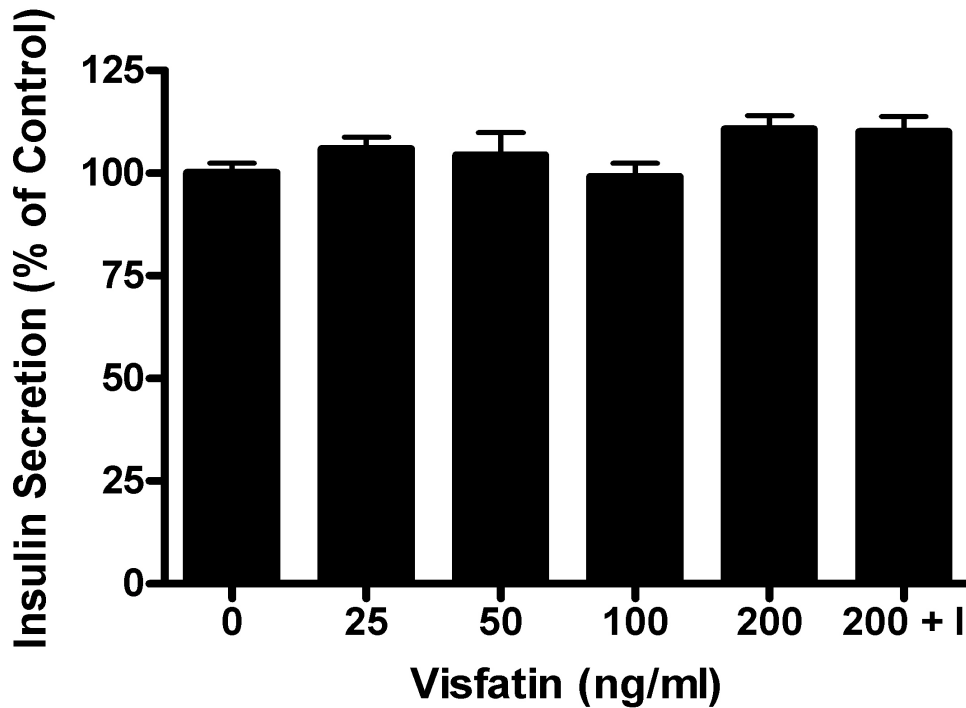


Figure 6.2.3 Effect of visfatin on insulin release from clonal pancreatic mouse β TC-6 cells at high glucose. The β -cells were incubated for 1 h at 37°C in serum free media containing 22 mM glucose and varying concentrations of visfatin (0 – 200ng/ml). The media from this last treatment was then collected and assayed for insulin content using a proprietary Mouse insulin ELISA. n=6.

6.3.2.4. NMN regulation of insulin secretion (low glucose)

Finally, the effect of NMN on insulin secretion was evaluated. Incubation with 10 and 100 μM NMN caused small but non-significant increase in insulin secretion at low glucose. 200ng/ml visfatin was used as a positive control and elicited a significant increase in insulin secretion compared to untreated control ($p < 0.05$), $n = 5$.

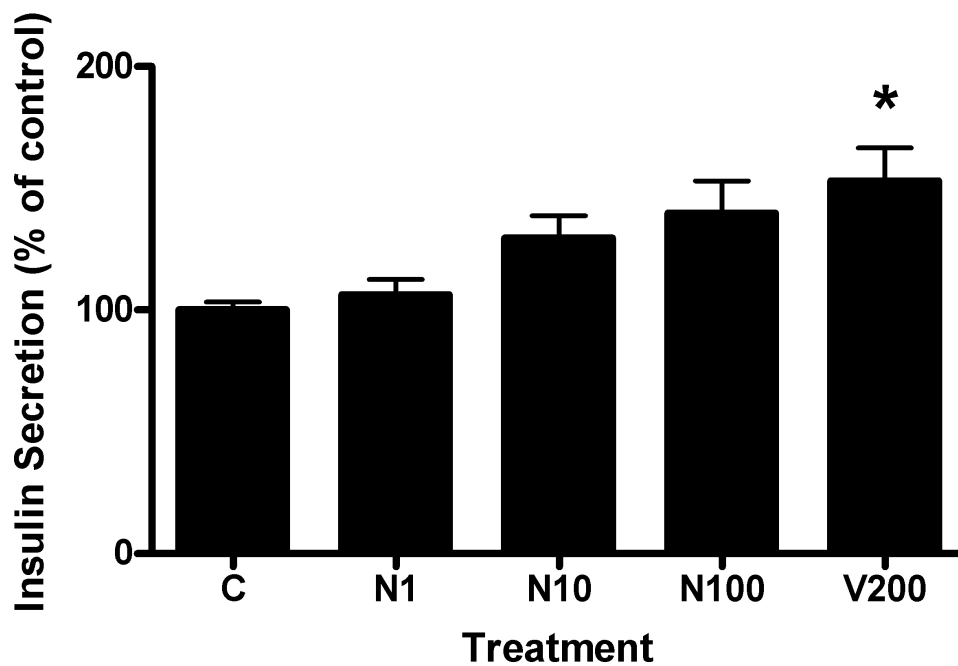


Figure 6.2.4 Effect of NMN on insulin release from clonal pancreatic mouse $\beta\text{TC-6}$ cells at low glucose. The β cells were incubated in for 1 h at 37°C in serum free media containing 2.25 mM glucose and varying concentrations of NMN (1 – 100 μM) and 200ng/ml visfatin. The media from this last treatment was then collected and assayed for insulin content using a proprietary Mouse insulin ELISA. Key: C=control, N1=1 μM NMN, N10=10 μM NMN, N100=100 μM NMN and V200=200ng/ml Visfatin. $n=5$.

6.4. Discussion

Visfatin has been recognized as a potential regulator of glucose homeostasis and mediator of obesity-associated insulin resistance (Chen *et al.*, 2006; Haider *et al.*, 2006b). Visfatin mRNA has previously been detected in human adipocytes (Berndt *et al.*, 2005). The results outlined in this study demonstrate the existence of the visfatin mRNA transcript and translated protein in the pancreatic β -cells. The high expression of visfatin mRNA and protein in the β -cells suggests that the functions of this adipokine are not restricted to the adipose tissue or muscle but also involves other endocrine organs and thus may act locally in the β -cells as a paracrine / autocrine compound.

The existence of visfatin mRNA and protein in the β -cells is of particular interest since elevated levels of visfatin has been reported in patients with type 2 diabetes (Chen *et al.*, 2006). Sun *et al.*, have recently reported that over-expression of visfatin in rats (in vivo) improves insulin sensitivity with notable increase in tyrosine phosphorylation of insulin receptor substrate-1 (Sun *et al.*, 2009). On the other hand, tumour necrosis alpha- α (TNF- α); an adipokine known to potently induces insulin resistance (Stephens & Pekala 1991; Spiegelman & Flier 2001) has been shown to suppresses visfatin gene expression in 3T3-L1 adipocytes (Kralisch *et al.*, 2005b). Thus, the high expression of visfatin in the β cells may have a possible regulatory effect on β -cell function. Further studies focusing on the identification of a potential cellular receptor are needed and the signalling pathways involved in its activity.

Initial reports regarding visfatin's insulin-mimetic effects such as lowering the plasma glucose levels and activation of insulin signalling pathway have since remained controversial due to conflicting reports (Fukuhara *et al.*, 2005; Xie *et al.*, 2007; Revollo *et al.*, 2007b; Sun *et al.*, 2009). In this study, the action of visfatin on insulin receptor activation was investigated. The results outlined in figure 6.1.3 show that visfatin treatment of clonal mouse β TC-6 cells resulted in increased insulin receptor tyrosine phosphorylation compared to untreated controls. This finding is consistent with a recent report by Xie H. and co-workers who demonstrated that visfatin induced tyrosine phosphorylation of insulin receptor (IR), insulin receptor substrate 1 (IRS-1)

and insulin receptor substrate 2 (IRS-2) in human primary osteoblasts (Xie *et al.*, 2007). The magnitude of the effects of visfatin at 50ng/ml on IR activation was similar to those seen with insulin and suggests that visfatin may have comparable effect to insulin receptor as insulin at this concentration.

Time dependant effect on IR phosphorylation by visfatin in β TC-6 cells was also determined using specific enzyme-linked immunosorbent assay (ELISA). Visfatin stimulation of the β -cells caused a significant phosphorylation of IR as illustrated in Figure 6.1.4 with the maximal effect observed after 15 minutes incubation and increased receptor phosphorylation was maintained for 45 minutes. The observed effect of visfatin on insulin receptor makes it a potential therapeutic target for insulin sensitizing drugs. In one study; rosiglitazone and fenofibrate, commonly used insulin sensitizing drugs have been shown to increase the expression of visfatin mRNA in visceral fat deposits of Otsuka Long-Evans Tokushima fatty (OLETF) rats (Choi *et al.*, 2005). These drugs increased intra-cellular visfatin expression however the increase in beta-cell insulin receptor phosphorylation illustrated in this study was due to administered (extracellular) visfatin.

However based on the effects of visfatin on insulin receptor (IR) phosphorylation illustrated in this study, it is interesting to speculate that the ability of these drugs to alleviate dyslipidemia, hyperglycaemia, and insulin resistance in animal models of obesity and T2D (Oliver Jr *et al.*, 2001; Wang *et al.*, 2003) could potentially be through regulation of visfatin expression. Characterizing the interaction process of visfatin-IR activation will be an important next step in an attempt to understand the pathways involved in the interaction and also identify possible novel targets for the development of anti-diabetic therapy. Further studies are also needed to elucidate the effects of intracellular visfatin on insulin receptor phosphosrylation.

As reviewed in the introduction chapter, the involvement of NAD⁺ biosynthesis in the regulation of β -cell function has been established by many groups. Visfatin has previously been shown to catalyse the rate-limiting step in the NAD⁺ biosynthetic pathway (Revollo *et al.*, 2004). Visfatin inhibitor FK866 non-competitively binds the enzymatic region of visfatin which normally binds nicotinamide thus preventing synthesis of NMN which is an intermediate in NAD⁺ biosynthesis (Kim *et al.*, 2006).

The involvement of this enzymatic action in visfatin induced phosphorylation of insulin receptor was examined. Briefly cells were treated with FK866 and IR activity analysed.

As illustrated in Figure 6.1.5, the ability of visfatin to stimulate IR phosphorylation was significantly inhibited effectively by co-incubation with FK866. Reduced phosphorylation of IR in the presence of FK866 compared with visfatin treatment alone demonstrates a possible inhibition of NMN biosynthesis suggesting visfatin enzymatic function and intracellular NAD⁺ content is involved in mediating this process. This observation suggests that visfatin elicits its actions on the insulin receptor (atleast in part) via the NAD biosynthetic pathway. To gain more insight into the mechanism of action of visfatin on IR activation, the cells were incubated with NMN and time-dependent effects on IR phosphorylation were analysed. As illustrated in Figure 6.1.6, β -cell stimulation with NMN resulted in a significant increase in phosphorylation of IR compared to basal. Moreover, the effect of NMN on IR phosphorylation correlated with the observed time course effect of visfatin on IR phosphorylation adding further weight to the suggestion that the NAD biosynthetic pathway is a key mechanism underpinning this observation. Based on the effects of FK866 on IR phosphorylation, it is clear that visfatin function in the β -cell is via its enzymatic activity.

Visfatin has previously been shown mediate some of its actions (e.g. angiogenesis) via activation of mitogen-activated protein kinase ERK1/2 dependent pathway (Kim *et al.*, 2007). The MAPK family of protein kinases are major signalling systems used by cells to transduce diverse intracellular signals and regulation of cellular processes. However, activation of MAPK by visfatin in the β -cells has not been previously described. As illustrated in Figure 6.1.7, visfatin induced activation of ERK1/2 in the β -cells with the maximal stimulatory effect observed after 15 minutes of incubation, however this effect subsided to near basal after 60 minutes.

Because of the potent effect of FK866 observed in reversing visfatin induced IR activation, the possible effect of this inhibitor in enzymatic activity of visfatin in ERK activation was examined. As illustrated in Figure 6.1.9, co-incubation with FK866 significantly blocked visfatin induced activation of ERK1/2 compared to visfatin

alone. The diminished phosphorylation of ERK1/2 by visfatin resulting from treatment with FK866 suggests that NMN or the NAD⁺ pathway are necessary for ERK1/2 activation in these cells. Treatment with NMN itself resulted in a significant activation of ERK1/2, further confirming the involvement of NMN in the ERK1/2 activation process.

Recently, studies have implied that visfatin may emerge as an element in the regulation of inflammation. Oki *et al.*, demonstrated that serum visfatin levels are positively correlated with the serum levels of interleukin-6 (IL-6) and C-reactive protein (CRP) both of which are inflammatory markers (Oki *et al.*, 2007). Other investigations have shown that visfatin induces the production of IL-6 and TNF- α in human monocytes (Moschen *et al.*, 2007). These the associations between markers of inflammation and visfatin indicate that circulating visfatin may reflect inflammation status. A report indicating an increase in serum visfatin with progressive β -cell deterioration suggests it may be involved with inflammatory processes in the β -cells (López-Bermejo *et al.*, 2006).

To gain insight into the mechanisms by which visfatin promotes inflammatory process, the effect on MAPK signalling was examined. p38 MAPKs are members of the MAPK family that are activated by a variety of environmental stresses and proinflammatory stimuli and involved in mediating various inflammatory processes (Furukawa *et al.*, 2003). Selective inhibition of p38 MAPK has previously been shown to reduce TNF- α and free fatty acids (FFAs) induced inflammation (Ju *et al.*, 2003; Chai *et al.*, 2007). As illustrated in Figure 6.2.1, visfatin did not induce p38 activation in the β -cells suggesting that p38 signalling does not mediate visfatin-induced inflammation.

Visfatin has previously been suggested to play a role in regulating insulin secretion (Revollo *et al.*, 2007b). The observations illustrated from this report were however based on intracellular actions of visfatin as opposed to administered visfatin. In the present study the role of visfatin in regulating insulin secretion in-vitro was examined. Between 5 and 100ng/ml, visfatin did not elicit any effect on insulin secretion at low glucose, however at the higher concentration of 200ng/ml there was a significant 50% increase in insulin secretion compared to untreated cells ($p < 0.01$). Although this is a

relatively small increase in insulin secretion, it is still within a range which makes it physiologically relevant. This observation demonstrates that at low glucose condition, visfatin is a potential secretagogue of insulin secretion in mouse β -cells.

The findings illustrated in the previous paragraph provide new insight into the molecular functions of visfatin in glucose metabolism. The present study provides evidence demonstrating that visfatin regulates insulin secretion in the pancreatic β -cells. The low concentrations of visfatin did not induce insulin release from the β -cells; however increasing the dosage of visfatin to 200ng/ml conveys beneficial effects on glucose homeostasis due to the enhanced insulin secretion. The high visfatin expression in the beta-cells and the regulatory effect on insulin secretion makes it a potential target for therapeutic interventions for the prevention and treatment of obesity induced insulin resistance.

The intracellular signalling pathways involved in the visfatin-induced modulation of insulin release were investigated using pharmacological inhibitors of NAD⁺ biosynthesis and MAPK activity, all of which are known to be important in the β -cell. Previous studies have shown that activation of MAPKs is closely involved in insulin transduction pathways and glucose stimulated insulin secretion (Frödin *et al.*, 1995). Co-incubation with visfatin inhibitor FK866 significantly blocked visfatin induced increase in insulin secretion ($p < 0.01$). Co-incubation with the ERK1/2 signalling pathway inhibitor UO126 however did not alter the effects of visfatin on insulin secretion. This result suggests that although MAPK may be activated by visfatin as illustrated in Figure 6.1.7, this pathway is not mechanistically involved in visfatin induced insulin release.

The observed effect of co-incubation with visfatin and FK866 suggests that visfatin regulation of insulin secretion is mediated by its ability to synthesize NMN since FK866 non-competitively binds the enzymatic region of visfatin which normally binds nicotinamide (the substrate in mammalian NAD biosynthesis). In order to confirm this observation, the β -cells were stimulated with NMN in similar conditions to visfatin and effect on insulin secretion determined. As illustrated in Figure 6.2.4, NMN did not significantly increase insulin secretion in the β TC-6 cells. This suggests the involvement of a more complex mechanism in mediating the actions of visfatin in

insulin secretion. It is thus possible that, in addition to blocking the ability of visfatin to bind nicotinamide as previously described (Kim *et al.*, 2006) FK866 also blocks other pathways which are yet to be identified e.g. additional metabolites to NMN.

The effect of visfatin incubation in the presence of high glucose concentrations was also examined. As illustrated in Figure 6.2.4, visfatin did not elicit any effect on insulin secretion at any of the treatment concentrations used. It is unclear why visfatin evoked a strong stimulatory effect on insulin secretion at low glucose and not at high glucose although it is possible that increases were masked by glucose-induced changes in basal secretion.

In conclusion, the observations outlined in this study suggest that visfatin has a regulatory role in beta-cell function and insulin receptor signalling, possibly through NAD biosynthetic activity. The high mRNA expression of visfatin in the β -cells and its ability to activate IR makes it a potential useful target for future drug therapies of T2D especially insulin-sensitizing drugs. The effect of visfatin on insulin secretion is evident from the results outlined in this study however the mechanism by which visfatin elicits this effect is unclear. Further investigation will be necessary to test potential pathways involved in this process.

Chapter 7

Final Discussion, Conclusion and Future Work

7.1. Final Discussion

Previously, the role of adipose tissue was considered to be little more than inert and passive storage for fat during times of caloric excess. However, in recent years it has become increasingly apparent that adipose tissue, besides storing the excess dietary energy in the form of triglycerides, is a very active secretory organ (Kim *et al.*, 2000). The number of adipose tissue secreted factors/adipokines has expanded rapidly ever since the initial discovery of adiponectin (Sirtori *et al.*, 1987) and leptin (Zhang *et al.*, 1994). Some adipokines have been shown to exert inflammatory response leading to the development of insulin resistance associated with obesity (Hotamisligil *et al.*, 1994; Pradhan *et al.*, 2001) while others have been shown to be protective against insulin resistance and T2DM (Berg *et al.*, 2001; Combs *et al.*, 2001; Steinberg *et al.*, 2002).

At the onset of this study, there was very little published data regarding the effects of resistin and visfatin in the beta cell, with only conflicting reports existing over the role of resistin in beta cell function. The initial experimental aim of this study was thus to clarify the role of resistin and visfatin in the regulation of beta cell function, viability and key genes involved in beta cell function. This would hopefully provide an insight into the roles of these adipokines in beta cell function (or dysfunction) and a basis for subsequent investigation.

As discussed in chapter three, resistin treatment at 20ng/ml which is the reported concentration in lean non-diabetics significantly increased β -cell viability over 24 hours however a higher dose of 40ng/ml which is the reported concentration in obesity and diabetic patients did not promote viability of the β -cells. This observed effect on viability was not associated with suppressed cell death under serum-deprived conditions, but rather with increased proliferation. This outcome was consistent with previous reports which indicated similar proliferative effects in endothelial cells (Verma *et al.*, 2003; Mu *et al.*, 2006). The action elicited by resistin is crucial for the beta cell function since dysregulation of β -cell proliferation has previously been shown to be a crucial feature in the pathogenesis of type 2 diabetes (Ackermann *et al.*, 2007). These effects are also critical to sustaining the secretory

function of the β -cells and maintain normoglycaemia. Adipose tissue is also one of the target organs for insulin. As such, these observations highlight a possible mechanism by which adipose tissue secreted factors contribute to β -cell survival thus fulfilling its primary role of insulin secretion to meet increased demand from enlarged adipose cell.

Visfatin did not have any effects on β -cell viability at the low concentration however supraphysiological visfatin concentrations significantly reduced β -cell viability. This shows that the low circulating concentration of visfatin do not regulate β -cell mass however the supraphysiological concentrations have a negative regulatory effect in-vitro. Previous reports have indicated that circulating visfatin concentrations is lower in healthy young subjects compared to those in patients with diabetes (Chan *et al.*, 2006; Haider *et al.*, 2006a; Haider *et al.*, 2006b; Krzyzanowska *et al.*, 2006a). It is thus possible that the low circulating visfatin concentrations do not elicit any effect on beta cell survival however subsequent increase in concentration as those observed in diabetes and obesity have pathological consequences.

In a recent study, serum visfatin concentrations were shown to increase with progressive β -cell deterioration (López-Bermejo *et al.*, 2006), suggesting this adipokine may be involved with inflammatory processes in the β -cells. Even though most reports of the circulating levels of visfatin are well below these concentrations (400 and 500ng/ml), some studies have suggested far higher circulating concentrations of visfatin (Chan *et al.*, 2007). The observed effect on cell viability is thus significant as progressive deterioration of β -cell function is often associated with a loss of β -cell mass (Prentki and Nolan, 2006) which subsequently affects secretory capability of the pancreatic islet.

Resistin has been demonstrated to exert a wide range of effects including regulation of metabolic pathways such as glucose and lipid metabolism (Steppan *et al.*, 2001; Palanivel *et al.*, 2006). Regulation of these processes involves controlling the proteins implicated in these pathways both at mRNA translation level and the respective gene expression level. Recent advances in genomic analysis technology have led to identification of genetic profile alterations of important genes involved in glucose metabolism and subsequent consequences in pathogenesis of T2D. As demonstrated

in previous studies, defects in the expression of genes involved in glucose homeostasis may be important in the aetiology of T2D (Garvey *et al.*, 1992; Pendergrass *et al.* 1998; Ducluzeau *et al.*, 2001).

In this study, resistin treatment resulted in significant alterations in the expression profiles of key genes involved in β -cell function. From the results illustrated in chapter 4, it is clear that the IL-6 and MAPK-8 mRNA expressions are profoundly increased in the β -cells after resistin treatment. Interleukin-6 is known to induce insulin resistance while MAPK-8 negatively affects insulin signalling. The observed alteration in the mRNA expression of these molecules by resistin highlights a possible pathway by which resistin induces insulin resistance in mouse beta cells. It is important to note that fatty acid induced insulin resistance is mediated by MAPK-8 (Solinas *et al.*, 2006) further illustrating the significance of this signalling molecule as a mediator of β -cell dysfunction.

Resistin treatment produced changes in the mRNA levels of a few selected enzymatic genes. Among the genes under investigation, the mRNA expression for glucose-6-phosphate dehydrogenase (G6pd2) and Glucose-6-phosphatase (G6pc) were significantly up-regulated. Upregulation of G6pc and overexpression of G6pd2 has previously been shown to result in increased hepatic glucose output and obesity induced lipid metabolism disorders respectively (Park *et al.*, 2005). In the beta cells, overexpression of the glucose-6-phosphatase has previously been shown to attenuate glucose sensitivity of insulin secretion (Iizuka *et al.*, 2000). Glucocorticoid treatment has also been shown to inhibit glucose-induced insulin release via stimulation of G6pc activity in the beta cells (Ling *et al.*, 1998). Glucose-6-phosphatase thus appears to be an important mediator of GSIS. Enlarged adipose tissue which leads to altered adipokine secretion in this case resistin may result in induction of these genes regardless of the metabolic state. The increased mRNA levels of these genes illustrates novel ways by which elevated concentrations of resistin regulates beta cell function and the pathological effect of increased adiposity on beta cell function.

Based on these observations, it is apparent that resistin modulates a variety of transcriptional changes in the beta cells, which represents the molecular basis of its action in the beta cells e.g. the effect on insulin receptor expression as illustrated in

chapter 3. The resistin concentration exerting these genetic alterations is similar to that reported in obese and T2D (Fehmann *et al.*, 2002; Zhang *et al.*, 2003) and thus highlights a possible mechanism by which the regulation of a cluster of genes involved in insulin action and glucose metabolism may be affected by altered adipokine production and secretion.

In addition to resistin, visfatin treatment resulted in significant changes in the expression profiles of key beta cell genes. Visfatin treatment resulted in down-regulation of Mapk8 mRNA and the up-regulation of Akt2. These are important signalling molecules in glucose homeostasis and alteration in expression or activity could have serious ramifications. Akt2-deficient mice have previously been shown to exhibit insulin resistance and mild diabetes (Cho *et al.*, 2001) while activation of this molecule in response to insulin positively influences glucose metabolism (Brazil and Hemmings, 2001). The 24 fold increase of Akt2 mRNA shows that visfatin has a high potency in regulating the gene expression level of Akt2 and may have a therapeutic advantage in improving insulin sensitivity in the beta-cells.

Conversely MAPK8 has been shown to increase the serine phosphorylation of insulin receptor substrate-1 (IRS-1) which in effect inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 (Aguirre *et al.*, 2000). From the results in Table 4.2, it is clear that visfatin significantly reduced Mapk8 mRNA expression by -1.122 fold. In a recent study, visfatin was shown to improve insulin sensitivity and insulin receptor substrate tyrosine phosphorylation in rodents (Sun *et al.*, 2009). This report is consistent with the results illustrated in chapter 6 (Figure 6.1.4) which shows that visfatin activates insulin receptor in the β -cells. The observed effects of visfatin have the potential to improve insulin sensitivity and reverse obesity induced insulin resistance. It is thus possible that the actions of visfatin as illustrated by Sun *et al.*, and in Figure 6.1.4 of this study could be mediated by induction of Akt2 gene and suppression of MAPK8 mRNA expression.

Visfatin treatment also resulted in an 8-fold increase in insulin mRNA. The transcription and subsequent translation of insulin gene is important in maintaining sufficient insulin supply after repeated glucose challenges. The upregulation of insulin mRNA by visfatin is of great significance as induction of insulin gene is a key step in

restoring normal blood glucose levels during hyperglycaemia. Consistent with the visfatin effect on insulin gene was a decrease in UCP-2 and PPAR- α mRNA expression in the β -cells all of which are known to be involved in suppression of insulin secretion in response to glucose (Chan *et al.*, 1999; Tordjman *et al.*, 2002). These observations shed light on the possible processes involved in visfatin induced insulin secretion as illustrated in Figure 6.2.2 of chapter 6. In addition to this, there was a significant increase in key transcription factors HNF4- α and Tcf2 as a result of visfatin treatment. This is significant and consistent with the observed effects on insulin and UCP-2 genes as both HNF4- α and Tcf2 are both involved in insulin gene transcription (Bernardo *et al.*, 2008). It also further highlights a possible pathway by which visfatin causes an increase in insulin secretion as observed in Figure 6.2.2.

The observations illustrated in chapter 5 paint a picture of a potential role of resistin in the pathophysiology of insulin resistance and T2D. The results outlined in the present study show that insulin receptor expression both at mRNA and protein level is downregulated by resistin. Several mechanisms by which obesity influences insulin resistance have been suggested in recent years. The reported physiological concentration of resistin (20ng/ml) did not have any effect on insulin receptor expression; however the elevated concentrations (40ng/ml) elicited this negative response (Figure 5.4 and 5.5). It is now clear from previous studies that increased adipose tissue mass results in altered adipokine production (Weyer *et al.*, 2000; Skurk *et al.*, 2007). The negative effects on insulin receptor expression by elevated resistin concentrations illustrate how increased adiposity negatively regulates insulin receptor expression and highlights a possible link between obesity and insulin resistance.

The result illustrated in Figure 5.1 show that resistin mRNA is highly expressed in the beta cells. The high expression of resistin in the beta cells and the effects on insulin receptor expression illustrate a potential role of this adipokine in the pathophysiology of T2D. Multiple defects in insulin signalling transduction pathways e.g. reduced insulin receptor expression levels and tyrosine kinase activity have been identified as major contributory factors to insulin resistance (Leng *et al.*, 2004; Musi *et al.*, 2006). It is thus possible that resistin induced insulin resistance in the pancreatic islets (Nakata *et al.*, 2007) is mediated by its ability to reduce insulin receptor expression in the beta cells as illustrated in this study. This is consistent with a previous report

which demonstrated that serum resistin is increased in T2D subjects (McTernan *et al.*, 2002a) highlighting a link between elevated levels and T2D. Initial studies by Stepan *et al.*, showed that circulating resistin levels are decreased by the anti-diabetic drug rosiglitazone (Stepan *et al.*, 2001). This makes resistin a potential target for diabetes therapy.

Previous studies involving visfatin and the insulin receptor have been controversial. Right in the middle of the study, the “Science” journal article (Fukuhara *et al.*, 2005) which outlined visfatin as an insulin mimetic adipokine and on which initial experiments of this study was based on was retracted. It should be stated however that only some of the experiments from this retracted paper were criticised. At this point, the experimental results from this study showed that visfatin stimulation resulted in activation of insulin receptor and the subsequent question was how. As time progressed various reports continued to emerge with some showing visfatin action to be similar to insulin (Moschen *et al.*, 2007; Xie *et al.*, 2007) while others suggesting the contrary (Pagano *et al.*, 2006). However it was not until Revollo *et al.*, illustrated the involvement of NAD⁺ biosynthetic pathway that visfatin function in the β -cells began to become clear (Revollo *et al.*, 2007b). Their study did not find evidence of visfatin insulin mimetic activity as previously reported by Fukuhara *et al.*, e.g. activation of insulin receptor (Fukuhara *et al.*, 2005) however they demonstrated that visfatin induced insulin secretion was via NAD⁺ biosynthesis.

This study therefore sought to evaluate whether the observed activation of insulin receptor was via NAD biosynthesis. As illustrated in Figure 6.1.3 and 6.1.4, visfatin activates insulin receptor in the beta-cell in a concentration and time dependant fashion. To evaluate the involvement of NAD⁺ biosynthesis in mediating the actions of visfatin in insulin receptor activation, the cells were co-incubated with visfatin and specific Nampt inhibitor FK866 and analyzed by phospho-specific insulin receptor sandwich ELISA. FK866 significantly reversed the effects of visfatin in activating insulin receptor while NMN treatment resulted in activation of insulin receptor, demonstrating that the reduced visfatin activity on insulin receptor is due to the lack of the NAD⁺ biosynthetic activity of Nampt. The results presented here demonstrate that enzymatic activity of visfatin and not the insulin mimetic activity as previously claimed by Fukuhara *et al.*, plays an essential role in activation of insulin receptor.

This adipokine is thus a potential pharmacological target in reversing insulin resistance.

Visfatin has previously been shown to activate MAPK in other cell types (Adya *et al.*, 2008b). To test whether this observation extends to the beta cells, β TC-6 cells were stimulated with visfatin and the effect on MAPK activity analysed using phospho ERK1/2 ELISA. The results in chapter 6 show that visfatin activates ERK1/2 with the maximal effect observed after 15 minutes. To test the involvement of NAD biosynthesis in this process, the cells were treated with NMN and in a separate experiment, co-incubated with the specific NAMPT inhibitor FK866. As illustrated in chapter 6, NMN stimulation resulted in activation of ERK1/2 in a similar fashion to visfatin however the visfatin induced phosphorylation of ERK1/2 was significantly reduced by FK866. This suggests that visfatin effects on ERK1/2 activity are mediated by NAD biosynthesis as FK866 non-competitively binds the enzymatic region of visfatin which normally binds nicotinamide. This finding is important to the beta cell function since increased ERK1/2 activity has previously been shown to correlate with glucose stimulated insulin secretion (Frödin *et al.*, 1995).

Most recently, it has been proposed that visfatin plays a key role in the regulation of insulin secretion (Revollo *et al.*, 2007b). In this study Revollo *et al.*, showed that NMN was able to restore insulin secretion in islets extracted from visfatin knockout mice. This conclusion was however drawn on findings based on intracellular actions of visfatin, as opposed to administered visfatin. The findings illustrated in chapter 6 of this study show that administered visfatin induces insulin secretion in the β -cells and thus suggest that visfatin has an vital role both as intra- and extracellular regulator of pancreatic beta-cell function. This observation indicate an important physiological role for visfatin as a potential regulator of insulin secretion and highlights a role by which adipose secreted factors induce insulin secretion to increase metabolic activity (glucose metabolism) and meet increased energy resulting from obesity.

As previously illustrated in chapter 6, the MAPK ERK1/2 are significantly activated by visfatin ($p=0.001$). The potential requirement for ERK activity and visfatin enzymatic activity in insulin secretion was examined. The cells were co-incubated with ERK1/2 inhibitor U0126 and Nampt inhibitor FK866. As illustrated in chapter 6,

U0126 co-incubation did not significantly alter the effects of 200ng/ml visfatin on insulin secretion compared to visfatin alone. Insulin secretion occurred normally in these experiments even when ERK activity was blocked demonstrating that visfatin induced insulin secretion is not mediated by MAPK signalling.

Co-incubation with FK866 however significantly blocked this increase in insulin secretion induced by visfatin ($p < 0.01$). This result implies that visfatin effect on insulin secretion is mediated by its ability to synthesize NMN however NMN on its own did not significantly increase insulin secretion in the beta cells, suggesting the possible existence of additional signalling pathway or the possibility that FK866 blocks visfatin in other ways. The nature of this postulated additional signal is still not known, although the results illustrated in chapter 6 have ruled out involvement of the MAPK ERK1/2 pathways in visfatin induced insulin secretion. This outcome suggests a more complex mechanism for the actions of visfatin on insulin secretion which remains to be established.

7.2. Conclusion

The identification of resistin mRNA in the beta cells show that the role of this adipokine is not restricted in the adipose tissue but also other endocrine organs. The effect of resistin in promoting cell viability illustrates the role of this adipokine in regulating beta cell mass. It is clear from this study that resistin promotes beta cell survival and may prove protective to the beta cells when subjected to stress. In this study, resistin downregulated insulin receptor expression both at transcription and translation level suggesting a potential link between increased adiposity and insulin resistance. The high resistin mRNA expression in the β -cells suggests that resistin might be having paracrine or autocrine effects.

Visfatin is also highly expressed in the beta cells and like resistin is not restricted to the adipose tissue but other endocrine organs. The high visfatin concentrations significantly reduced β -cell viability suggesting that the elevated concentrations as those observed in obesity and T2D have a negative regulatory effect on beta cell survival. Visfatin stimulation of the beta cells resulted in activation of the insulin receptor via NAD⁺ biosynthesis. This result demonstrates that the ability of visfatin to activate insulin receptor is not due to the insulin mimetic properties as previously suggested by Fukuhara *et al.*, but rather enzymatic. Visfatin treatment of the beta cells in low glucose induced insulin secretion however the process seems to be mediated by a more complex mechanism which may involve a known or unknown signalling molecule. It is clear from this study that resistin has a potential role linking obesity and insulin resistance while visfatin could potentially improve insulin sensitivity due to its effects on insulin receptor and also play a critical role in regulating insulin secretion in the beta cells.

7.3. Future work

It is slightly clear based on the results of this study how resistin and visfatin might be working in the beta cells or in any of the cell types discussed in the introductory chapter. These adipokines are presumably acting by binding to their respective putative receptors or by making NMN in the case of visfatin. Further work is thus needed to clearly identify these receptors. To further unravel the physiological roles of these adipokines, in the case of resistin; future studies should involve the use of beta cell-specific resistin knockout mice to determine the contribution of this adipokine in beta cell function and the phenotype of type 2 diabetes e.g. insulin resistance. Additional experiments examining the subcellular localisation of visfatin and resistin in the beta-cell will also be vital in understanding the roles of these adipokines in beta cell function.

Although SOCS-3 and MAPK signals are known to mediate major actions of resistin in various cell types, it is possible that many other pathways exist in the resistin signalling. Due to the effect of resistin on insulin receptor expression as illustrated in chapter 5, it would be important to clarify the mechanism by which putative resistin receptor and insulin receptor signal transduction systems could cross-talk with each other. Further analysis will be necessary to clarify this potential interaction.

Resistin treatment resulted in increased viability of the beta cells. Various signalling molecules are involved in cell proliferation and the different forms of cell death including apoptosis and necrosis. These factors play a crucial role in beta cell survival. Additional studies will be necessary to further delineate molecular mechanisms responsible for resistin-induced increase in viability of the beta cells.

The data in chapter 6 show that visfatin induces insulin secretion. This effect was reversed by visfatin inhibitor FK866. As previously illustrated, FK866 non-competitively binds the enzymatic region of visfatin which normally binds nicotinamide thus preventing synthesis of NMN which is an intermediate in NAD biosynthesis. Although the data outlined show that NMN on its own did not induce insulin secretion, the results reported here provide evidence that at least one additional

signalling pathway is necessary for visfatin function in inducing insulin secretion. Further studies should be performed to identify the exact pathways involved in visfatin signal. It is also possible that unknown or other known intermediates which are inhibited by FK866 are involved in visfatin induced insulin secretion. Additional studies should be employed to clarify this.

Glucose-stimulated biphasic insulin secretion involves at least two glucose sensing pathways: the K_{ATP} channel-dependent and K_{ATP} channel-independent pathways. Future work should examine whether visfatin and its reaction product NMN have any significant effects on K_{ATP} channels or other downstream components involved in insulin secretion as illustrated in Figure 1.0.

The results in chapter 5 and 6 show that resistin and visfatin are highly expressed in the beta cells. Additional studies are needed to identify the factors regulating intracellular resistin and visfatin. Since resistin exerts pathological effects at elevated concentrations corresponding with those observed in obesity and T2D, selective depletion of this adipokine might have a powerful therapeutic impact. Since the effect of resistin is not localised to the adipose tissue but rather endocrine, identifying factors that reduce the expression and release from adipocytes could potentially alter circulating levels and have positive impact in reversing insulin resistance associated with increased adiposity.

Visfatin effect in activating insulin receptor makes it a potential target for insulin sensitizing pharmacological agents. The described relationships of rodent visfatin expression and the observed effect on insulin receptor and MAPK phosphorylation illustrated in this study may not readily be translated to humans. Therefore, further studies are needed to clarify the role of this adipokine in human metabolism e.g. experiments on human islets.

Chapter 8

References

Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, Minnemann T, Shulman GI and Kahn BB. (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* 409, 729-33.

Accili D. Lilly lecture (2003) the struggle for mastery in insulin action: from triumvirate to republic. *Diabetes*; 2004 Jul; 53(7):1633-42.

Adya R, Tan BK, Chen J, Randeve HS. (2008a) Nuclear factor-kappaB induction by visfatin in human vascular endothelial cells: its role in MMP-2/9 production and activation. *Diabetes Care*. Apr; 31(4):758-60.

Adya R, Tan BK, Punn A, Chen J, Randeve HS. (2008b) Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis. *Cardiovascular Research*; May 1; 78(2):356-65.

Aguirre V, Uchida T, Yenush L, Davis R, White MF. (2000) The c-Jun NH (2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser (307). *Journal of Biological Chemistry* Mar 24; 275(12):9047-54.

Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF. (2002) Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *Journal of Biological Chemistry* 277(2): 1531-7

Ailhaud G, Grimaldi P, and Negrel R. (1992) Cellular and molecular aspects of adipose tissue development. *Annual Review of Nutrition* Vol. 12: 207-233.

Akerfeldt MC, Howes J, Chan JY, Stevens VA, Boubenna N, McGuire HM, King C, Biden TJ, Laybutt DR. (2008) Cytokine-induced beta-cell death is independent of endoplasmic reticulum stress signalling. *Diabetes*; Nov; 57(11):3034-44.

Alba A, Puertas MC, Carrillo J, Planas R, Ampudia R, Pastor X, Bosch F, Pujol-Borrell R, Verdaguer J, Vives-Pi M. (2004) IFN beta accelerates autoimmune type 1 diabetes in non-obese diabetic mice and breaks the tolerance to beta cells in nondiabetic-prone mice. *The Journal of Immunology* 173: 6667-6675.

Alberti KGMM, Zimmet P, Straw J. (2007) International diabetes federation: a consensus on T2D prevention. *Diabetic Medicine*; 24:451-63.

American Diabetes Association, (2000). Consensus statement: T2D in children and adolescents. *Diabetes Care* 23, 381-389.

- Amos A.F, D.J. McCarty and P. Zimmet. (1997) The rising global burden of diabetes and its complications: estimates and projections to the year 2010. *Diabetic Medicine* 14, S7–S85.
- Anello M, Lupi R, Spampinato D, Piro S, Masini M and U. Boggi. (2005) Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. *Diabetologia* 48
- Arnush MR, Heitmeier AL, Scarim MH, Marino PT, Manning and JA Corbett. (1998). IL-1 produced and released endogenously within human islets inhibits β cell function, *Journal of Clinical Investigation* 102, pp. 516–526
- Ashcroft FM, Proks P, Smith PA, Ammala C, Bokvist K, Rorsman P. (1994) Stimulus secretion coupling in pancreatic β cells. *Journal of Cellular Biochemistry* Volume 55, Issue SUPPL., Pages 54-65
- Atkinson MA, Maclaren NK. (1994) The pathogenesis of insulin-dependent diabetes mellitus. *New England Journal of Medicine* 24:1428–1436
- Azuma K, Katsukawa F, Oguchi S, Murata M, Yamazaki H, Shimada A, Saruta T. (2003) Correlation between serum resistin level and adiposity in obese individuals. *Obesity Research* Aug; 11(8):997-1001
- Baker JM, Myers MG, Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skolnik E Y, Schlessinger J and White MF. (1992) Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.* 11, 3469-3479
- Banerjee, RR., Rangwala SM, Shapiro JS, Rich AS, Rhoades B, Qi Y, Wang J, Rajala MW, Poci A, Scherer PE, Steppan CM, Ahima RS, Obici S, Rossetti L, and Lazar MA. (2004) Regulation of fasted blood glucose by resistin. *Science*; 303: 1195–1198
- Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, Vidal H and Hainque B. (2000) Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *Journal of Clinical Endocrinology and Metabolism* 85 3338 – 3342
- Bastard JP, Maachi M, Tran Van Nhieu J, Jardel C, Bruckert E, Grimaldi A, Robert JJ, Capeau J, Hainque B. (2002) Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. *Journal of Clinical Endocrinology and Metabolism* 87: 2084.
- Beale EG and Tishler EJ. (1992) Expression and regulation of cytosolic phosphoenolpyruvate carboxykinase in 3T3-L1 adipocytes. *Biochemical Biophysical Research Communication* 189, 925-930
- Bell GI, Pictet RL, Rutter WJ, Cordell B, Tischer E, Goodman HM. (1980) Sequence of the human insulin gene. *Nature* 284:26–32,

- Bergenstal RM, Kendall DM. (2001) Comprehensive management of patients with T2D: establishing priorities of care. *The American Journal of Managed Care*; Aug; 7 (10 Supplementary):S327-43; quiz S344-8.
- Bernardo AS, Hay CW, Docherty K (2008) Pancreatic transcription factors and their role in the birth, life and survival of the pancreatic beta cell. *Molecular and Cellular Endocrinology* 294:1-9
- Barnard RJ and Youngren JF. (1992) Regulation of glucose transport in skeletal muscle. *FASEB Journal*; Volume 6, Issue 14, Pages 3238-3244
- Berndt J, Klötting N, Kralisch S, Kovacs P, Fasshauer M, Schön MR, Stumvoll M, Blüher M. (2005) Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. *Diabetes* 54:2911–2916
- Berg AH, Combs TP, Du X, Brownlee M and Scherer PE. (2001) The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nature Medicine* 7, 947-953
- Boden G, Chen X, DeSantis RA and Kendrick Z. (1993) Effects of age and body fat on insulin resistance in healthy men. *Diabetes Care*, Volume 16, Issue 5 728-733,
- Bonner-Weir S. (2000) Islet growth and development in the adult. *Journal of Molecular Endocrinology*; 24:297–302,
- Bordone L, Motta MC, Picard F, Robinson A, Jhala US, Apfeld J, McDonagh T, Lemieux M, McBurney M, Szilvasi A, Easlson EJ, Lin SJ, Guarente L. (2006) Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells. 1: *PLoS Biology*. Feb; 4(2):e31.
- Borst SE, Conover CF. (2005) High-fat diet induces increased tissue expression of TNF-alpha. *Life Sciences* Sep 9; 77(17):2156-65.
- Bos JL. (1995) A target for phosphoinositide 3-kinase: Akt/PKB. 1: *Trends in Biochemical Sciences*. Nov; 20(11):441-2
- Brazil, DP, Hemmings, BA. (2001) Ten years of protein kinase B signalling: a hard Akt to follow. *Trends in Biochemical Sciences*; 26:657-664.
- Bresolin N, Bet L, Moggio M, Meola G, Fortunato F, Comi G, Adobbati L, Geremia L, Pittalis S, Scarlato G. (1989) Muscle glucose-6-phosphate dehydrogenase deficiency. *Journal of Neurology* May; 236(4):193-8
- Brown JE, Onyango DJ, Dunmore SJ. (2007) Resistin down-regulates insulin receptor expression, and modulates cell viability in rodent pancreatic beta-cells. *FEBS Letters*; Jul 10; 581(17):3273-6.
- Brown JE, Thomas S, Digby JE, Dunmore SJ. (2002) Glucose induces and leptin decreases expression of uncoupling protein-2 mRNA in human islets. *FEBS Letters* Feb 27; 513(2-3):189-92.

Bruning JC, Winnay J, Bonner-Weir S, Taylor SI, Accili, D. & Kahn CR. (1997) Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles *Cell* 88, 561-572.

Buchanan TA, Xiang AH, Peters RK, Kjos SL, Berkowitz K, Marroquin A, Goico J, Ochoa C, Azen SP. (2000) Response of pancreatic beta-cells to improved insulin sensitivity in women at high risk for T2D. *Diabetes* 49:782 –788

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) β -Cell deficit and increased β -cell apoptosis in humans with type-2 diabetes mellitus. *Diabetes*, 52: 102 –110

Butler PC, Meier JJ, Butler AE, Bhushan A. (2007) The replication of beta cells in normal physiology, in disease and for therapy. *Nature Clinical Practice Endocrinology and Metabolism* Nov; 3(11):758-68

Calvo MN, Bartrons R, Castaño E, Perales JC, Navarro-Sabaté A, Manzano A. (2006) PFKFB3 gene silencing decreases glycolysis, induces cell-cycle delay and inhibits anchorage-independent growth in HeLa cells. 1: *FEBS Letters*. May 29; 580(13): 3308-14.

Campfield LA, Smith FJ, Guisez Y, Devos R and Burn P. (1995). Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269, 546-549

Carey DG, Jenkins AB, Campbell LV, Freund J, and Chisholm DJ. (1996). Abdominal fat and insulin resistance in normal and overweight women: direct measurements reveal a strong relationship in subjects at both low and high risk of NIDDM. *Diabetes* 45: 633 –638,

Chai W, Liu Z. (2007) p38 mitogen-activated protein kinase mediates palmitate-induced apoptosis but not inhibitor of nuclear factor-kappaB degradation in human coronary artery endothelial cells. *Endocrinology*; Apr; 148(4):1622-8.

Chan CB, MacDonald PE, Saleh MC, Johns DC, Marbàn E, Wheeler MB. (1999) Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action. *Diabetes* 48: 1482–1486

Chan SJ, Seino S, Gruppuso PA, Schwartz R, and Steiner DF. (1987). A mutation in the B chain coding region is associated with impaired proinsulin conversion in a family with hyperproinsulinemia. *Proceedings of the National Academy of Sciences of the United States of America*, , 84:2194–2197.

Cheatham B, Volchuk A, Kahn CR, Wang L, Rhodes CJ, Klip A. (1996) Insulin-stimulated translocation of GLUT4 glucose transporters requires SNARE-complex proteins. *Proceedings of the National Academy of Science USA*; Dec 24; 93(26):15169-73.

Cheatham B and Kahn CR. (1995) Insulin action and the insulin signalling network. *Endocrine Reviews*; 16,117-142.

Chen H, Wertheimer SJ, Lin CH, Katz SL, Amrein KE, Burn P, Quon MJ. (1997). Protein-tyrosine phosphatases PTP1B and syp are modulators of insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Journal of Biological Chemistry* Mar 21; 272(12):8026-31

Chen MP, Chung FM, Chang DM, Tsai JC, Huang HF, Shin SJ and Lee YJ. (2006) Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with T2D mellitus. *Journal of Clinical Endocrinology and Metabolism* 91, pp. 295–299.

Chiasson JL, Josse RG, Gomis R, Hanefeld M, Karasik A and Laakso M. (2002) Acarbose for prevention of T2D mellitus: the STOP-NIDDM randomised trial. *Lancet* 359:2072 –2077,

Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ. (2001) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science*; Jun 1; 292 (5522):1728-31.

Choi KC, Ryu OH, Lee KW, Kim HY, Seo JA, Kim SG, Kim NH, Choi DS, Baik SH, Choi KM. (2005) Effect of PPAR-alpha and -gamma agonist on the expression of visfatin, adiponectin, and TNF-alpha in visceral fat of OLETF rats. *Biochemical and Biophysical Research Communications* Volume 336: 747–753,

Choi SE, Choi KM, Yoon IH, Shin JY, Kim JS, Park WY, Han DJ, Kim SC, Ahn C, Kim JY, Hwang ES, Cha CY, Szot GL, Yoon KH, Park CG. (2004) IL-6 protects pancreatic islet beta cells from pro-inflammatory cytokines-induced cell death and functional impairment in vitro and in vivo. *Transplant Immunology*. Jun-Jul; 13(1):43-53

Clement S, Krause U, Desmedt F, Tanti JF, Behrends J, Pesesse X, Sasaki T, Penninger J, Doherty M, Malaisse W, Dumont JE, Le Marchand-Brustel Y, Erneux C, Hue L, Schurmans S (2001) The lipid phosphatase SHIP2 controls insulin sensitivity. *Nature* 409:92–97,

Coffinier C, Barra J, Babinet C, Yaniv M. (1999) Expression of the vHNF1/HNF1 beta homeoprotein gene during mouse organogenesis. *Mechanisms of Development* 89 :(1–2)211–213

Cohen G, Ilic D, Raupachova J, Hörl WH. (2008) Resistin inhibits essential functions of polymorphonuclear leukocytes. 1: *Journal of Immunology*. Sep 15; 181(6):3761-8

Colditz GA, Willett WC, Rotnitzky A, Manson JE. (1995) Weight gain as a risk factor for clinical diabetes mellitus in women. *Annals of Internal Medicine*; 122:481-6.

Colditz, GA, Willett WC, Stampfer MJ, Manson JE, Hennekens CH, Arky RA, Speizer FE. (1990) Weight as a risk factor for clinical diabetes in women. *American Journal of Epidemiology* 132:501–513.

Combs TP, Berg AH, Obici S, Scherer PE, and Rossetti L. (2001) Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *Journal of Clinical Investigation* 108, 1875-1881

Combs, TP, Pajvani, UB, Berg, AH, Lin Y, Jelicks LA, Laplante M, Nawrocki AR, Rajala MW, Parlow AF, Cheeseboro L. (2004) A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity. *Endocrinology* 145:367–383

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF. (1996) Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine*; 334: 292.

Cook KS, Min HY, Johnson D, Chaplinsky RJ, Flier JS, Hunt CR, Spiegelman BM. (1987) Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. *Science*; Jul 24; 237: 402-5.

Cornelius P, Enerback S, Bjursell G, Olivecrona T, and Pekala PH. (1988) Regulation of lipoprotein lipase mRNA content in 3T3-L1 cells by tumour necrosis factor. *Biochemical Journal* 249, 765-769

Cornelius P, MacDougald OA and Lane MD. (1994) Regulation of adipocyte development. *Annual Review of Nutrition*; 14, 99-129

Coughlan A, McCarty DJ, Jorgensen LN, Zimmet P. (1997) The epidemic of NIDDM in Asian and Pacific Island populations: prevalence and risk factors. *Hormone and Metabolic Research*; 29: 323–331

Cowie CC, Port FK, Wolfe RA, Savage PJ, Moll PP, Hawthorne VM. (1989) Disparities in incidence of diabetic end-stage renal disease according to race and type of diabetes. *New England Journal of Medicine*; Oct 19; 321(16):1074-9.

Curat CA, Wegner V, Sengenès C, Miranville A, Tonus C, Busse R and Bouloumie A. (2006) Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin, *Diabetologia* 49 pp. 744–747

Czech MP, Corvera S. (1999) Signalling mechanisms that regulate glucose transport. *Journal of Biological Chemistry* Jan 22; 274(4):1865-8

Daval M, Foufelle F, Ferré P. (2006) Functions of AMP-activated protein kinase in adipose tissue. *Journal of Physiology* Jul 1; 574(Pt 1):55-62.

Degawa-Yamauchi M, Bovenkerk JE, Juliar BE, Watson W, Kerr K, Jones R, Zhu Q, Considine RV. (2003) Serum resistin (FIZZ3) protein is increased in obese humans. *Journal of Clinical Endocrinology and Metabolism* 88:5452–5455

Del Guerra S, Lupi R, Marselli L, Masini M, Bugliani M, Sbrana S, Torri S, Pollera M, Boggi U, Mosca F, Del Prato S, Marchetti P. (2005) Functional and molecular defects of pancreatic islets in human T2D. *Diabetes* 54:727–735

Del Prato S, Wishner WJ, Gromada J, Schluchter BJ. (2004) Beta-cell mass plasticity in type 2 diabetes. *Diabetes Obesity and Metabolism*; Sep; 6(5):319-31

Desbuquois B, Tozzo E, Collinet M, Lopez S, Bortoli S, Amessou M. (1993) Regulation of insulin receptor expression and its gene. *Annales Endocrinologie (Paris)*; 54 (6):373-84.

Dogru T, Sonmez A, Tasci I, Bozoglu E, Yilmaz MI, Genc H, Erdem G, Gok M, Bingol N, Kilic S, Ozgurtas T, Bingol S. (2007) Plasma visfatin levels in patients with newly diagnosed and untreated T2D mellitus and impaired glucose tolerance. *Diabetes Research in Clinical Practice* 76:24–29

Doliba NM, Wehrli SL, Vatamaniuk MZ, Qin W, Buettger CW, Collins HW, Matschinsky FM. (2007) Metabolic and ionic coupling factors in amino acid-stimulated insulin release in pancreatic beta-HC9 cells. *American Journal of Physiology Endocrinology and Metabolism*; Jun; 292(6):E1507-19.

Dombrowski L, Roy D, Marette A. (1998) Selective impairment in GLUT-4 translocation to transverse tubules in skeletal muscle of streptozotocin-induced diabetic rats. *Diabetes*; Jan; 47(1):5-12

Donath MY, Gross DJ, Cerasi E and Kaiser N. (1999) Hyperglycaemia-induced beta-cell apoptosis in pancreatic islets of *Psammomys obesus* during development of diabetes. *Diabetes*, Volume 48, Issue 4 738-744.

Dunstan DW, Zimmet PZ, Welborn TA, De Courten MP, Cameron AJ, Sicree RA, Dwyer T, Colagiuri S, Jolley D, Knuiman M, Atkins R, Shaw JE. (2002) The rising prevalence of diabetes and impaired glucose tolerance: the Australian Diabetes, Obesity and Lifestyle Study. *Diabetes Care* May; 25(5):829-34.

Dvorak R, Starling RD, Callés-Escandon J, Sims EA, Poehlman ET. (1997) Drug therapy for obesity in the elderly. *Drugs Aging*; Nov; 11(5):338-51

Efrat S, Surana M, Fleischer N. (1991) Glucose induces insulin gene transcription in a murine pancreatic beta-cell line. *Journal of Biological Chemistry* Jun 15; 266(17):11141-3

Engeli JJ, Gorzelnik K, Luft FC and Sharma AM. (2002) Resistin gene expression in human adipocytes is not related to insulin resistance. *Obesity Research* 10 (1): 1-5

Eizirik DL, Korbitt GS, Hellerstrom C. (1992) Prolonged exposure of human pancreatic islets to high glucose concentrations in vitro impairs the β -cell function. *Journal of Clinical Investigations* 90:1263–1268

Evans, D.J. Murray R and Kissebahet AH. (1984) Relationship of body fat tomography to insulin sensitivity and metabolic profile in premenopausal woman. *Metabolism* 36, 68–75

Exton JH, Park CR. (1968) Control of gluconeogenesis in liver. II. Effects of glucagon, catecholamines, and adenosine 3',5'-monophosphate on gluconeogenesis in the perfused rat liver. *Journal of Biological Chemistry* Aug 25; 243(16):4189-96.

Fagot-Campagna A, Narayan KM, Imperatore G. (2001) Type 2 diabetes in children. *BMJ*. Feb 17; 322(7283):377-8.

Fan Hong-qi, Gu Nan, Liu Feng, Fei Li, Pan Xiao-qin, Guo Mei, Chen Rong-hua, Guo Xi-rong. (2007) Prolonged exposure to resistin inhibits glucose uptake in rat skeletal muscles. *Acta Pharmacologica Sinica*; Mar; 28 (3): 410–416

Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM, O'Rahilly S. (2002) Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *Journal of Clinical Investigation* Oct; 110(8):1093-103.

Fehmann HC, Heyn J. (2002) Plasma resistin levels in patients with type 1 and type 2 diabetes mellitus and in healthy controls. *Hormone and Metabolic Research*, 34: 671–3.

Fernandez-Real, J. M., M. Broch, J. Vendrell, C. Gutierrez, R. Casamitjana, M. Pugeat, C. Richart, and W. Ricart. (2000) Interleukin-6 gene polymorphism and insulin sensitivity. *Diabetes* 49:517-520

Ferrannini E. Insulin resistance versus insulin deficiency in non-insulin-dependent diabetes mellitus: problems and prospects. 1: *Endocrine Reviews*. (1998) Aug; 19(4): 477-90.

Ferrannini E, A. Gastaldelli, Y. Miyazaki, M. Matsuda, A. Mari, R.A. DeFronzo. (2005) Beta-cell function in subjects spanning the range from normal glucose tolerance to overt diabetes: a new analysis. *Journal of Clinical Endocrinology Metabolism*; 90 493–500.

Ferri C, Bellini C, Desideri G, Valenti M, De Mattia G, Santucci A, Hollenberg NK, Williams GH. (1999) Relationship between insulin resistance and nonmodulating hypertension: linkage of metabolic abnormalities and cardiovascular risk. *Diabetes* 48:1623-30.

Festa A, Williams K, Hanley AJ, Haffner SM. (2008) Beta-cell dysfunction in subjects with impaired glucose tolerance and early type 2 diabetes: comparison of surrogate markers with first-phase insulin secretion from an intravenous glucose tolerance test. *Diabetes*; Jun; 57(6):1638-44.

Feve B, Emorine L J, Briend-Sutren MM, Lasnie F, Strosberg AD, and Pairault J. (1990) Differential regulation of beta 1- and beta 2-adrenergic receptor protein and mRNA levels by glucocorticoids during 3T3-F442A adipose differentiation. *Journal of Biological Chemistry*

Fink, RI, Huecksteadt T, and Karaoghlanian Z. (1980) The effects of aging on glucose metabolism in adipocytes from Fischer rats. *Endocrinology* 118: 1139-1147,

Flegal, K.M., Carroll, M.D., Ogden, C.L., and Johnson, C.L. (2002) Prevalence and trends in obesity among US adults, 1999-2000. *JAMA*; 288:1723–1727.

Flier JS, Cook KS, Usher P, Spiegelman BM. (1987) Severely impaired adiponin expression in genetic and acquired obesity. *Science* 237:405–408

Flier SN, Kulkarni RN, Kahn CR. (2001) Evidence for a circulating islet growth factor in insulin resistant states. *Proceedings of the National Academy of Science USA* 98:7475 –7480,

Flodström M, Welsh N, Eizirik DL. (1996) Cytokines activate the nuclear factor kappa B (NF-kappa B) and induce nitric oxide production in human pancreatic islets. *FEBS Letters*; Apr 29; 385(1-2):4-6.

Flores JC, Hirschhorn J, Altshuler D. (2003) The inherited basis of diabetes mellitus: implications for the genetic analysis of complex traits. *Annual Review of Genomics and Human Genetics*, 4: 257-291.

Ford ES, Williamson DF, Liu S. (1997) Weight change and diabetes incidence: findings from a national cohort of US adults. *American Journal of Epidemiology*, 146:214-222.

Frank SJ. (2001) Growth hormone signalling and its regulation: preventing too much of a good thing. *Growth Hormone and IGF Research* 11 201–212

Frödin M, Sekine N, Roche E, Filloux C, Prentki M, Wollheim CB, Van Obberghen E. (1995) Glucose, other secretagogues, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin-secreting beta-cell line, INS-1. *Journal of Biological Chemistry*; Apr 7; 270(14):7882-9.

Frohnert BI, Nui TY, Bernlohr DA. (1999) Identification of a functional peroxisome proliferator-activated responsive element in the murine fatty acid transport protein gene. *Journal of Biological Chemistry* 274: 3970–3977

Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, and Lodish HF. (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proceedings of the National Academy of Science USA* 98, 2005-2010

Fried SK, Bunkin DA, Greenberg AS. (1998) Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *Journal of Clinical Endocrinology and Metabolism*; 83: 847.

Fujinami A, Obayashi H, Ohta K, Ichimura T, Nishimura M, Matsui H, Kawahara Y, Yamazaki M, Ogata M, Hasegawa G, Nakamura N, Yoshikawa T, Nakano K, Ohta M. (2004) Enzyme-linked immunosorbent assay for circulating human resistin:

Resistin concentrations in normal subjects and patients with type 2 diabetes. *Clinica Chimica Acta*; 339: 57–63.

Fujita H, Fujishima H, Morii T, Koshimura J, Narita T, Kakei M, Ito S. (2002) Effect of metformin on adipose tissue resistin expression in db/db mice. *Biochemical and Biophysical Research Communications* Volume 298, Issue 3, 1 November, Pages 345-349

Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y, Shimomura I. (2005) Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science*; 307: 426–430

Furukawa F, Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sugano Y, Yamagata H, Matsuushita M, Seki T, Inagaki Y, Nishizawa M, Fujisawa J, Inoue K. (2003) p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. *Hepatology*; 38,879-889

Gabriely I, Ma XH, Yang XM, Atzmon G, Rajala MW, Berg AH, Scherer P, Rossetti L, Barzilai N. (2002) Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process? *Diabetes*; 10: 2951–2958.

Gammeltoft S. (1984) Insulin receptors: binding kinetics and structure-function relationship of insulin. *Physiological Reviews*; Oct; 64(4):1321-78

Garten A, Petzold S, Körner A, Imai SI, Kiess W. (2008) Nampt: linking NAD biology, metabolism and cancer. *Trends in Endocrinology & Metabolism*;

Gerich JE. (1998) The genetic basis of T2D mellitus: impaired insulin secretion versus impaired insulin sensitivity. *Endocrinology Review* 19:491 –503,

Gerstmayr B, Küsters D, Gebel S, Müller T, Van Miert E, Hofmann K, Bosio A. (2003) Identification of RELM gamma, a novel resistin-like molecule with a distinct expression pattern. *Genomics*; Jun; 81(6):588-95.

Gesta S, Tseng YH, Kahn CR. (2007) Developmental origin of fat: tracking obesity to its source. *Cell*; 131:242e256.

Goodner CJ, Koerker DJ, Weigle DS, McCulloch DK. (1989) Decreased insulin- and glucagon-pulse amplitude accompanying beta-cell deficiency induced by streptozocin in baboons. *Diabetes* 38:925–931,

Gloyn AL. (2003) The search for T2D genes. *Ageing Research Reviews*; 2: 111-127.

Granata R, Trovato L, Garbarino G, Taliano M, Ponti R, Sala G, Ghidoni R, Ghigo E (2004) Dual effects of IGFBP-3 on endothelial cell apoptosis and survival: involvement of the sphingolipid signalling pathways. *FASEB J* 18:1456–1458

Grempler R, Leicht S, Kischel I, Eickelmann P, Redemann N. (2007) Inhibition of SH2-domain containing inositol phosphatase 2 (SHIP2) in insulin producing INS1E cells improves insulin signal transduction and induces proliferation. *FEBS Letters*; Dec 22; 581(30):5885-90.

Groop LC and Ferrannini E. (1993) Insulin action and substrate competition. In: E. Ferrannini, Editor, *Baillière's Clinical Endocrinology and Metabolism*, Baillière Tindall, London pp. 1007–1032.

Guerre-Millo M. (2004) Adipose tissue and adipokines, for better or worse. *Diabetes & Metabolism*; Volume 30, Issue 1, February, Pages 13-19

Haider DG, Schaller G, Kapiotis S, Maier C, Luger A and Wolzt M. (2006a) The release of the adipokine visfatin is regulated by glucose and insulin, *Diabetologia* 49, pp. 1909–1914.

Haider DG, Holzer G, Schaller G, Weghuber D, Widhalm K, Wagner O, Kapiotis S, Wolzt M. (2006b) The adipokine visfatin is markedly elevated in obese children. *Journal of Pediatric Gastroenterology and Nutrition*; 2006 Oct; 43(4):548-9.

Haider DG, Schindler K, Schaller G, Prager G, Wolzt M, Ludvik B. (2006c) Increased plasma visfatin concentrations in morbidly obese subjects are reduced after gastric banding. *Journal of Clinical Endocrinology and Metabolism*; Apr; 91(4):1578-81.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*; Jul 28; 269(5223):543-6

Hamilton-Shield JP. (2007) Overview of neonatal diabetes. *Endocrine Development*; 12:12–23.

Hammarstedt A, Pihlajamaki J, Rotter Sopasakis V, Gogg S, Jansson PA, Laakso M and Smith U. (2006) Visfatin is an adipokine, but it is not regulated by thiazolidinediones. *Journal of Clinical Endocrinology and Metabolism* 91 pp 1181–1184.

Han Cho, James Mu, Jason K. Kim, Joanne L. Thorvaldsen, Qingwei Chu, E. Bryan Crenshaw III, Klaus H. Kaestner, Marisa S. Bartolomei, Gerald I. Shulman, Morris J. Birnbaum. (2001) Insulin Resistance and a Diabetes Mellitus-Like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKB). *Science*; June Vol. 292.

Hansen, L. (2003) Candidate genes and late-onset T2D mellitus. Susceptibility genes or common polymorphisms? *Danish Medical Bulletin*, 50: 320-346.

Hansmann F, Mordier S, Iynedjian PB. (2006) Insulin induction of glucokinase and fatty acid synthase in hepatocytes: analysis of the roles of sterol-regulatory-element-binding protein-1c and liver X receptor. *Biochemical Journal*; Oct 15; 399(2):275-83.

- Harris TE, Persaud SJ and Jones MJ. (1996) Atypical isoforms of PKC and insulin secretion from pancreatic β -cells: evidence using Gö 6976 and Ro 31-8220 as PKC inhibitors. *Biochemical and Biophysical Research Communications*; 227, pp. 672–676.
- Hattersley AT, Turner RC, Permutt MA, Patel P, Tanizawa Y, Chiu KC, O’Rahilly S, Watkins PJ, Wainscoat JS. (1992) Linkage of type 2 diabetes to the glucokinase gene. *Lancet* 339: 1307–1310.
- Hedeskov CJ. (1980) Mechanism of glucose-induced insulin secretion. *Physiological Reviews*; Apr; 60(2):442-509
- Henquin JC. (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* Nov; 49(11):1751-60.
- Hiles ID, Otsu M, Volinia S, Fry MJ, Gout I, Dhand R, Panayotou G, Ruiz-Larrea F, Thompson A, Totty NF. (1992) Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell* Aug 7; 70(3):419-29
- Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. (2002) A central role for JNK in obesity and insulin resistance. *Nature*; Nov 21; 420(6913):333-6
- Holcomb IN, Kabakoff RC, Chan B, Baker TW, Gurney A, Henzel W, Nelson C, Lowman HB, Wright BD, Skelton NJ, Frantz GD, Tumas DB, Peale FV Jr, Shelton DL, Hébert CC. (2000) FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *The EMBO Journal* 19, 4046–4055
- Horikawa Y, Iwasaki N, Hara M, Furuta H, Hinikio Y, Cockburn BN, Linder T, Yamagata K, Ogata M, Tomonaga O, Kuroki H, Kasahara T, Iwamoto Y, Bell GI. (1997) Mutation in hepatocyte nuclear factor-1 gene (TCF-2) associated with MODY. *Nature Genetics* 17: 384 – 385
- Hossain P, Kavar B, El Nahas M. (2007) Obesity and diabetes in the developing world—a growing challenge. *New England Journal of Medicine*; 356:213-5.
- Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. (1994) Tumor necrosis factor alpha inhibits signalling from the insulin receptor. *Proceedings of the National Academy of Science USA*; 91:4854-8.
- Hotamisligil GS, Shargill NS, Spiegelman BM. (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259, 87-91
- Hu FB, Manson JE, Stampfer MJ, Colditz G, Liu S, Solomon CG, Willett WC. (2001) Diet, lifestyle, and the risk of T2D mellitus in women. *New England Journal of Medicine*; 345:790-7.

Hunt CR, Ro JH, Dobson DE, Min HY and Spiegelman BM. (1986) Adipocyte P2 gene: developmental expression and homology of 5'-flanking sequences among fat cell-specific genes Proceedings of the National Academy of Science USA. 83, 3786-3790

Icks A, Trautner C, Haastert B, Berger M, Giani G. (1997) Blindness due to diabetes: population-based age- and sex-specific incidence rates. Diabetic Medicine Jul; 14(7):571-5

Imai SI, Armstrong CM, Kaerberlein M, Guarente L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature Volume 403, Issue 6771, 17 February,

Ishihara H, Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki JI, Oka Y. (1993) Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. Diabetologia, Nov; 36(11):1139-45

Ishihara H, Sasaoka T, Hori H, Wada T, Hirai H, Haruta T, Langlois WJ, Kobayashi M (1999) Molecular cloning of rat SH2-containing inositol phosphatase 2 (SHIP2) and its role in the regulation of insulin signaling. Biochemical and Biophysical Research Communications 260:265–272,

Jazet IM, Pijl H, Meinders AE. Adipose tissue as an endocrine organ: impact on insulin resistance. Netherlands Journal of Medicine 2003; 61: 194-212

Jia SH, Li Y, Parodo J, Kapus A, Fan L, Rotstein OD, Marshall JC. (2004) Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. Journal of Clinical Investigations 113: 1318–1327

Ju H, Behm DJ, Nerurkar S, Eybye ME, Haimbach RE, Olzinski AR, Douglas SA, Willette RN (2003) p38 MAPK inhibitors ameliorate target organ damage in hypertension: part 1. p38 MAPK-dependent endothelial dysfunction and hypertension. Journal of Pharmacology and Experimental Therapeutics; 307:932–938

Kaburagi Y, Yamauchi T, Yamamoto-Honda R, Ueki K, Tobe K, Akanuma Y, Yazaki Y, Kadowaki T. (1999) The mechanism of insulin-induced signal transduction mediated by the insulin receptor substrate family. Endocrine Journal Mar; 46 Suppl: S25-34

Kahn BB. (1992) Facilitative glucose transporters: regulatory mechanisms and dysregulation in diabetes. Journal of Clinical Investigation; 89, 1367-1374

Kahn BB and Flier JS. (2000) Obesity and insulin resistance. Journal of Clinical Investigation; 106:473–481.

Kahn SE. (2001) Clinical review 135: the importance of β -cell failure in the development and progression of T2D. Journal of Clinical Endocrinology and Metabolism 86:4047–4058

- Kahn SE, Prigeon RL, McCulloch DK, Boyko EJ, Bergman RN, Schwartz MW, Neifing JL, Ward WK, Beard JC, Palmer JP *et al.*, (1993) Quantification of the relationship between insulin sensitivity and B-cell function in human subjects. Evidence for a hyperbolic function. *Diabetes*; 42:1663–1672
- Kainulainen H, Breiner M, Schürmann A, Martinen A, Virjo A, Joost HG. (1994). In vivo glucose uptake and glucose transporter proteins GLUT1 and GLUT4 in heart and various types of skeletal muscle from streptozotocin-diabetic rats. *Biochimica et Biophysica Acta*. Feb 22; 1225(3):275-82
- Kanai F, Ito K, Todaka M, Hayashi H, Kamohara S, Ishii K, Okada T, Hazeki O, Ui M, Ebina Y. (1993) Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase. *Biochemical and Biophysical Research Communication*; Sep 15; 195(2):762-8.
- Kasuga M. (2006) Insulin resistance and pancreatic beta cell failure. *Journal of Clinical Investigation*. 2006 Jul; 116(7):1756-60.
- Kawate R, Talan MI, Engel BT. (1994) Sympathetic nervous activity to brown adipose tissue increases in cold-tolerant mice. *Physiology and Behaviour*; May; 55(5):921-5
- Kelly KL, Ruderman NB and Chen KS. (1992) Phosphatidylinositol-3-kinase in isolated rat adipocytes. Activation by insulin and subcellular distribution. *Journal of Biological Chemistry*; 267, 3423-3428
- Kershaw EE, Flier JS. (2004) Adipose tissue as an endocrine organ. *Journal of Clinical Endocrinology and Metabolism*; Jun; 89(6):2548-56.
- Khan JA, Tao X, Tong L. (2006) Molecular basis for the inhibition of human NMPRTase, a novel target for anticancer agents, *Nature Structural and Molecular Biology*; 13, pp. 582–588.
- Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G. (2003) Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. 1: *Diabetes*. Jul; 52(7):1779-85
- Kersten S. (2001) Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep*. Apr; 2(4):282-6.
- Kieffer TJ, Heller RS, Leech CA, Holz GG, Habener JF. (1997) Leptin suppression of insulin secretion by the activation of ATP-sensitive K⁺ channels in pancreatic beta-cells. *Diabetes*, Jun; 46(6):1087-93.
- Kim MK, Lee JH, Kim H, Park SJ, Kim SH, Kang GB, Lee YS, Kim JB, Kim KK, Suh SW, Eom SH. (2006) Crystal structure of visfatin/pre-B cell colony-enhancing factor 1/nicotinamide phosphoribosyltransferase, free and in complex with the anti-cancer agent FK-866. *Journal of Molecular Biology* 362, 66–77

Kim KH, Lee K, Moon YS and Sul HS. (2001) A cysteine rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. *Journal of Biological Chemistry*; 276, 11252–11256.

Kim S, Moustaid-Moussa N. (2000) Secretary, endocrine and autocrine/paracrine function of the adipocyte. *Journal of Nutrition*; 130:3110Se3115.

Kim SR, Bae YH, Bae SK, Choi KS, Yoon KH, Koo TH, Jang HO, Yun I, Kim KW, Kwon YG, Yoo MA, Bae MK. (2008) Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF-kappaB activation in endothelial cells. *Biochimica et Biophysica Acta*; May; 1783(5):886-95.

Kim SR, Bae SK, Choi KS, Park SY, Jun HO, Lee JY, Jang HO, Yun I, Yoon KH, Kim YJ, Yoo MA, Kim KW, Bae MK. (2007) Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2. : *Biochemical and Biophysical Research Communications*. May 25; 357(1):150-6.

Kirpichnikov D, McFarlane SI, Sowers JR. (2002) Metformin: an update. 1: *Annals of Internal Medicine*. Jul 2; 137(1):25-33

Kjems LL, Kirby BM, Welsh EM, Veldhuis JD, Straume M, McIntyre SS, Yang D, Lefebvre P, and Butler PC (2001) Decrease in beta-cell mass leads to impaired pulsatile insulin secretion, reduced postprandial hepatic insulin clearance, and relative hyperglucagonemia in the minipig. *Diabetes* 50:2001–2012,

Kloppel G, Mattias L, Habich K, Oberholzer M, Heitz PU. (1985) Islet pathology and the pathogenesis of type 1 and T2D mellitus revisited. *Survey and Synthesis of Pathology Research* 4:110 –125,

Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM. (2002) Reduction in the incidence of T2D with lifestyle intervention or metformin. *New England Journal of Medicine* 3 46:393 –403,

Kovacikova M, Vitkova M, Klimcakova E, Polak J, Hejnova J, Bajzova M, Kovacova Z, Viguerie N, Langin D, Stich V. (2008) Visfatin expression in subcutaneous adipose tissue of pre-menopausal women: relation to hormones and weight reduction. *European Journal of Clinical Investigation*; Jul; 38(7):516-22

Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M, Fasshauer M. (2005a) Interleukin-6 is a negative regulator of visfatin gene expression in 3T3-L1 adipocytes. *American Journal of Physiology Endocrinology Metabolism*; 289: E586–E590

Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M and Fasshauer M. (2005b) Hormonal regulation of the novel adipocytokine visfatin in 3T3-L1 adipocytes. *Journal of Endocrinology* 185, R1-R8

Krehl WA, Trepley LJ, Sarma PS, Elvehjem CA. (1945) Growth retarding effect of corn in niacin-low rations and its counteraction by tryptophan. *Science*; 101:489–490.

- Krentz AJ, Friedmann PS. (2006) Type 2 diabetes, psoriasis and thiazolidinediones. : International Journal of Clinical Practice. Mar; 60(3):362-3.
- Krook A, O’Rahilly S. (1996) Mutant insulin receptors in syndromes of insulin resistance. Baillieres Clinical Endocrinology Metabolism; 10:97-122
- Krzyzanowska K, Krugluger W, Mittermayer F, Rahman R, Haider D, Shnawa N, Scherthaner G. (2006a) Increased visfatin concentrations in women with gestational diabetes mellitus. Clinical Science (London) 110:605–609
- Krzyzanowska K, Mittermayer F, Krugluger W, Kopp HP and Scherthaner G. (2006b) Increase in Visfatin after Weight Loss Induced by Gastroplastic Surgery. Obesity 14, 1886–1889
- Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, Yano W, Froguel P, Nagai R, Kimura S, Kadowaki T, Noda T. (2002) Disruption of adiponectin causes insulin resistance and neointimal formation. Journal of Biological Chemistry. 277:25863–25866
- Kucera, G. L., and Rittenhouse S. E. (1990) Human platelets form 3-phosphorylated phosphoinositides in response to alpha-thrombin, U46619, or GTP gamma S. Journal of Biological Chemistry. 265, (10):5345-8.
- Kudva YC, Butler PC: Insulin secretion in type-2 diabetes mellitus. In Clinical Research in Diabetes and Obesity. Volume 2: Diabetes and Obesity. Draznin B, Rizza R, Eds. Totowa, NJ, Humana Press,1997 , p.119 –136
- Kulkarni RN, Brüning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR. (1999) Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in T2D. Cell 96:329-339.
- Kulkarni RN. (2002) Receptors for insulin and insulin-like growth factor-I and insulin receptor substrate-1 mediate pathways that regulate islet function. Biochemical Society Transactions; 30: 317-322,
- Kumar S, Boehm J, Lee JC. (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nature Reviews. Drug Discovery. Sep; 2(9):717-26.
- Kusminski CM, McTernan PG, Kumar S. (2005) Role of resistin in obesity, insulin resistance and Type II diabetes. Clinical Science (London); Sep; 109(3):243-56.
- Kutlu B, Burdick D, Baxter D, Rasschaert J, Flamez D, Eizirik DL, Welsh N, Goodman N, Hood L. (2009) Detailed transcriptome atlas of the pancreatic beta cell. BMC Medical Genomics; Jan 15; 2:3
- Kwon G, Corbett JA, Rodi CP, Sullivan P, McDaniel ML. (1995) Interleukin-1 beta-induced nitric oxide synthase expression by rat pancreatic beta-cells: evidence for the involvement of nuclear factor kappa B in the signaling mechanism. Endocrinology; Nov; 136(11):4790-5.

- Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV, Biden TJ. (2007) Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia*. Apr; 50(4):752-63.
- Leahy JL. (2005) Pathogenesis of type 2 diabetes mellitus. *Archives of Medical Research*; May-Jun; 36(3):197-209.
- Leng Y, Karlsson HK, Zierath JR. (2004) Insulin signalling defects in type 2 diabetes. *Reviews in Endocrine and Metabolic Disorders*; May; 5(2):111-7
- Levy JR, Davenport B, Clore JN, Stevens W. (2002) Lipid metabolism and resistin gene expression in insulin-resistant Fischer 344 rats. *American Journal of Physiology* 282:E626–E633
- Li B, Nolte LA, Ju JS, Han DH, Coleman T, Holloszy JO, Semenkovich CF. (2000) Skeletal muscle respiratory uncoupling prevents diet-induced obesity and insulin resistance in mice. *Nature Medicine* 6: 1115–1120
- Lihn AS, Richelsen B, Pedersen SB, Haugaard SB, Rathje GS, Madsbad S, Andersen O. (2003) Increased expression of TNF-alpha, IL-6, and IL-8 in HALS: implications for reduced adiponectin expression and plasma levels. *American Journal of Physiology Endocrinology and Metabolism*; Nov; 285(5):E1072-80
- Lillioja S, Mott DM, Howard BV, Bennett PH, Yki-Järvinen H, Freymond D, Nyomba BL, Zurlo F, Swinburn B, Bogardus C. (1988) Impaired glucose tolerance as a disorder of insulin action. Longitudinal and cross-sectional studies in Pima Indians. *New England Journal of Medicine*; May 12; 318(19):1217-25.
- Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E, Knowler WC, Bennett PH, and Bogardus C. (1993) Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus: prospective studies of Pima Indians. *New England Journal of Medicine* 329: 1988-1992,
- Linhart HG, Ishimura-Oka K, DeMayo F, Kibe T, Repka D, Poindexter B, Bick RJ, Darlington GJ. (2001) C/EBPalpha is required for differentiation of white, but not brown, adipose tissue. *Proceedings of the National Academy of Science USA*; Oct 23; 98(22):12532-7.
- Lipsett M, Finegood DT. (2002) Beta-cell neogenesis during prolonged hyperglycemia in rats. *Diabetes*. Jun; 51(6):1834-41.
- Liu F, Dallas-Yang Q, Castriota G, Fischer P, Santini F, Ferrer M, Li J, Akiyama TE, Berger JP, Zhang BB, Jiang G. (2009) Development of a novel GLUT4 translocation assay for identifying potential novel therapeutic targets for insulin sensitization. *Biochemical Journal*. Mar 1; 418(2):413-20.
- Liu F, Yang T, Wang B, Zhang M, Gu N, Qiu J, Fan HQ, Zhang CM, Fei L, Pan XQ, Guo M, Chen RH, Guo XR. (2008) Resistin induces insulin resistance, but does not

affect glucose output in rat-derived hepatocytes. *Acta Pharmacologica Sinica*; Jan; 29(1):98-104.

López-Bermejo A, Chico-Julià B, Fernández-Balsells M, Recasens M, Esteve E, Casamitjana R, Ricart W, Fernández-Real JM. (2006) Serum visfatin increases with progressive beta-cell deterioration. *Diabetes*; Oct; 55(10):2871-5.

Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y. (2002) Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nature Medicine*; 8:731–737.

Magni G, Amici A, Emanuelli M, Raffaelli N, Ruggieri S. (1999) Enzymology of NAD⁺ synthesis. *Advances in Enzymology and Related Areas of Molecular Biology*. Volume 73, Pages 135-182

Marion E, Kaisaki PJ, Pouillon V, Gueydan C, Levy JC, Bodson A, Krzentowski G, Daubresse JC, Mockel J, Behrends J, Servais G, Szpirer C, Kruys V, Gauguier D, Schurmans S. (2002) The gene INPPL1, encoding the lipid phosphatase SHIP2, is a candidate for type 2 diabetes in rat and man. : *Diabetes*. Jul; 51(7):2012-7.

Martin PR, Shea RJ and Mulks MH. (2001) Identification of a plasmid-encoded gene from *Haemophilus ducreyi* which confers NAD independence. *Journal of Bacteriology*; February, p. 1168-1174, Vol. 183.

Matschinsky F, Liang Y, Kesavan P, Wang L, Froguel P, Velho G, Cohen D, Permutt MA, Tanizawa Y, Jetton TL. (1993) Glucokinase as pancreatic β cell glucose sensor and diabetes gene. *Journal of Clinical Investigation* 92:2092–2098

Mayfield J. (1998) Diagnosis and classification of diabetes mellitus: new criteria. 1: *American Family Physician*. Oct 15; 58(6):1355-62, 1369-70

McTernan CL, McTernan PG, Harte AL, Levick PL, Barnett AH, Kumar S. (2002a) Resistin, central obesity, and type 2 diabetes. *Lancet* Jan 5; 359(9300):46-7

McTernan PG, McTernan CL, Chetty R, Jenner K, Fisher FM, Lauer MN, Crocker J, Barnett AH, Kumar S. (2002b) Increased resistin gene and protein expression in human abdominal adipose tissue. *Journal of Clinical Endocrinology and Metabolism*; May; 87(5):2407

Meier JJ, Butler AE, Galasso R, Rizza RA, Butler PC. (2006) Increased islet beta cell replication adjacent to intrapancreatic gastrinomas in humans; *Diabetologia*. Nov; 49(11):2689-96.

Miura A, Yamagata K, Kakei M, Hatakeyama H, Takahashi N, Fukui K, Nammo T, Yoneda K, Inoue Y, Sladek FM, Magnuson MA, Kasai H, Miyagawa J, Gonzalez FJ, Shimomura I. (2006) Hepatocyte Nuclear Factor-4 α Is Essential for Glucose-stimulated Insulin Secretion by Pancreatic beta-Cells. *Journal of Biological Chemistry*; 281: 5246–5257

- Mohamed-Ali V, Pinkney JH, Coppack SW. (1998) Adipose tissue as an endocrine and paracrine organ. : International Journal of Obesity and Related Metabolic Disorders. Dec; 22(12):1145-58.
- Moore GB, Chapman H, Holder JC, Lister CA, Piercy V, Smith SA, Clapman JC (2001) Differential regulation of adipocytokine mRNA by rosiglitazone in db/db mice. Biochemical and Biophysical Research Communications 286:735–741
- Morash BA, Wilkinson D, Ur E, Wilkinson M. (2002) Resistin expression and regulation in mouse pituitary. FEBS Letters Volume 526, Issue 1-3, 28 August, Pages 26-30
- Moschen AR, Kaser A, Enrich B, Mosheimer B, Theurl M, Niederegger H and Tilg H. (2007) Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. Journal of Immunology; 178, pp. 1748–1758.
- Moustaid N and Sul HS. (1991) Regulation of expression of the fatty acid synthase gene in 3T3-L1 cells by differentiation and triiodothyronine. Journal of Biological Chemistry 266, 18550-18554
- Mu H, Ohashi R, Yan S, Chai H, Yang H, Lin P, Yao Q, Chen C. (2006) Adipokine resistin promotes in vitro angiogenesis of human endothelial cells. Cardiovascular Research; Apr 1; 70(1):146-57.
- Muse ED, Obici S, Bhanot S, Monia BP, McKay RA, Rajala MW, Scherer PE, Rossetti L. (2004) Role of resistin in diet-induced hepatic insulin resistance. Journal of Clinical Investigations; Jul; 114(2):232-9.
- Musi N, Goodyear LJ. (2006) Insulin resistance and improvements in signal transduction. Endocrine; Feb; 29(1):73-80
- Myers SR, Diamond MP, Adkins-Marshall BA, Williams PE, Stinsen R and Cherrington AD. (1991) Effects of small changes in glucagon on glucose production during a euglycemic, hyperinsulinemic clamp. Metabolism 40:66–71,
- Nagaev I, Smith U. (2001) Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. Biochemical Biophysical Research Communications; 285:561-564
- Nakata M, Okada T, Ozawa K, Yada T. (2007) Resistin induces insulin resistance in pancreatic islets to impair glucose-induced insulin release. Biochemical and Biophysical Research Communications, Feb 23; 353(4):1046-51.
- Nanjo K, Sanke T, Miyano M, Okai K, Sowa R, Kondo M, Nishimura S, Iwo K, Miyamura K, Given BD, et al (1986) Diabetes due to secretion of a structurally abnormal insulin (insulin Wakayama): clinical and functional characteristics of [LeuA3] insulin. Journal of Clinical Investigation 77:514–519,

Niederwanger A, Kranebitter M, Ciardi C, Tatarczyk T, Patsch JR, Pedrini MT. (2007) Resistin impairs basal and insulin-induced glycogen synthesis by different mechanisms. *Molecular and Cellular Endocrinology*; Jan 15; 263(1-2):112-9.

Nigro J, Osman N, Dart AM, Little PJ. (2006) Insulin resistance and atherosclerosis. : *Endocrine Reviews*. May; 27(3):242-59.

Nishizuka Y, Hayaishi O. (1963) Studies on the biosynthesis of nicotinamide adenine dinucleotide. I. Enzymatic synthesis of niacin ribonucleotides from 3-hydroxyanthranilic acid in mammalian tissues. *Journal of Biological Chemistry*; 238:3369–3377.

Nordlie RC, Bode AM, Foster JD. (1993) Recent advances in hepatic glucose 6-phosphatase regulation and function. *Proceedings of the Society for Experimental Biology and Medicine*; Jul; 203(3):274-85.

Olefsky JM. (1990) The insulin receptor: A multi-functional protein. *Diabetes*; 39:1009-1016

Ofei F, Hurel S, Newkirk J, Sopwith M, Taylor R. (1996) Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes*; 45:881-5.

Ognjanovic S and Bryant-Greenwood GD. (2002) Pre-B-cell colony-enhancing factor, a novel cytokine of human fetal membranes, *American Journal of Obstetrics and Gynaecology* 187, pp. 1051–1058

Ognjanovic S, Bao S, Yamamoto SY, Garibay-Tupas J, Samal B, Bryant-Greenwood GD. (2001) Genomic organization of the gene coding for human pre-B-cell colony enhancing factor and expression in human fetal membranes. *Journal of Molecular Endocrinology* Volume 26, Issue 2, Pages 107-117

Ohsugi M, Cras-Méneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA. (2005) Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signalling in gene expression, proliferation, insulin content, and secretion. *Journal of Biological Chemistry*; Feb 11; 280(6):4992-5003.

Okar DA, Manzano A, Navarro-Sabate A, Riera L, Bartrons R, Lange AJ. (2001) PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2, 6-bisphosphate. *Trends Biochemical Science* 26:30 –35,

Oki K, Yamane K, Kamei N, Nojima H, Kohno N. (2007) Circulating visfatin level is correlated with inflammation, but not with insulin resistance. *Clinical Endocrinology (Oxf)*; Nov; 67(5):796-800.

Okita K, Yang Q, Yamagata K, Hangenfeldt KA, Miyagawa J, Kajimoto Y, Nakajima H, Namba M, Wollheim CB, Hanafusa T, Matsuzawa Y. (1999) Human insulin gene is a target gene of hepatocyte nuclear factor-1alpha (HNF-1alpha) and HNF-1beta. *Biochemical and Biophysical Research Communications*, Sep 24; 263(2):566-9.

Oliver WR Jr, Shenk JL, Snaith MR, Russell CS, Plunket KD, Bodkin NL, Lewis MC, Winegar DA, Sznaidman ML, Lambert MH, Xu HE, Sternbach DD, Klierwer SA, Hansen BC, Willson TM. (2001) A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. 1: Proceedings of the National Academy of Science of the United States of America. Apr 24; 98(9):5306-11.

O'Rahilly S, Turner RC, Matthews DR. (1988) Impaired pulsatile secretion of insulin in relatives of patients with non-insulin-dependent diabetes. *New England Journal of Medicine* 318:1225–1230

Oshima MM, Taketo and M. Oshima. (2006) Destruction of pancreatic β -cells by transgenic induction of prostaglandin E2 in the islets. *Journal of Biological Chemistry*; 281, pp. 29330–29336

Otani K, Kulkarni RN, Baldwin AC, Krutzfeldt J, Ueki K, Stoffel M, Kahn CR, Polonsky KS. (2004) Reduced beta-cell mass and altered glucose sensing impair insulin-secretory function in betaIRKO mice. *American Journal of Physiology. Endocrinology and Metabolism*; 286 (1)

Ou D, Metzger DL, Wang X, Huang J, Pozzilli P, Tingle AJ. (2002) TNF-related apoptosis-inducing ligand death pathway-mediated human beta-cell destruction. *Diabetologia*; Dec; 45(12):1678-88.

Pagano C, Pilon C, Olivieri M, Mason P, Fabris R, Serra R, Milan G, Rossato M, Federspil G, Vettor R. (2006) Reduced plasma visfatin/pre-B cell colony enhancing factor in obesity is not related to insulin resistance in humans, *Journal of Clinical Endocrinology and Metabolism*; 91, pp. 3165–3170

Palanivel R, Maida A, Liu Y and Sweeney G. (2006) Regulation of insulin signalling, glucose uptake and metabolism in rat skeletal muscle cells upon prolonged exposure to resistin. *Diabetologia* 49: 183–190

Pan XR, Li GW, Hu YH, Wang JX, Yang WY, An ZX, Lin J, Xiao JZ, Cao HB, Liu PA, Jiang XG, Jiang YY, Wang JP, Zheng H, Zhang H, Bennett PH, Howard BV. (1997) Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. The Da Qing IGT and Diabetes Study. *Diabetes Care*; 20:537–544,

Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L. (1995) Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO Journal*; 14:5209-5215

Park J, Rho HK, Kim KH, Choe SS, Lee YS, Kim JB. (2005) Overexpression of Glucose-6-Phosphate Dehydrogenase Is Associated with Lipid Dysregulation and Insulin Resistance in Obesity. *Molecular and Cellular Biology* June; 25(12): 5146–5157

- Parker PJ and Waterfield MD. (1992) Phosphatidylinositol 3-kinase: a novel effector. *Cell Growth and Differentiation*; 3, 747-752
- Paulauskis JD, Sul HS. (1989) Hormonal regulation of mouse fatty acid synthase gene transcription in liver. *Journal of Biological Chemistry*; Jan 5; 264(1):574-7.
- Peraldi P, Hotamisligil GS, Buurman WA, White MF and Spiegelman BM. (1996) Tumor Necrosis Factor (TNF)- Inhibits Insulin Signaling through Stimulation of the p55 TNF Receptor and Activation of Sphingomyelinase. *Journal of biological Chemistry*; Volume 271, Number 22,
- Pessin JE, Thurmond DC, Elmendorf JS, Coker KJ, and Okada S. (1999) Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. *Journal of Biological Chemistry*; 274:2593-2596
- Pilkis SJ, Claus TH, Kurland IJ, Lange AJ. (1995) 6-Phosphofructo-2-kinase/fructose-2, 6-bisphosphatase: a metabolic signalling enzyme. *Annual Review of Biochemistry* 64:799 –835,
- Poitout V, Robertson RP. (2008) Glucolipototoxicity: fuel excess and beta-cell dysfunction. *Endocrine Reviews*. May; 29(3):351-66
- Polonsky KS. (2000) Dynamics of insulin secretion in obesity and diabetes. *International Journal of Obesity and Related Metabolic Disorders* 24 (Suppl. 2):S29-S31,
- Polonsky KS, Given BD, Hirsch LJ, Tillil H, Shapiro ET, Beebe C, Frank BH, Galloway JA, Van Cauter E. (1988) Abnormal patterns of insulin secretion in non-insulin-dependent diabetes mellitus. *New England Journal of Medicine* 318:1231–1239
- Pontiroli AE, Pizzocri P, Librenti MC, Vedani P, Marchi M, Cucchi E, Orena C, Paganelli M, Giacomelli M, Ferla G, Folli F. (2002) Laparoscopic adjustable gastric banding for the treatment of morbid (grade 3) obesity and its metabolic complications: a three-year study. *Journal of Clinical Endocrinology Metabolism*; 87: 3555–3561
- Porte D Jr. (1991) Banting lecture 1990. Beta-cells in type II diabetes mellitus. *Diabetes* 40 (2) pp. 166–180
- Porte D Jr, Seeley RJ, Woods SC, Baskin DG, Figlewicz DP, Schwartz MW. (1998) Obesity, diabetes and the central nervous system. *Diabetologia*; 41:863–881.
- Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. (2001) C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *The journal of American Medical association*; Jul 18; 286(3):327-34.
- Preiss J and Handler P. (1957) Enzymatic synthesis of nicotinamide mononucleotide. *Journal of Biological Chemistry*; 225, pp. 759–770.

- Preiss J, Handler P. (1958) Biosynthesis of diphosphopyridine nucleotide .I. Identification of intermediates. *Journal of Biological Chemistry*; 233:488–492
- Qi Y, Nie Z, Lee YS, Singhal NS, Scherer PE, Lazar MA, Ahima RS. (2006) Loss of resistin improves glucose homeostasis in leptin deficiency. *Diabetes*; Nov; 55(11):3083-90.
- Qiao LY, Zhande R, Jetton TL, Zhou G, Sun XJ. (2002) In vivo phosphorylation of insulin receptor substrate 1 at serine 789 by a novel serine kinase in insulin-resistant rodents. *Journal of Biological Chemistry*; 277:26530 -26539
- Rabe K, Lehrke M, Parhofer KG and Broedl UC. (2008) Adipokines and Insulin Resistance. *Molecular Medicine*; November–December; 14(11-12): 741–751.
- Rajala MW, Obici S, Scherer PE and Rossetti L. (2003) Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. *Journal of Clinical Investigation*; 111: 225–230
- Rameh LE, Cantley LC (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *Journal of Biological Chemistry*; 274:8347–8350
- Rangwala SM, Rich AS, Rhoades B, Shapiro JS, Obici S, Rossetti L, Lazar MA. (2004) Abnormal glucose homeostasis due to chronic hyperresistinemia. *Diabetes*; Aug; 53(8):1937-41.
- Rakatzi I, Mueller H, Ritzeler O, Tennagels N, Eckel J. (2004) Adiponectin counteracts cytokine- and fatty acid-induced apoptosis in the pancreatic beta-cell line INS-1. *Diabetologia*; Feb; 47(2):249-58.
- Reber M, Cereghini S. (2001) Variant hepatocyte nuclear factor 1 expression in the mouse genital tract. *Mechanisms of Development*; 100 :(1)75–78
- Retnakaran R, Hanley AJ, Raif N, Hirning CR, Connelly PW, Sermer M, Kahn SE, Zinman B. (2005) Adiponectin and beta cell dysfunction in gestational diabetes: pathophysiological implications. *Diabetologia*; May; 48(5):993-1001.
- Reed BC and Lane MD. (1980) Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes *Proceedings of the National Academy of Science USA*; 77, 285-289
- Ren J, Semenkovich CF, Gulve EA, Gao J, Holloszy JO. (1994) Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin stimulated glycogen storage in muscle. *Journal of Biological Chemistry*; 269:14396-14401.
- Resnick HE, Valsania P, Halter JB, Lin X. (2000) Relation of weight gain and weight loss on subsequent diabetes risk in overweight adults. *Journal of Epidemiology and Community Health*; 54:596-602.

- Revollo JR, Grimm AA, Imai, SI. (2007a) The regulation of nicotinamide adenine dinucleotide biosynthesis by Nampt/PBEF/visfatin in mammals, *Current Opinion in Gastroenterology*; Volume 23, Issue 2, March, Pages 164-170
- Revollo JR, Grimm AA, Imai S. (2004) The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *Journal of Biological Chemistry*; Dec 3; 279(49):50754-63.
- Revollo JR, Körner A, Mills KF, Satoh A, Wang T, Garten A, Dasgupta B, Sasaki Y, Wolberger C, Townsend RR, Milbrandt J, Kiess W, Imai S. (2007b) Nampt /PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metabolism* 6(5):363-75
- Robertson RP, Harmon J, Tran PO, Poitout V. (2004) Beta-cell glucose toxicity lipotoxicity and chronic oxidative stress in type 2 diabetes. *Diabetes*; Feb; 53 Supplementary 1:S119-24.
- Rongvaux A, Andris F, Van Gool F, Leo O. (2003) Reconstructing eukaryotic NAD metabolism. *BioEssays*. Volume 25, Issue 7, 1 July, Pages 683-690
- Rongvaux A, Shea RJ, Mulks MH, Gigot D, Urbain J, Leo O and Andris F. (2002) Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *European Journal of Immunology*; 32 pp 3225–3234.
- Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ and Spiegelman BM. (2002) C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes and Development*; Jan 1; 16 (1):22-6
- Ross SR, Graves RA, Spiegelman BM. (1993) Targeted expression of a toxin gene to adipose tissue: transgenic mice resistant to obesity. *Genes and Development* 7:1318–1324,
- Rotter V, Nagaev I, Smith U. (2003) Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumour necrosis factor-alpha, over-expressed in human fat cells from insulin-resistant subjects. *Journal of Biological Chemistry* Nov 14; 278(46):45777-84. 2003
- Saldeen J. (2000) Cytokines Induce Both Necrosis and Apoptosis via a Common Bcl-2-Inhibitable Pathway in Rat Insulin-Producing Cells. *Endocrinology*; Vol. 141, No. 6
- Saltiel AR, Kahn CR. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* Dec 13; 414(6865):799-806.
- Samal B, Sun Y, Stearns G, Xie C, Suggs S and McNiece I. (1994) Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Molecular and Cellular Biology*; 14, 1431–1437
- Sartipy P, Loskutoff DJ. (2003) Monocyte chemoattractant protein-1 in obesity and insulin resistance. *Proceedings of the National Academy of Science USA*; 100: 7265

Satoh H, Nguyen MT, Miles PD, Imamura T, Usui I, Olefsky JM (2004) Adenovirus-mediated chronic “hyper-resistinemia” leads to in vivo insulin resistance in normal rats. *Journal of Clinical Investigations* 114:224–231

Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV, O’Rahilly S. (2001) Resistin / FIZZ3 expression in relation to obesity and peroxisome proliferator – activated receptor- γ action in humans. *Diabetes* 50: 2199-2202

Scharfmann R and Czernichow P. (1996) Differentiation and growth of pancreatic beta cells. *Diabetes and Metabolism*; 22(4):223-8.

Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, Deeb S, Staels B, Auwerx J. (1996). PPAR and PPAR activators direct a tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* 15: 5336–5348

Schrauwen P. (2007) High-fat diet, muscular lipotoxicity and insulin resistance. *Proceedings of Nutrition Society Feb*; 66(1):33-41.

Schweiger M, Schreiber R, Haemmerle G, Lass A, Fledelius C, Jacobsen P, Tornqvist H, Zechner R, Zimmermann R. (2006) Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. *Journal of Biological Chemistry*; Dec 29; 281(52):40236-41

Sethi JK, Vidal-Puig A. (2005) Visfatin: the missing link between intra-abdominal obesity and diabetes? *Trends in Molecular Medicine*; Volume 11, Issue 8, August

Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems JA, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DE, Eisenbarth G, Segal M, Preiksaitis J, Korbitt GS, Barton FB, Viviano L, Seyfert-Margolis V, Bluestone J, Lakey JR. (2006) International trial of the Edmonton protocol for islet transplantation. *New England Journal of Medicine*; Sep 28; 355(13):1318-30.

Shepherd M, Shields B, Ellard S, Rubio-Cabezas O, Hattersley AT. (2009) A genetic diagnosis of HNF1A diabetes alters treatment and improves glycaemic control in the majority of insulin-treated patients. *Diabetic Medicine*; Apr; 26(4):437-41

Shepherd PR & Kahn BB (1999) Glucose transporters and insulin action—implications for insulin resistance and diabetes mellitus. *New England Journal of Medicine*; 341, 248–257

Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS. (1998) Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes and Development* 12:3182–3194,

Siiteri PK. (1987) Adipose tissue as a source of hormones. *American Journal of Clinical Nutrition*; 45: 277–282.

Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ. (2005) Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway. *Biochemical and Biophysical Research Communication*. Sep 9; 334(4):1092-101

Singhal NS, Patel RT, Qi Y, Lee YS, Ahima RS. (2008) Loss of resistin ameliorates hyperlipidemia and hepatic steatosis in leptin-deficient mice. *American Journal of Physiology, Endocrinology and Metabolism*; Aug; 295(2):E331-8.

Skurk T, Alberti-Huber C, Herder C, Hauner H. (2007) Relationship between adipocyte size and adipokine expression and secretion. *Journal of Clinical Endocrinology and Metabolism*; Mar; 92(3):1023-33.

Skyler JS, Insulin therapy in T2D mellitus. In: R.A. DeFronzo, Editor, *Current Therapy of Diabetes Mellitus*, Mosby-Year Book, St. Louis, MO (1998), pp. 108–116.

Sleeman MW, Wortley KE, Lai KMV, Gowen LC, Kintner J, Kline WO, Garcia K, Stitt TN, Yancopoulos GD, Wiegand SJ, Glass DJ 2005 Absence of the lipid phosphatase SHIP2 confers resistance to dietary obesity. *Nature Medicine*; 11:199–205

Solinas G, Naugler W, Galimi F, Lee MS, Karin M. (2006) Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. *Proceedings of the National Academy of Science USA*; Oct 31; 103(44):16454-9.

Sommer G, Garten A, Petzold S, Beck-Sickinger AG, Blüher M, Stumvoll M, Fasshauer M. (2008) Visfatin/PBEF/Nampt: structure, regulation and potential function of a novel adipokine. *Clinical Science*; Jul; 115(1):13-23.

Sosa-Pineda B, Chowdhury K, Torres M, Oliver G and Gruss P. (1997) The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. *Nature*; Mar 27; 386 (6623):399-402

Spiegelman BM, Choy L, Hotamisligil GS, Graves RA and Tontonoz P. (1993) Regulation of Adipocyte Gene expression in differentiation and syndromes of Obesity/Diabetes *Journal of Biological Chemistry*. 268, 6823-6826

Spiegelman BM, Frank M and Green H. (1983) Molecular cloning of mRNA from 3T3 adipocytes. Regulation of mRNA content for glycerophosphate dehydrogenase and other differentiation- dependent proteins during adipocyte development. *Journal of Biological Chemistry*; 258, 10083-10089

Spiegelman BM, Flier JS. (2001) Obesity and the regulation of energy balance. *Cell*; Feb 23; 104(4):531-43.

Steinberg GR, Parolin ML, Heigenhauser GJ and Dyck DJ. (2002) Leptin increases FA oxidation in lean but not obese human skeletal muscle: evidence of peripheral leptin resistance. *American Journal of Physiology Endocrinology and Metabolism* 283 pp. E187–E192

Stephens JM & Pekala PH. (1991) Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor- α . *Journal of Biological Chemistry*; 266 21839–21845.

Stephens JM and Vidal-Puig AJ. (2006) An update on visfatin/pre-B cell colony-enhancing factor, an ubiquitously expressed, illusive cytokine that is regulated in obesity, *Current Opinion in Lipidology*; 17 pp. 128–131

Stephens LR, Jackson TR, Hawkins PT. (1993) Agonist-stimulated synthesis of phosphatidylinositol (3,4,5)-trisphosphate: a new intracellular signalling system? *Biochimica et Biophysica Acta*; Oct 7; 1179(1):27-75.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. (2001) The hormone resistin links obesity to diabetes. *Nature* 409, 307–312.)

Steppan CM, Lazar MA. (2002) Resistin and obesity-associated insulin resistance. *Trends Endocrinology Metabolism* 13:18–23.

Steppan CM, Lazar MA. (2004) The current biology of resistin. *Journal of Internal Medicine* 255:439–447.

Steppan CM, Wang J, Whiteman EL, Birnbaum MJ, Lazar MA. (2005) Activation of SOCS-3 by resistin. *Molecular and Cellular Biology*; Feb; 25(4):1569-75

Stith RD, Luo J. (1994) Endocrine and carbohydrate responses to interleukin-6 in vivo. *Circulatory Shock*; 44:210-215

Stoffers DA, Ferrer J, Clarke WL, Habener JL. (1997) Early-onset type 2 diabetes mellitus (MODY4) linked to IPF-1. *Nature Genetics* 1 7: 138–139

Straub SG, Sharp GW. (2002) Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes/Metabolism Research and Reviews*; Nov-Dec; 18(6):451-63

Sun Q, Li L, Li R, Yang M, Liu H, Nowicki MJ, Zong H, Xu J, Yang G. (2009) Overexpression of visfatin/PBEF/Nampt alters whole-body insulin sensitivity and lipid profile in rats. *Annals of Medicine*; 41(4):311-20

Tan BK, Chen J, Brown J, Adya R, Ramanjaneya M, Menon V, Bailey CJ, Lehnert H, Randeve HS. (2009) In vivo and ex vivo regulation of visfatin production by leptin in human and murine adipose tissue: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. *Endocrinology*; Aug; 150(8):3530-9.

Tang QQ, Zhang JW, Daniel Lane M. (2004) Sequential gene promoter interactions by C/EBPbeta, C/EBPalpha, and PPARgamma during adipogenesis. *Biochemical and Biophysical Research Communications*; Volume 318, Issue 1, 21 May, Pages 213-218

Taylor SI, Arioglu E. (1998) Syndromes associated with insulin resistance and acanthosis nigricans. *Journal of Basic and Clinical Physiology and Pharmacology*; 9:419-439.

Tordjman K, Standley KN, Bernal-Mizrachi C, Leone TC, Coleman T, Kelly DP, Semenkovich CF. (2002) PPARalpha suppresses insulin secretion and induces UCP2 in insulinoma cells. *Journal of Lipid Research*; Volume 43, (6):936-43.

Thams P, Anwar MR, Capito K. (2005) Glucose triggers protein kinase A-dependent insulin secretion in mouse pancreatic islets through activation of the K⁺ATP channel-dependent pathway. *European Journal of Endocrinology*. Apr; 152(4):671-7.

Tontonoz P, Kim JB, Graves RA, Spiegelman BM. (1993) ADD1: A novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Molecular and Cellular Biology*; 13:4753–4759.

Trayhurn P, Wood IS. (2004) Adipokines: inflammation and the pleiotropic role of white adipose tissue. *British Journal of Nutrition*; Sep; 92(3):347-55.

Traynor-Kaplan AE, Thompson BL, Harris AL, Taylor P, Omann GM and Sklar LA. (1989) Transient increase in phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol trisphosphate during activation of human neutrophils. *Journal of Biological Chemistry*; 264(26):15668-73

Tsiotra PC, Tsigos C and Raptis SA. (2001) TNF and leptin inhibit basal and glucose-stimulated insulin secretion and gene transcription in the HIT-T15 pancreatic cells. *International Journal of Obesity* 25, 1018--1026

Tuomilehto J, Lindstrom J, Eriksson JG, Valle TT, Hamalainen H, Ilanne-Parikka P, Keinanen-Kiukkaanniemi S, Laakso M, Louheranta A, Rastas M, Salminen V, Uusitupa M. (2001) Prevention of T2D mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *New England Journal of Medicine*; 344:1343 –1350,

Ueki K, Yamamoto-Honda R, Kaburag, Y, Yamauchi T, Tobe K, Burgering BM, Coffey PJ, Komuro I, Akanuma Y, Yazaki Y, and Kadowaki T. (1998). Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. *Journal of Biological Chemistry*; 273(9), 5315-5322

UK Prospective Diabetes Study Group, U.K. (1995) prospective diabetes study 16: overview of 6 years' therapy of type II diabetes: a progressive disease. *Diabetes*; 44, pp. 1249–1258.

UK Prospective Diabetes Study (UKPDS) Group (1998) Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with T2D (UKPDS 33). *Lancet* 352:837–853

- UK Prospective Diabetes Study (UKPDS) Group (1998) Effect of intensive blood-glucose control with metformin on complications in overweight patients with T2D (UKPDS 34). *Lancet* 352:854–865
- Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. (1997) Protection from obesity-induced insulin resistance in mice lacking TNF- α function. *Nature* 389:610–4.
- Van der Veer E, Nong Z, O'Neil C, Urquhart B, Freeman D, Pickering JG. (2005) Pre-B-cell colony-enhancing factor regulates NAD⁺-dependent protein deacetylase activity and promotes vascular smooth muscle cell maturation. *Circulation Research*; Jul 8; 97(1):25-34.
- Van Dielen FM, van't Veer C, Buurman WA, Greve JW (2002) Leptin and soluble leptin receptor levels in obese and weight-losing individuals. *Journal of Clinical Endocrinology and Metabolism* 87:1708–1716
- Varma V, Yao-Borengasser A, Rasouli N, Bodles AM, Phanavanh B, Lee MJ, Starks T, Kern LM, Spencer III HJ, McGehee Jr RE, Fried SK, Kern PA (2007) Human visfatin expression: relationship to insulin sensitivity, intramyocellular lipids, and inflammation. *Journal of Clinical Endocrinology and Metabolism* 92:666–672
- Vaxillaire M, Froguel P. (2006) Genetic basis of maturity-onset diabetes of the young. *Endocrinol Metab Clin North Am* 35:371–384
- Vázquez-Vela ME, Torres N, Tovar AR. (2008) White adipose tissue as endocrine organ and its role in obesity. *Archives of Medical Research* Nov; 39(8):715-28.
- Vázquez MJ, González CR, Varela L, Lage R, Tovar S, Sangiao-Alvarellos S, Williams LM, Vidal-Puig A, Nogueiras R, López M, Diéguez C. (2008) Central resistin regulates hypothalamic and peripheral lipid metabolism in a nutritional-dependent fashion. *Endocrinology*; Sep; 149(9):4534-43.
- Verma S, Li SH, Wang CH, Fedak PW, Li RK, Weisel RD, Mickle DA. (2003) Resistin promotes endothelial cell activation: further evidence of adipokine-endothelial interaction. *Circulation*; 108:736– 40.
- Vernon RG, Barber MC, Finley E. (1991) Modulation of the activity of acetyl-CoA carboxylase and other lipogenic enzymes by growth hormone, insulin and dexamethasone in sheep adipose tissue and relationship to adaptations to lactation. *Biochemical Journal*; Mar 1; 274 (Pt 2):543-8.
- Virkamäki A, Ueki K, Kahn CR. (1999) Protein-protein interactions in insulin signalling and the molecular mechanisms of insulin resistance. *Journal of Clinical Investigation*; 103:931-943.
- Virtanen I, Banerjee M, Palgi J, Korsgren O, Lukinius A, Thornell LE, Kikkawa Y, Sekiguchi K, Hukkanen M, Konttinen YT, Otonkoski T. (2008) Blood vessels of human islets of Langerhans are surrounded by a double basement membrane. *Diabetologia*; Jul; 51(7):1181-91.

Virtue S, Vidal-Puig A (2008) It's not how fat you are, it's what you do with it that counts. *PLoS Biology* Sep 23; 6(9):e237.

Volchuk A, Wang Q, Ewart HS, Liu Z, He L, Bennett MK, Klip A. (1996) Syntaxin 4 in 3T3-L1 adipocytes: regulation by insulin and participation in insulin-dependent glucose transport. *Molecular Biology of the Cell*; Jul; 7(7):1075-82

Wang H, Maechler P, Antinozzi PA, Hagenfeldt KA, Wollheim CB. (2000) Hepatocyte nuclear factor 4 α regulates the expression of pancreatic beta -cell genes implicated in glucose metabolism and nutrient-induced insulin secretion. *Journal of Biological Chemistry*; Nov 17; 275(46):35953-9.

Wang L, Coffinier C, Thomas MK, Gresh L, Eddu G, Manor T, Levitsky LL, Yaniv M, Rhoads DB. (2004) Selective deletion of the Hnf1beta (MODY5) gene in beta-cells leads to altered gene expression and defective insulin release. *Endocrinology*; Aug; 145(8):3941-9

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR and Darlington GJ. (1995) Impaired energy homeostasis in C/EBP α knockout mice. *Science*; 269, 1108–1112.

Wang T, Zhang X, Bheda P, Revollo JR, Imai S and Wolberger C. (2006) Structure of Nampt/PBEF/visfatin, a mammalian NAD⁺ biosynthetic enzyme. *Nature Structural and Molecular Biology*; 13, 661–662

Wang YX, Lee CH, Tjep S, Yu RT, Ham J, Kang H, Evans RM. (2003) Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. *Cell*; Apr 18; 113(2):159-70

Warren-Perry MG, Manley SE, Ostrega D, Polonsky K, Mussett S, Brown P, Turner RC: A novel point mutation in the insulin gene giving rise to hyperproinsulinemia. *Journal of Clinical Endocrinology and Metabolism*; 82:1629–1631, 1997

Way JM, Gorgun CZ, Tong Q, Uysal KT, Brown KK, Harrington WW, Oliver Jr WR, Willson TM, Klier SA, Hotamisligil GS 2001 Adipose tissue resistin expression is severely suppressed in obesity and stimulated by PPAR agonists. *Journal of Biological Chemistry*; 276:25651–25653

Westerlund J, Ortsäter H, Palm F, Sundsten T, Bergsten P. Glucose-regulated pulsatile insulin release from mouse islets via the K_(ATP) channel-independent pathway. *European Journal of Endocrinology*; 2001 Jun; 144(6):667-75

Weyer C, Bogardus C, Mott DM, Pratley RE. (1999). The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of T2D mellitus. *Journal of Clinical Investigation*; 104:787-794.

Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. (2000) Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia* Dec; 43(12):1498-506.

WHO Expert Committee. Report of a WHO Expert Committee. (1995) World Health Organ Tech Rep Ser; 854: 1–452

Will JC, Williamson DF, Ford ES, Calle EE, Thun MJ. (2002) Intentional weight loss and 13-year diabetes incidence in overweight adults. *American Journal of Public Health*; 92:1245-1248.

Xie H, Tang SY, Luo XH, Huang J, Cui RR, Yuan LQ, Zhou HD, Wu XP and Liao EY. (2007) Insulin-Like Effects of Visfatin on Human Osteoblasts. *Calcified Tissue International*. 80 pp. 201–210.

Xu J, Long YS, Gozal D, Epstein PN. (2009) Beta-cell death and proliferation after intermittent hypoxia: role of oxidative stress. *Free Radical Biology and Medicine*. Mar 15; 46(6):783-90

Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, Fajans SS, Signorini S, Stoffel M, Bell GI. (1996) Mutations in the hepatocyte nuclear factor-4 gene in maturity onset diabetes of the young (MODY1). *Nature*; 384:458–460,

Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine* August; 7(8):887-8.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Medicine*; 8:1288–1295,

Yang WS, Lee WJ, Funahashi T, Tanaka S, Matsuzawa Y, Chao CL, Chen CL, Tai TY, Chuang LM (2001) Weight reduction increases plasma levels of an adipose-derived antiinflammatory protein, adiponectin. *Journal of Clinical Endocrinology and Metabolism*; 86:3815–3819

Yannakoulia M, Yiannakouris N, Bluher S, Matalas AL, Klimis-Zacas D, Mantzoros CS. (2003) Body fat mass and macronutrient intake in relation to circulating soluble leptin receptor, free leptin index, adiponectin, and resistin concentrations in healthy humans. *Journal of Clinical Endocrinology and Metabolism*.88: 1730–6.

Yokoyama C., Wang X., Briggs M.R., Admon A., Wu J., Hua X., Goldstein J.L., Brown M.S. (1993) SREBP-1, a basic helix-loop-helix leucine zipper protein that controls transcription of the LDL receptor gene. *Cell*; 75:187–197.

Yonezawa T, Haga S, Kobayashi Y, Takahashi T, Obara Y. (2006) Visfatin is present in bovine mammary epithelial cells, lactating mammary gland and milk, and its expression is regulated by cAMP pathway. *FEBS Letters*; Dec 11; 580(28-29):6635-43

Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI. (2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *Journal of Biological Chemistry*; Dec 27; 277(52):50230-6

Zawalich WS, Zawalich KC, Kelley GG. (1995) Regulation of insulin release by phospholipase C activation in mouse islets: differential effects of glucose and neurohumoral stimulation. *Endocrinology*; Nov; 136(11):4903-9

Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grujic D, Hagen T, Vidal-Puig AJ, Boss O, Kim YB, Zheng XX, Wheeler MB, Shulman GI, Chan CB, Lowell BB. (2001) Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell*; Jun 15; 105(6):745-55.

Zhang JL, Qin YW, Zheng X, Qiu JL, Zou DJ. (2003) Serum resistin level in essential hypertension patients with different glucose tolerance. *Diabetic Medicine*; 20: 828–31

Zhang S, Kim KH. (1995) TNF-alpha inhibits glucose-induced insulin secretion in a pancreatic beta-cell line (INS-1). *FEBS Letters*; Dec 18; 377(2):237-9.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432

Zierath JR, Krook A, Wallberg-Henriksson H. (2000) Insulin action and insulin resistance in human skeletal muscle. *Diabetologia* Jul; 43(7):821-35.

Zimmet P, Alberti KG, Shaw J. (2001) Global and societal implications of the diabetes epidemic. *Nature*; Dec 13; 414(6865):782-7.

Zurlo F, Larson K, Bogardus C, Ravussin E. (1990) Skeletal muscle metabolism is a major determinant of resting energy expenditure. *Journal of Clinical Investigation*; Nov; 86(5):1423-7

Appendices

Appendix 1

Appendix 1.1 Equipment

Block heater	Wolf Laboratories, York, UK
Centrifuges	M.S.E Mistral 2000, Fisons Instrumentation, U.K.
CO ₂ incubator	Jensons Scientific Inc. Bridgeville, PA 15017, USA
Coulter counter Z1	Beckman Coulter limited High Wycombe, UK
Genequant Pharmacia	Biotech Inc. NJ, USA
Hybaid PCR Express thermocycler	Hybaid, Action Court, Middlesex, UK
ImageQuant™ TL image analysis software	GE Healthcare Life Sciences Amersham, Bucks, UK
Inverted microscope Celti,	Optical Instruments, Belgium
Labsystems Multiskan MS	Labsystems, Finland
Magnetic Stirrer Hotplate	Stuart Scientific, Beacon Road, Staffordshire, UK

Mini-PROTEAN 3 cell Electrophoresis system	Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
Mini Trans-Blot cell	Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
PhosphorImager™ technology	GE Healthcare Life Sciences Amersham, Bucks, UK
Platform Shaker STR6	Stuart Scientific, Beacon Road, Staffordshire, UK
PowerPac 1000 Power Supply	Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
iCycler Real-Time PCR Detection System	Bio-Rad Laboratories Inc. Hercules, CA 94547, USA
Rocker	Wolf Laboratories, York, UK
Vortex	Sigma-Aldrich Company Ltd, Dorset, UK
Water baths	Grant Instruments (Cambridge) Ltd, Cambridge UK

Appendix 1.2 Specialised kits

CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit.	Promega UK, Southampton, UK
Mouse insulin secretion ELISA kit	Mercodia, Sweden
Mouse Diabetes RT ² Profiler™ PCR Array	SABiosciences Corporation MD 21703, USA
Rat insulin secretion ELISA kit	Mercodia, Sweden
RayBio Cell-Based Phospho-Insulin Receptor B ELISA kit	RayBiotech, Inc. USA
RayBio Cell-Based ERK1/2 (activated) ELISA kit	RayBiotech, Inc. USA
RayBio Cell-Based p38 MAPK (Thr 180/Tyr 182) ELISA kit	RayBiotech, Inc. USA

Appendix 1.3 Reagents and chemicals

Acetone	BDH Laboratories Supplies, Poole, England
Albumin from bovine serum	Sigma-Aldrich Company Ltd, Dorset, UK
Ammonium persulphate	Sigma-Aldrich Company Ltd, Dorset, UK
AMV reverse transcriptase	Promega UK, Southampton, UK
AMV RT buffer	Promega UK, Southampton, UK
Beta mercaptoethanol	Bio-Rad Laboratories Ltd Hemel Hempstead, UK
Bionic™ Buffer, 10x	Sigma-Aldrich Company Ltd, Dorset, UK
Complete, Mini Protease Inhibitor Cocktail Tablets	Roche Diagnostics GmbH, Mannheim, Germany
D (+) Glucose solution 45%	Sigma-Aldrich Company Ltd, Dorset, UK
DC Protein Assay Kit	Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
DMSO	Sigma-Aldrich Company Ltd, Dorset, UK
dNTP mix	Promega UK, Southampton, UK

ECL Plus™ Western Blotting Detection Reagent	GE Healthcare UK Ltd Amersham, Bucks, UK
Ethanol	BDH Laboratories Supplies, Poole, England
Ethidium bromide	Sigma-Aldrich Company Ltd, Dorset, UK
Fetal calf serum	PAA Laboratories Ltd, Yeovil, UK
Hanks Balanced salt solution	Sigma-Aldrich Company Ltd, Dorset, UK
'Hi-Pure' Low EEO agarose gels	BioGene Ltd., Kimbolton, UK
Human resistin	Cambridge Biosciences Ltd Cambridge, UK
Hydrochloric Acid	BDH Laboratories Supplies, Poole, England
Hyper Ladder IV – 100bp ladder	Bioline Ltd, London, UK
L-glutamine	Sigma-Aldrich Company Ltd, Dorset, UK
Magnesium sulphate	BDH Laboratories Supplies, Poole, England
Methanol	BDH Laboratories Supplies, Poole, England

β Nicotinamide mononucleotide	Sigma-Aldrich Company Ltd, Dorset, UK
Non-essential amino acid solution	Sigma-Aldrich Company Ltd, Dorset, UK
Nuclease-Free Water	Promega UK, Southampton, UK
Oligo (dT) 15 Primer	Promega UK, Southampton, UK
Phosphate buffered saline	Sigma-Aldrich Company Ltd, Dorset, UK
Phosphatase inhibitor cocktail	Sigma-Aldrich Company Ltd, Dorset, UK
Penicillin–Streptomycin solution Hybri-Max®	Sigma-Aldrich Company Ltd, Dorset, UK
Pre-Aliquoted Reddy Mix™ PCR Master-Mix	Abgene Limited, Epsom, UK
Protein standard	Sigma-Aldrich Company Ltd, Dorset, UK
Protogel (30% solution at 37.5:1 ratio)	Geneflow UK Staffordshire, UK
Protogel resolving buffer	Geneflow UK Staffordshire, UK
Protogel stacking buffer	Geneflow UK Staffordshire, UK

2xqPCR Mastermix for iCycler 12.5ml plus SYBR Green	Primerdesign Ltd Southampton, UK
RNaseZap® RNase Decontamination Solution	Applied Biosystems, Warrington, UK
RNasin Ribonuclease Inhibitor	Promega UK, Southampton, UK
RPMI 1640 without Glucose	PAA Laboratories Ltd, Yeovil, UK
RPMI 1640 without L-Glutamine	PAA Laboratories Ltd, Yeovil, UK
Recombinant human visfatin	Axxora UK LTD
SeeBlue® Plus2 Pre-Stained Standard	Invitrogen Ltd., Paisley, UK
Sodium bicarbonate	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium pyruvate solution	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium Chloride	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium phosphate monobasic dihydrate	BDH Laboratories Supplies, Poole, England
SV Total RNA Isolation System	Promega UK, Southampton, UK
TEMED	Sigma-Aldrich Company Ltd, Dorset, UK

Tissue culture plasticware	Becton-Dickinson Franklin Lakes, NJ
Tris buffered saline	Sigma-Aldrich Company Ltd, Dorset, UK
Tris buffered saline (with tween)	Sigma-Aldrich Company Ltd, Dorset, UK
Tris/Glycine/SDS running buffer	Geneflow Ltd, Staffordshire, UK
Tris/Glycine transfer buffer	Geneflow Ltd, Staffordshire, UK
Triton X	Sigma-Aldrich Company Ltd, Dorset, UK
Trizma® hydrochloride solution	Sigma-Aldrich Company Ltd, Dorset, UK
Trizma® HCL	Sigma-Aldrich Company Ltd, Dorset, UK
Trizma® Base	Sigma-Aldrich Company Ltd, Dorset, UK
Trypsin/EDTA	Sigma-Aldrich Company Ltd, Dorset, UK
Tween-20	Pharmacia Biotech,
Urea	Sigma-Aldrich Company Ltd, Dorset, UK

Appendix 2

Appendix 2.1 Effects of resistin on gene expression profile in mouse β TC-6 cells

RT-real time PCR array analysis of transcripts expressed in β TC-6 cells exposed to resistin resulted in altered patterns of expression. The table below represents the genes that were not significantly regulated by resistin ($p>0.05$).

	Description	Fold Change	p value
Acly	ATP citrate lyase	-1.7817	0.084041219
Adra R a1a	Adrenergic receptor, alpha 1a	-1.1755	0.677983311
Adra R b3	Adrenergic receptor, beta 3	1.6624	0.117926367
Agt	angiotensinogen	1.9096	0.388713508
Akt2		1.0717	0.090975081
Aqp2	Aquaporin 2	1.9096	0.393317552
Ccl5	Chemokine (C-C motif) ligand 5; small inducible cytokine A5	4.8121	0.316747233
Ccr2	Chemokine (C-C) receptor 2	2.0945	0.056861898
CeaCam	Carcinoembryonic antigen-related cell adhesion molecule pseudogene 1	1.4472	0.577056158
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	2.4060	0.18613699
DPPIV	Dipeptidyl-peptidase 4	8	0.563424508
Dusp4	Dual specificity phosphatase 4	1.9096	0.811762895
Enpp1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	1.3503	0.994929123
Fbp1	Fructose bisphosphatase 1	2.8284	0.734777383
Foxg1	Forkhead box G1	-1.4142	0.546800032
Foxp3	Forkhead box P3	1.0092	0.179655246
Gcg R	Glucagon receptor	1.0968	0.109701782
GLP-1 R	Glucagon-like peptide 1 receptor	4.1891	0.833419789
Gsk3b	Musculus glycogen synthase kinase 3 beta	1.4810	0.316033459
Hmox1	Musculus heme oxygenase (decycling) 1	-1.0472	0.109697203

ICAM-1	Intercellular adhesion molecule	1.8660	0.536402527
Ide	Insulin degrading enzyme	2.5198	0.799804479
IfnG	Interferon gamma	4.3872	0.744246832
Igfbp5	Insulin-like growth factor binding protein 5	1.8234	0.568768424
Ikkkb	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	2	0.371528346
Il10	Interleukin 10	-1.4472	0.294852614
Il12b	Interleukin 12B	3.0314	0.773175579
Il4 Ra	Interleukin 4 receptor, alpha	2.9621	0.863078818
Ipf1	Insulin promoter factor 1, homeodomain transcription factor	-3.7321	0.25693434
IRS1	Insulin receptor substrate 1	1.3819	0.888963482
Neurod1	Neurogenic differentiation 1	2.700699892	0.543960627
Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	2.094588246	0.114422573
Nos3	Musculus nitric oxide synthase 3, endothelial cell	-1.231144413	0.560528488
NRF1	Nuclear respiratory factor 1	1.175547906	0.67802319
Nsf	N-ethylmaleimide sensitive fusion protein	1.274560627	0.176024388
Parp1		28.50875898	0.452436054
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic	2.193649959	0.134575435
Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	2.193649959	0.659040069
PPARa	Peroxisome proliferator activated receptor alpha	1.447269237	0.164535421
PPARg	Peroxisome proliferator activated receptor gamma	3.646889954	0.380643637
PPARg c1a		54.4426983	0.393620986
Ptpn1	Protein tyrosine phosphatase, non- receptor type 1	1.25992105	0.685085441
Pygl	Liver glycogen phosphorylase	-1.447269237	0.215295252
Rab4a	RAB4A, member RAS oncogene family	-1.481097552	0.185240692
Sell	Selectin, lymphocyte	-1.350349946	0.800747199
Serpine1	Mouse plasminogen activator inhibitor,	1.289370308	0.06079973

	Serine proteinase inhibitor		
Slc14a2	Solute carrier family 14 (urea transporter), member 2	2.351095813	0.272247246
Snap23	Synaptosomal-associated protein 23	-1.047294123	0.558413254
Snap25	Synaptosomal-associated protein 25	-2.143546925	0.128924982
Sod2	superoxide dismutase 2, mitochondrial	1.4142	0.935066398
Srebf1	Sterol regulatory element binding factor 1	1.7211	0.954531541
Stxbp1	Syntaxin binding protein 1	-11.0553	0.148731951
Stxbp4	Syntaxin binding protein 4	2.9621	0.136042844
Tgfb1	Transforming growth factor, beta 1	54.4426	0.152194079
Tnf	tumor necrosis factor	2.3510	0.163261352
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	1.6624	0.548745715
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	2.4060	0.174416588
Trib3	Tribbles homolog 3	2.3510	2.97294E-05
UCP2	Uncoupling protein 2, mitochondrial	-1.2893	0.876142543
Vamp2	Vesicle-associated membrane protein 2	1.2893	0.244660209
Vapa	Vesicle-associated membrane protein, associated protein A	-30.5549	0.130899437

Appendix 2.2 Effects of visfatin on gene expression profile in mouse β TC-6 cells

RT-real time PCR array analysis of 84 genes was performed to reveal genes regulated by visfatin. The table below represents the genes that were not significantly regulated by visfatin ($p > 0.05$).

	Description	Fold Change	p value
Ace 1	Angiotensin-converting enzyme	-3.7321	0.015655069
Acly	ATP citrate lyase	2.7006	0.471153199
Adra R a1a	Adrenergic receptor, alpha 1a	4.9245	0.517866023
Adra R b3	Adrenergic receptor, beta 3	1.2311	0.334403221
Agt	angiotensinogen	1.8234	0.844951734
Aqp2	Aquaporin 2	1.2599	0.337708525
Ccl5	Chemokine (C-C motif) ligand 5; small inducible cytokine A5	7.2937	0.051644769
Ccr2	Chemokine (C-C) receptor 2	3.1748	0.329699176
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	4.3872	0.407346191
Ctla4	Cytotoxic T-lymphocyte-associated protein 4	8.5741	0.166988611
Dusp4	Dual specificity phosphatase 4	3.6468	0.419052334
Enpp1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	1.5157	0.663494285
Fbp1	Fructose biphosphatase 1	-2	0.158159302
Foxc2	Forkhead box C2	1.7411	0.831267123
Foxg1	Forkhead box G1	1.4142	0.800735881
Foxp3	Forkhead box P3	-1.2893	0.087497875
G6pc	Glucose-6-phosphatase, catalytic	4.9245	0.074838271
G6pd2	Glucose-6-phosphate dehydrogenase 2	1.9096	0.774508188
Gcg	Glucagon	1.7817	0.651687656
Gcg R	Glucagon receptor	2.2973	0.873211439
GLP-1 R	Glucagon-like peptide 1 receptor	1.0717	0.284345238
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	2.9621	0.171942962

Gsk3b	Musculus glycogen synthase kinase 3 beta	-1.4472	0.265892181
Hmox1	Musculus heme oxygenase (decycling) 1	3.6468	0.202419341
ICAM-1	Intercellular adhesion molecule	-1.2599	0.363088348
Ide	Insulin degrading enzyme	1.0968	0.396278215
IfnG	Interferon gamma	2.7638	0.586450912
Igfbp5	Insulin-like growth factor binding protein 5	2.7638	0.844364914
Ikbkb	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	1.5157	0.726549287
Il10	Interleukin 10	7.1271	0.481530346
Il12b	Interleukin 12B	4.8121	0.663889265
Il4 Ra	Interleukin 4 receptor, alpha	12.4091	0.103260036
Il6	Interleukin 6	2.3510	0.692378847
Inpp1	Inositol polyphosphate phosphatase-like 1	2.4622	0.370523986
Ipf1	Insulin promoter factor 1, homeodomain transcription factor	2.0467	0.921952374
IRS1	Insulin receptor substrate 1	4.2870	0.320091065
Mapk14	Mitogen-activated protein kinase 14	2.6390	0.771075372
Neurod1	Neurogenic differentiation 1	1.1224	0.118191636
Nos3	Musculus nitric oxide synthase 3,	8.9796	0.35973144
NRF1	Nuclear respiratory factor 1	1.5874	0.39052759
Nsf	N-ethylmaleimide sensitive fusion protein	2.2449	0.901014369
Parp1		1.1755	0.168815644
Pax4	Paired box gene 4	16	0.113507305
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic	3.4822	0.108129377
Pfkfb3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	3.4822	0.451151128
Pik3cd	Phosphatidylinositol 3-kinase catalytic delta polypeptide	2.0945	0.966682432
Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	2.2973	0.947380411
PPARg	Peroxisome proliferator activated receptor gamma	2.8945	0.761844161
PPARg c1a		5.5276	0.404103527
Ptpn1	Protein tyrosine phosphatase, non-	2.2973	0.904054495

	receptor type 1		
Pygl	Liver glycogen phosphorylase	2	0.776799629
Rab4a	RAB4A, member RAS oncogene family	1.1755	0.284139537
Retn	Resistin	3.5635	0.499490948
Sell	Selectin, lymphocyte	15.2774	0.119007467
Serpine1	Mouse plasminogen activator inhibitor, Serine proteinase inhibitor	1.2893	0.552680071
Slc14a2	Solute carrier family 14 (urea transporter), member 2	2.3510	0.874060481
Slc2a4	Solute carrier family 2 (facilitated glucose transporter),member 4	2.7006	0.624193569
Snap23	Synaptosomal-associated protein 23	-1.0472	0.417365703
Snap25	Synaptosomal-associated protein 25	-2.1435	0.12800747
Sod2	superoxide dismutase 2, mitochondrial	1.4142	0.691779363
Srebf1	Sterol regulatory element binding factor 1	1.7211	0.8131873
Stxbp1	Syntaxin binding protein 1	-11.0553	0.318665471
Stxbp4	Syntaxin binding protein 4	2.9621	0.405292492
Tgfb1	Transforming growth factor, beta 1	54.4426	0.155133222
Tnf	tumor necrosis factor	2.3510	0.89080638
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	1.6624	0.736695019
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	2.4060	0.861448053
Trib3	Tribbles homolog 3	2.3510	0.952761409
Vamp2	Vesicle-associated membrane protein 2	1.2893	0.331494111
Vamp3	Vesicle-associated membrane protein 3	-6.3496	0.307442214
Vapa	Vesicle-associated membrane protein, associated protein A	-30.5549	0.254341088
Vegfa	Vascular endothelial growth factor A	-4.7021	0.389972132

Appendix 3

Publications and conference presentations

Published papers

Brown JE, Onyango DJ, Dunmore SJ. (2007) Resistin down-regulates insulin receptor expression, and modulates cell viability in rodent pancreatic beta-cells. *FEBS Letters*, Jul 10; 581(17):3273-6.

Brown JE, Onyango DJ, Ramanjaneya M, Conner AC, Patel ST, Dunmore SJ and Randeve HS. (2009) Visfatin regulates insulin secretion, insulin receptor signalling and mRNA expression of diabetes related genes in mouse pancreatic beta-cells. *Journal of Molecular Endocrinology* (2009) - In press DOI: 10.1677/JME-09-0071

Conference presentations

Onyango DJ, Brown JE, Dunmore SJ. Visfatin is expressed in the pancreatic beta cells. *Diabetes UK Annual Professional Conference, Glasgow 2007*

Dunmore SJ, Onyango DJ, Brown JE. Visfatin decreases cell viability, activates insulin receptor and alters expression of key genes in a clonal beta-cell line. 44th *European Association for the Study of Diabetes Annual Meeting, Rome 2008*