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

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Date: 13th November 2009
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 (βTC-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.
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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  
Ca²⁺           Calcium ions  
[Ca²⁺]i                                                                                     Intracellular calcium  
cDNA               Complementary DNA  
Cl⁻           Chloride  
CO₂                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
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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 insulitis. 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
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-4α (MODY-1), Hnf-1α (MODY-3), Pdx-1 (MODY-4), Hnf-1β (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)-1β, 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 (Hedskov, 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 Ca²⁺ influx into the β-cell. A rise in free cytosolic Ca²⁺ 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), p60dok, 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 P(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 catalyse 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/EBPα “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).
The major transcriptional factors involved in the adipogenic include proteins belonging to the CCAAT/enhancer binding protein family (C/EBP), peroxisome proliferator-activated receptor-\(\gamma\) (PPAR-\(\gamma\)) 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-\(\gamma\). PPAR-\(\gamma\) then induces C/EBP-\(\alpha\) 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 bloodstream 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 Feve 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 (over-nutrition) 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:  = inhibition,  = 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).

![Diagram of pathways leading to insulin resistance associated with obesity induced by adipokines, free fatty acids, and chronic inflammation in adipose tissue.]

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 lipoatrophic 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
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 RELMα 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 promotes 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-\(\gamma\) 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-\(\gamma\) 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-1β, 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 ß-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 ß-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 βTC-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-mercaptoethanol 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

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>24.22g</td>
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<tr>
<td>NaCl</td>
<td>80g</td>
</tr>
<tr>
<td>Water</td>
<td>986ml</td>
</tr>
<tr>
<td>HCL (Absolute – add in the hood)</td>
<td>14ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.6</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Tris buffered saline</td>
<td>100ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>900ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>500µl</td>
</tr>
</tbody>
</table>

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 βTC-6 cells

The insulin-secreting rat β-cell line BRIN-BD11 cells and mouse βTC-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 µg/ml streptomycin. The cells were seeded at 3 x 10^6 cells per flask and maintained in 175cm^3 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 x 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 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. 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, βTC-6 cells were seeded at a concentration of 2 x 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Strand</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Actin</td>
<td>Sense</td>
<td>GCTGTATTCCCCCTCCATCGTG</td>
</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>CGTCCCAGTTGGTAACAATGCC</td>
</tr>
<tr>
<td>rat GAPDH</td>
<td>Sense</td>
<td>GGAGTCTACTGGCGTCTTTCA</td>
</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>ATGAGCCCTCCACGAT</td>
</tr>
<tr>
<td>Mouse Insulin receptor</td>
<td>Sense</td>
<td>AATGGCAACATCACACACTACC</td>
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<tr>
<td></td>
<td>Anti-sense</td>
<td>CAGCCCTTTGAGACAATAATCC</td>
</tr>
<tr>
<td>Mouse GcgR</td>
<td>Sense</td>
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</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>AACCAGCAATAGTGGGTATGATG</td>
</tr>
<tr>
<td>Rat resistin</td>
<td>Sense</td>
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</tr>
<tr>
<td></td>
<td>Anti-sense</td>
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</tr>
<tr>
<td>Mouse visfatin</td>
<td>Sense</td>
<td>GCCTCCTGGATTTTCTTCTTTG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AACAATACCCACCCACACAA</td>
</tr>
</tbody>
</table>

**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-guage 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)v primer, dNTP mix and avian monoblastoma 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 (CT) after which fluorescence exceeds the background threshold minus the CT for the housekeeping control (ΔCT). 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 $2^{-\Delta\Delta CT}$, 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 $2^{-\Delta\Delta Ct}$ method, in which Ct 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 4⁰C 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 95⁰C for 5 minutes to reduce and denature the protein. Samples were stored at -20⁰C 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 (40µg) 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 $2 \times 10^4$ 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 µg 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 (Mercodia, Sweden).

For visfatin and NMN, glucose responsive clonal rodent pancreatic beta-cell line βTC-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 (Mercodia, 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 βTC-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 βTC-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≤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 βTC-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 βTC-6 cell total cell number. Total cell count determined using a Coulter counter. The experiment was repeated three times. * p≤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 βTC-6 cells were cultured with or without resistin for 48 hours. Sub-confluent βTC-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 βTC-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 βTC-6 cells from serum-depletion induced cell death. βTC-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 βTC-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 βTC-6 cells.

![Figure 3.1.5 Resistin treatment results in a non-significant increase in cell viability after 72 hours. βTC-6 cells (1 x 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 βTC-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.

![Graph showing cell viability over 24 hours with visfatin concentration](image)

**Figure 3.1.6 Visfatin has no effect on βTC-6 cell viability over 24 hours.**

βTC-6 cells (2 x 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 βTC-6 cells with visfatin for 48 hours at concentrations from 0 to 50ng/ml resulted in no significant effect on cell viability of βTC-6 cells.

Figure 3.1.7 Low visfatin concentration does not promote cell viability over 48 hours. βTC-6 cells (1 x 10⁴ 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 βTC-6 cells. This result shows that prolonged exposure of the cells to visfatin does not promote survival of the mouse βTC-6 cells.

![Graph showing cell viability vs visfatin concentration](image)

**Figure 3.1.8 Effects of visfatin on cell viability of βTC-6 cells over 72 hours.** βTC-6 cells (1 x 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 to 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.

![Graph showing the effect of visfatin concentrations on β-cell viability](image)

Figure 3.1.9 Visfatin decreases βTC-6 cell viability at high concentrations. βTC-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.

![Graph showing cell viability vs visfatin concentration](image)

**Figure 3.2.0 Visfatin decreases βTC-6 cell viability at high concentrations over 48 hours.** βTC-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≤0.05 and ** p≤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 βTC-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.

![Graph showing the effect of visfatin on βTC-6 cell viability](image)

**Figure 3.2.1 Visfatin decreases βTC-6 cell viability at high concentrations over 72 hours.** βTC-6 cells (2 x 10⁴ 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≤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 βTC-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. βTC-6 cells (2 x 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).
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 βTC-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. βTC-6 cells (2 x 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.

![Graph showing cell viability over NMN concentration.]

**Figure 3.2.4 Exposure to NMN results in a non-significant decrease β cell viability over 72 hours.** βTC-6 cells (2 x 10⁴ 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 βTC-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 βTC-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
βTC-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 βTC-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 βTC-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 ß-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 βTC-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 βTC-6 cells

RT-real time PCR array analysis of transcripts expressed in βTC-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.

<table>
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<tr>
<th>Description</th>
<th>Fold Change</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>Ace 1         Angiotensin-converting enzyme</td>
<td>-1.382</td>
<td>0.00153</td>
</tr>
<tr>
<td>CD28         CD28 antigen</td>
<td>1.447</td>
<td>0.00295</td>
</tr>
<tr>
<td>Ctf4         Cytotoxic T-lymphocyte-associated protein 4</td>
<td>3.102</td>
<td>0.0287</td>
</tr>
<tr>
<td>Foxc2        Forkhead box C2</td>
<td>2.297</td>
<td>0.0345</td>
</tr>
<tr>
<td>G6pc         Glucose-6-phosphatase, catalytic</td>
<td>1.350</td>
<td>0.00101</td>
</tr>
<tr>
<td>G6pd2        Glucose-6-phosphate dehydrogenase 2</td>
<td>3.102</td>
<td>0.00706</td>
</tr>
<tr>
<td>Gcg          Glucagon</td>
<td>-1.047</td>
<td>0.0392</td>
</tr>
<tr>
<td>Gpd1         Glycerol-3-phosphate dehydrogenase 1 (soluble)</td>
<td>3.102</td>
<td>0.0310</td>
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<tr>
<td>HNF4a        Hepatic nuclear factor 4</td>
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<td>0.0457</td>
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<tr>
<td>Il6          Interleukin 6</td>
<td>1.447</td>
<td>0.0261</td>
</tr>
<tr>
<td>Inpp1       Inositol polyphosphate phosphatase-like 1</td>
<td>2.194</td>
<td>0.0381</td>
</tr>
<tr>
<td>Ins1         Insulin I</td>
<td>1.023</td>
<td>0.0443</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Fold Change</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Mapk14</td>
<td>Mitogen-activated protein kinase 14</td>
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<tr>
<td>Mapk8</td>
<td>Mitogen activated protein kinase 8</td>
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</tr>
<tr>
<td>Pax4</td>
<td>Paired box gene 4</td>
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<tr>
<td>Pfkfb3</td>
<td>6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3</td>
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<tr>
<td>Pik3cd</td>
<td>Phosphatidylinositol 3-kinase catalytic delta</td>
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</tr>
<tr>
<td>Retn</td>
<td>Resistin</td>
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</tr>
<tr>
<td>Slc2a4</td>
<td>Solute carrier family 2 (facilitated glucose transporter),member 4</td>
<td>2.701</td>
</tr>
<tr>
<td>Stx4a</td>
<td>syntaxin 4A (placental)</td>
<td>17.549</td>
</tr>
<tr>
<td>Tcf2</td>
<td>Transcription factor 2</td>
<td>32</td>
</tr>
<tr>
<td>Vamp3</td>
<td>Vesicle-associated membrane protein 3</td>
<td>-6.349</td>
</tr>
<tr>
<td>Vegfa</td>
<td>Vascular endothelial growth factor A</td>
<td>-4.702</td>
</tr>
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**Table 4.1 RT-PCR array data for β-cells treated with resistin.** Gene Expression Analysis of resistin treated βTC-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, Inppl1 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 Inppl1 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 deacreses 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 βTC-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.

<table>
<thead>
<tr>
<th>Description</th>
<th>Fold Change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace 1  Angiotensin-converting enzyme</td>
<td>-3.732</td>
<td>0.0157</td>
</tr>
<tr>
<td>Akt2  Protein kinase B, beta</td>
<td>24.251</td>
<td>0.000668</td>
</tr>
<tr>
<td>CD28  CD28 antigen</td>
<td>97.006</td>
<td>0.00251</td>
</tr>
<tr>
<td>CeaCam  Carcinoembryonic antigen-related cell adhesion molecule pseudogene 1</td>
<td>-2.828</td>
<td>0.0178</td>
</tr>
<tr>
<td>DPPIV  Dipeptidyl-peptidase 4</td>
<td>1.0968</td>
<td>0.0751</td>
</tr>
<tr>
<td>HNF4a  Hepatic nuclear factor 4</td>
<td>16</td>
<td>0.0118</td>
</tr>
<tr>
<td>Ins1  Insulin I</td>
<td>8.979</td>
<td>0.0369</td>
</tr>
<tr>
<td>Mapk8  Mitogen activated protein kinase 8</td>
<td>-1.122</td>
<td>0.0386</td>
</tr>
<tr>
<td>Nfkb1  Nuclear factor of kappa light chain gene enhancer in B-cells, p105</td>
<td>40.317</td>
<td>0.0189</td>
</tr>
<tr>
<td>PPARa  Peroxisome proliferator activated receptor alpha</td>
<td>-1.259</td>
<td>0.0434</td>
</tr>
<tr>
<td>Stx4a  Syntaxin 4A (placental)</td>
<td>17.549</td>
<td>0.0352</td>
</tr>
<tr>
<td>Tcf2  Transcription factor 2</td>
<td>32</td>
<td>0.00313</td>
</tr>
<tr>
<td>UCP2  Uncoupling protein 2, mitochondrial</td>
<td>-1.289</td>
<td>0.00479</td>
</tr>
</tbody>
</table>
Table 4.2 RT-PCR array data for β cells treated with visfatin. Gene Expression Analysis of visfatin treated mouse βTC-6 cells using Mouse Diabetes RT² Profiler™ PCR Array. The Superarray system compared the relative levels of mRNA expressed in βTC-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\textsubscript{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\textsubscript{ATP} channel activity with subsequent reduced insulin secretion. Down-regulation of UCP-2 mRNA by visfatin 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 βTC-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 (Inpp11) 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)P₃ from PI(4,5)P₂ upon stimulation by a variety of ligands (Hiles et al., 1992; Stephens et al., 1993). PI (3,4,5)P₃ 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)P₃ to produce PI(3,4)P₂, in effect disrupting the PI3 kinase pathway and negatively regulating insulin signalling (Ishihara et al., 1999; Sleeman et al., 2005).

Resistin up-regulation of Inpp11 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 Inpp11 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 Pfkb3 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 glycogenolytic 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 Pfkb3 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 1β (HNF1β) 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-1β (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 Hnf1β 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-4α) 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-κB) 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-κB activation (Kim et al., 2008). In another study involving endothelial cells, NF-κB 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-κB in the IL-1β induced nitric oxide synthase expression and signaling mechanism has previously been demonstrated (Kwon et al., 1995). Additional studies have shown that cytokines IL-1β, interferon-gamma (IFN-γ)
and TNF-α activate NF-κB 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 NFkB 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 suggests that resistin previously described as a adipose tissue specific protein is also found in pancreatic β-cells.

![Resistin mRNA expression graph](image)

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, 1μg of mRNA from clonal rat BRIN-BD11 cells was reverse transcribed to cDNA and used in a 25 μl 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 βTC-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).

![Graph showing resistin did not have any effect on insulin receptor gene expression](image)

**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 ± 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 βTC-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 βTC-6 cells are decreased by high resistin concentrations

To further investigate the effect of resistin at concentrations reported in obese type 2 diabetics, βTC-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.

βTC-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 βTC-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 βTC-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 βTC-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 βTC-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; Kovacikova 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-1β (IL-1β), tumour necrosis factor-alpha (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 βTC-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](image)

**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 βTC-6 cells samples and quantified. 1 µg of total mRNA was DNAsed to exclude the possibility of genomic DNA contamination and reverse transcribed (RT) and amplified in a 25 µl 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).

![Visfatin Protein is expressed in the beta cells](image)

**Figure 6.1.2 Visfatin Protein is expressed in the beta cells.** Western blot analysis of protein extracts from clonal mouse βTC-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 βTC-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 βTC-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 βTC-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 ± 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. βTC-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 βTC-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.

![Graph showing time-dependent activation of ERK1/2 by visfatin]

**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.

![Graph showing ERK1/2 phosphorylation over time](image)

**Figure 6.1.8 Time response curves of ERK 1/2 activation by NMN.** The cells were stimulated with 100µM 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 ½ 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, βTC-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.](image)

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.

![Graph showing effect of visfatin on insulin release from clonal pancreatic mouse βTC-6 cells at low glucose.](image)

**Figure 6.2.2** Effect of visfatin on insulin release from clonal pancreatic mouse βTC-6 cells at low glucose. The exposure of the mouse βTC-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 U0126 (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 βTC-6 cells at elevated glucose concentrations.

Figure 6.2.3 Effect of visfatin on insulin release from clonal pancreatic mouse βTC-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 βTC-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 – 100µM) 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=1µM NMN, N10=10µM NMN, N100=100µM 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 βTC-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 βTC-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 (at least 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-alpha 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 βTC-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 adipsin (Siiteri 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 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 Steppan et al., showed that circulating resistin levels are decreased by the anti-diabetic drug rosiglitazone (Steppan 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 mimic 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, βTC-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 \( \beta \)-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 \( \beta \)-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 $K_{\text{ATP}}$ channel-dependent and $K_{\text{ATP}}$ channel-independent pathways. Future work should examine whether visfatin and its reaction product NMN have any significant effects on $K_{\text{ATP}}$ 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


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## Appendices

### Appendix 1

#### Appendix 1.1 Equipment

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<tr>
<th>Equipment</th>
<th>Manufacturer</th>
</tr>
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<td>Block heater</td>
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<td>Centrifuges</td>
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<td>CO₂ incubator</td>
<td>Jensons Scientific Inc. Bridgeville, PA 15017, USA</td>
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<tr>
<td>Coulter counter Z1</td>
<td>Beckman Coulter limited High Wycombe, UK</td>
</tr>
<tr>
<td>Genequant Pharmacia</td>
<td>Biotech Inc. NJ, USA</td>
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<td>Hybaid PCR Express thermocycler</td>
<td>Hybaid, Action Court, Middlesex, UK</td>
</tr>
<tr>
<td>ImageQuant™ TL image analysis software</td>
<td>GE Healthcare Life Sciences Amersham, Bucks, UK</td>
</tr>
<tr>
<td>Inverted microscope Celti,</td>
<td>Optical Instruments, Belgium</td>
</tr>
<tr>
<td>Labsystems Multiskan MS</td>
<td>Labsystems, Finland</td>
</tr>
<tr>
<td>Magnetic Stirrer Hotplate</td>
<td>Stuart Scientific, Beacon Road, Staffordshire, UK</td>
</tr>
<tr>
<td>Equipment</td>
<td>Supplier/Location</td>
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<td>---------------------------------------</td>
<td>--------------------------------------------</td>
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<td>Electrophoresis system</td>
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</tr>
<tr>
<td>PhosphorImager™ technology</td>
<td>GE Healthcare Life Sciences, Amersham, Bucks, UK</td>
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<td>PowerPac 1000 Power Supply</td>
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<td>Wolf Laboratories, York, UK</td>
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<td>Vortex</td>
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<td>Water baths</td>
<td>Grant Instruments (Cambridge) Ltd, Cambridge UK</td>
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### Appendix 1.2 Specialised kits

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<td>Cell Proliferation Assay (MTS) kit.</td>
<td>Southampton, UK</td>
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<tr>
<td>Mouse insulin secretion ELISA kit</td>
<td>Mercodia, Sweden</td>
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<tr>
<td>Mouse Diabetes RT² Profiler™ PCR Array</td>
<td>SABiosciences Corporation</td>
</tr>
<tr>
<td></td>
<td>MD 21703, USA</td>
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<tr>
<td>Rat insulin secretion ELISA kit</td>
<td>Mercodia, Sweden</td>
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<td>RayBio Cell-Based Phospho-Insulin Receptor B</td>
<td>RayBiotech, Inc. USA</td>
</tr>
<tr>
<td>ELISA kit</td>
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</tr>
<tr>
<td>RayBio Cell-Based ERK1/2 (activated)</td>
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<tr>
<td>ELISA kit</td>
<td></td>
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<td>RayBio Cell-Based p38 MAPK (Thr 180/Tyr 182)</td>
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<td>ELISA kit</td>
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## Appendix 1.3 Reagents and chemicals

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<tr>
<td>Ammonium persulphate</td>
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<tr>
<td>AMV reverse transcriptase</td>
<td>Promega UK, Southampton, UK</td>
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<tr>
<td>AMV RT buffer</td>
<td>Promega UK, Southampton, UK</td>
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<td>Beta mercaptoethanol</td>
<td>Bio-Rad Laboratories Ltd, Hemel Hempstead, UK</td>
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<tr>
<td>Bionic™ Buffer, 10x</td>
<td>Sigma-Aldrich Company Ltd, Dorset, UK</td>
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<tr>
<td>Complete, Mini Protease Inhibitor Cocktail Tablets</td>
<td>Roche Diagnostics GmbH, Mannheim, Germany</td>
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<td>D (+) Glucose solution 45%</td>
<td>Sigma-Aldrich Company Ltd, Dorset, UK</td>
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<td>DC Protein Assay Kit</td>
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<td>DMSO</td>
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<tr>
<td>ECL Plus™ Western Blotting Detection Reagent</td>
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<td>Ethanol</td>
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<td>Ethidium bromide</td>
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<td>Fetal calf serum</td>
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<td>Hanks Balanced salt solution</td>
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<tr>
<td>‘Hi-Pure’ Low EEO agarose gels</td>
<td>BioGene Ltd., Kimbolton, UK</td>
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<tr>
<td>Human resistin</td>
<td>Cambridge Biosciences Ltd, Cambridge, UK</td>
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<tr>
<td>Hydrochloric Acid</td>
<td>BDH Laboratories Supplies, Poole, England</td>
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<td>Hyper Ladder IV – 100bp ladder</td>
<td>Bioline Ltd, London, UK</td>
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<tr>
<td>L-glutamine</td>
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<td>Magnesium sulphate</td>
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<tr>
<td>Methanol</td>
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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 MixTM 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  
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Appendix 2

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

RT-real time PCR array analysis of transcripts expressed in βTC-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).

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<tr>
<th>Description</th>
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<tr>
<td>Acly ATP citrate lyase</td>
<td>-1.7817</td>
<td>0.084041219</td>
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<tr>
<td>Adra R alpha 1a Adrenergic receptor, alpha 1a</td>
<td>-1.1755</td>
<td>0.677983311</td>
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<td>Adra R beta 3 Adrenergic receptor, beta 3</td>
<td>1.6624</td>
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<tr>
<td>Agt angiotensinogen</td>
<td>1.9096</td>
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<tr>
<td>Akt2</td>
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<tr>
<td>Aqp2 Aquaporin 2</td>
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<tr>
<td>Ccl5 Chemokine (C-C motif) ligand 5; small inducible cytokine A5</td>
<td>4.8121</td>
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<td>Ccr2 Chemokine (C-C) receptor 2</td>
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<td>CeaCam Carcinoembryonic antigen-related cell adhesion molecule pseudogene 1</td>
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<td>Cebpa CCAAT/enhancer binding protein (C/EBP), alpha</td>
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<td>DPPIV Dipeptidyl-peptidase 4</td>
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<tr>
<td>Dusp4 Dual specificity phosphatase 4</td>
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<td>Enpp1 Ectonucleotide pyrophosphatase/phosphodiesterase 1</td>
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<td>Fbp1 Fructose bisphosphatase 1</td>
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<td>Foxg1 Forkhead box G1</td>
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<tr>
<td>Foxp3 Forkhead box P3</td>
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<td>Gcg R Glucagon receptor</td>
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<td>Gene</td>
<td>Description</td>
<td>Adj. Fold Change</td>
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Appendix 2.2 Effects of visfatin on gene expression profile in mouse βTC-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).

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<th>p value</th>
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Appendix 3

Publications and conference presentations

Published papers


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*