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The Effects and Mechanisms of Saponins of *Panax Notoginseng* on Glucose and Lipid Metabolism in 3T3-L1 Cells

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Declaration

This thesis titled 'The Effects and Mechanisms of Saponins of *Panax Notoginseng* on Glucose and Lipid Metabolism in 3T3-L1 Cells" is of original work. The work presented in this thesis was carried out under the supervision of A/Prof Xianqin Qu at University of Technology, Sydney. The thesis has not been submitted by the candidate for the award of any other degree.

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Abstract

Type 2 diabetes is a metabolic disorder which has been posed as a serious health problem in our present society. In light of the escalating prevalence of the disease and widespread evidence of progression to cardiovascular complications, there is a need to discover new agents to successfully treat this multifacet disorder.

Resistance to insulin-mediated glucose uptake and deregulation of lipid metabolism are early and major hallmarks in the development of type 2 diabetes. The aim of this thesis was to determine the effects and mechanisms of saponins of *Panax notoginseng* (SPN) on glucose and lipid metabolism. 3T3-L1 adipocytes was utilized to study whether this naturally occurring agent, which has been used in the treatment of ischemic cardio-cerebral vascular disease throughout China for years, improves insulin-mediated glucose uptake and lowers lipid levels *in vivo*.

3T3-L1 adipocytes were cultured and treated with 100 nM insulin alone or with concentrations of 10, 50, 100 and 200 μg/ml SPN, respectively. [^{3}H]2-deoxyglucose glucose uptake, GLUT4 immunofluorescence imaging and glycogen synthesis assay were carried out to determine the effects of SPN on glucose metabolism. In addition, lipid staining and lipolysis assay were carried to study the effects of SPN on lipid metabolism.

The results in Chapter 2 indicate that SPN consist of properties that improve glucose metabolism in 3T3-L1 cells. SPN significantly increased insulin-mediated glucose uptake

in a dose-dependent manner. Immunofluorescence imaging and analysis showed that SPN increased GLUT4 in the plasma membrane. Furthermore, glycogen synthesis augmented, whereby the incorporation of D-[U-^{14}C] glucose into glycogen was enhanced with SPN treatment in 3T3-L1 cells. These findings suggest that SPN may have a direct effect upon lowering glucose levels in type 2 diabetes.

Furthermore, results in Chapter 3 of this thesis illustrates that SPN also exert effects that regulate lipid metabolism. Lipid staining showed SPN significantly decreased lipid content 3T3-L1 adipocytes. In addition, SPN significantly inhibited lipolysis in vitro, indicating that this naturally occurring agent may decrease free fatty acid delivery to the liver thereby subsequently reducing hepatic glucose production.

In effort to discover a new anti-diabetic agent that achieves both treatment of type 2 diabetes and prevention of diabetic complications, this study supports that SPN is a potential agent that is capable of directly lowering blood glucose and lipid levels in type 2 diabetic patients. Further research with both in animal models and clinical trials, as described in Chapter 4, is needed to establish that SPN has high potential to be used as an agent for diabetes and its vascular complications. These findings may prove to be highly valuable to our society.

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Abbreviations

ADRAC	Australian Adverse Drug Reactions Advisory Committee
AGI	Alpha-glucosidase inhibitors
AMPK	AMP-activated protein kinase
ARR	absolute risk reductions
BMI	Body mass index
CHM	Chinese Herbal Medicine
EGIR	European Group for the Study of Insulin Resistance
FFA	free fatty acids
GLUT1	glucose transporter 1
GLUT2	glucose transporter 2
GLUT3	glucose transporter 3
GLUT4	glucose transporter 4
HDL	high-density lipoprotein
HSL	hormone-sensitive lipase
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL-6	interleukin-6
IRS	insulin receptor substrate
ISDN	isosorbide dinitrate
LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MI	myocardial infarction
MLC	myosin light chain
NCEP	National Cholesterol Education Program
NEFA	nonesterified free fatty acids
PDH	pyruvate dehydrogenase
PFK	phosphofructokinas
PI3-K	phosphoinositide 3-kinase
PKC	protein kinase- C
PPARγ	peroxisome proliferator activated receptor-γ
PVD	peripheral vascular disease
SPN	saponins of *Panax notoginseng*
T2D	Type 2 diabetes
TG	triglycerides
TNF-α	tumor necrosis factor α
TZD	thiazolidinedione

WHO	World Health Organisation
WTH	Waist-to-Hip

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Chapter 1

Introduction

CHAPTER 1: INTRODUCTION

1.1 Metabolism

Metabolism involves a comprehensive scope of chemical reactions that occurs in living cells. These processes are ultimately the basis of life, allowing cells to grow and reproduce, maintain their structures, and respond to their environments. With the rate of metabolism disorder occurrence on the rise, there is a severe need for research in this area.

1.1.1 Metabolic Syndrome

Metabolic syndrome is a term that exemplifies a combination of medical disorders that increases one's risk for cardiovascular disease and may lead to diabetes. Fasting hyperglycaemia (type 2 diabetes, impaired fasting glucose, impaired glucose tolerance or insulin resistance), high blood pressure, central obesity, decreased high-density lipoprotein (HDL) cholesterol and elevated triglycerides (TG) and uric acid levels are some of the common features of metabolic dysfunction (Cohn *et al.*, 2004). However, at present, no consensus exists for specific thresholds for establishing the diagnosis of each of these traits as components of the syndrome (Meigs *et al.*, 2003).

Associated diseases and signs are: fatty liver (especially in concurrent obesity), progressing to non-alcoholic fatty liver disease, polycystic ovarian syndrome,

hemochromatosis (iron overload) and acanthosis nigricans (a skin condition featuring dark patches).

A study was done to assess the trend between 1992 and 2002 in prevalence of the metabolic syndrome defined by the National Cholesterol Education Program (NCEP) and International Diabetes Federation (IDF). Results showed that between the 10 year period, the occurrence of metabolic syndrome in women increased 32.2 to 36.2% based on the NCEP definition (p=0.045) and 38.0 to 42.3% based on the IDF definition. (p=0.036), which supports the belief that metabolic syndrome is significantly becoming an increasing problem in our society (Hu *et al.*, 2006).

The definition of metabolic syndrome has been proposed by various academic societies and groups including WHO, NECP, European Group for the Study of Insulin Resistance (EGIR) and IDF (Ebara *et al.*, 2006). Figure 1.1 illustrates the diagnostic criteria of metabolic syndrome.

	WHO (1999)	EGIR (1999)	NCEP ATP III (2001)
BLOOD PRESSURE	≥ 140/90 mmHg	≥ 140/90 mmHg or antihypertensive medication	≥ 130/85 mmHg
	Hypertension		
TRIGLYCERIDE LEVEL	≥ 1.695 mmol/L	≥ 2.0 mmol/L	≥ 1.695 mmol/L
	Dislipidaemia		
HDL-C LEVEL	0.9 mmol/L (male) ≤ 1.0 mmol/L (female)	< 1.0 mg/dL	< 40 mg/dL (male) < 50 mg/dL (female)
	Dislipidaemia		
WAIST CIRCUMFERENCE	> 0.90 (male) > 0.85 (female) *(waist:hip ratio)*	≥ 94 cm (male) ≥ 80 cm (female)	≥ 102 cm (male) ≥ 88 cm (female)
	Central Obesity		
OTHERS	Microalbuminuria: urinary albumin excretion ratio ≥ 20 mg/min	Fasting plasma glucose ≥ 6.1 mmol/L	Fasting plasma glucose ≥ 6.1 mmol/L (110 mg/dl)

Figure 1.1 The diagnostic criteria of Metabolic Syndrome.

1.1.2 Insulin Resistance

Insulin resistance indicates a state in which the cells of the body become defiant to the effects of insulin, which causes anomalous response and a reduction to a given amount of insulin. As a consequence, higher levels of insulin are required in order for insulin to have its effects. Clinically, resistance is seen both endogenously and

exogenously, with both the body's own insulin, and if insulin is given through injection.

Similar to metabolic syndrome, it is a condition that has become widespread in the recent years but nonetheless the exact cause is still indistinct. The probable causes of insulin resistance include metabolic syndrome, obesity, pregnancy, infection, severe illness and stress.

Development of insulin resistance results in compensatory hyperinsulinemia, a state that is maintained until pancreatic secretory defects occur. However, once β-cell dysfunction occurs, inability to compensate for the increased insulin resistance results in hyperglycemia, and the diagnosis of type 2 diabetes is made on clinical grounds (Cefalu, 2001). The associated clinical and laboratory abnormalities that represent this syndrome consist of type 2 diabetes, central obesity, dyslipidemia (increased TG, decreased HDL, and increased LDL), hypertension, increased prothrombotic and antifibrinolytic factors (i.e., hypercoagulability), and a predilection for heart disease (Cefalu, 2001).

1.2 Type 2 Diabetes

1.2.1 Definition of Type 2 Diabetes

Diabetes mellitus is a chronic disease that is growing in prevalence worldwide (Zimmet *et al.*, 2001). Type 2 diabetes mellitus is a group of disorders characterized by hyperglycemia and associated with microvascular (ie, retinal, renal, possibly

neuropathic), macrovascular (ie, coronary, peripheral vascular), and neuropathic (ie, autonomic, peripheral) complications. At present, the genetics of type 2 diabetes are complex and not completely understood.

Evidence supports inherited components for both pancreatic beta cell failure and insulin resistance. Considerable debate exists regarding the primary defect in type 2 diabetes mellitus. Most patients have both insulin resistance and some degree of insulin deficiency. Nonetheless, research has revealed that insulin resistance itself is not the solitary cause for type 2 diabetes mellitus as many people with insulin resistance (particularly patients who are obese) do not develop glucose intolerance (Ligaray, 2007).

Also, insulin deficiency is responsible for the development of hyperglycaemic state. Patients may have high insulin levels, but the insulin concentrations are inappropriately low for the level of glycemia.

Interestingly, type 2 diabetes is a metabolic disorder that results from complex interactions of multiple factors and is characterized by 2 major defects: decreased secretion of insulin by the pancreas and resistance to the action of insulin in various tissues (muscle, liver and adipose). This results in impaired glucose uptake. The precise molecular mechanism of insulin resistance is not clearly understood, but studies have found that insufficiency in the post-insulin receptor intracellular signalling pathways are believed to play a major role (The Diabetes Control and Complications Trial Research Group, 1993).

Insulin resistance, which is commonly present prior to the onset of diabetes, is determined by a number of factors, including genetics, age, obesity and, later in the disease, hyperglycemia itself. In addition, excess visceral adiposity, dyslipidemia and hypertension often accompany insulin resistance.

Other clinical findings and manifestations may include impaired fibrinolysis, increased platelet aggregation, vascular inflammation, endothelial dysfunction and premature atherosclerosis (Cheng *et al.*, 2005).

1.2.2 Clinical Features of Type 2 Diabetes

In 1988 Reaven showed insulin resistance to be the central problem resulting in a clustering of cardiovascular risk factors including hypertension, glucose intolerance and hyperlipidaemia. More recently, the significance of central obesity has been recognised as the fourth characteristic of the "deadly quartet." For some, obesity has been identified as the major driving force behind the clustering risk factors (Wilson *et al.*, 1999).

Insulin resistance, a major cause of type 2 diabetes is a state in which a given concentration of insulin is associated with a subnormal glucose response due to the insensitivity of peripheral tissues, resulting in secondary hyperinsulinaemia (Lombard *et al.*, 2002).

Extensive research in this area has revealed that Impaired Fasting Glucose (IFG) and Impaired Glucose Tolerance (IGT) are common precursors of type 2 diabetes. The Adult Treatment Panel III of the National Cholesterol Education Program has identified metabolic syndrome as a constellation of lipid and non-lipid risk factors for coronary artery disease. The syndrome is characterized by insulin resistance, atherogenic dyslipidemia (high TG level, LDL cholesterol level, and small, dense LDL cholesterol particles), hypertension, abdominal obesity, and pro-thrombotic and pro-inflammatory states. Metabolic syndrome is diagnosed when three or more of the risk factors (Rao *et al.*, 2004).

Current knowledge suggests that the development of impaired glucose tolerance is initiated by insulin resistance and is worsened by the compensatory hyperinsulinemia. Others suggest that the progression from normal glucose tolerance to type 2 diabetes is characterized by dual defects of insulin resistance and insulin secretory defect caused by β-cell dysfunction. The progression to type 2 diabetes is further influenced by genetics and environmental or acquired factors such as a sedentary lifestyle and dietary habits that promote obesity.

Interestingly, in recent years, central obesity has become the leading risk factor and clinical feature of type 2 diabetes. Abdominal obesity is recognized to be the most prevalent manifestation of metabolic syndrome and is a marker of 'dysfunctional adipose tissue', and is of great importance in clinic diagnosis (Despres, 1996).

Insulin resistance is frequently observed in obese subjects and has been established as an independent risk factor for the development of both type 2 diabetes and coronary

artery disease. Although it is established that hyperinsulinemia, insulin resistance, and other obesity-related metabolic abnormalities are significantly associated with overall accumulation of fat in the body, there is now substantial evidence that the specific distribution of fat is important. Excessive accumulation of fat in the upper body's so-called truncal region, or central obesity, is a better predictor of morbidity than excess fat in the lower body, the so-called lower body segment obesity (Cefalu, 2001).

A perusal into the diagnostic guidelines such as, NCEP-ATP III, recommend doctors and clinicians to measure waist circumference rather than body mass index (BMI). This portrays and indicates the importance of abdominal obesity in conjunction to metabolic syndrome and insulin resistant states.

The subsistence of metabolic syndrome implies a shift from a pathophysiological concept based on metabolic abnormalities resulting from an insulin-resistant state to an epidemiological construct based on abdominal obesity and crude correlates of the features of insulin resistance (Despres, J., *et al.* 1996).

Clinically, measurement of waist circumference signifies the aggregate capacity of the actual amount of total and abdominal fat accumulation and is a crucial correlate of the complexities found among obese and overweight patients. (Kissebah *et al.*, 1982) Unlike the BMI, waist circumference is not profoundly influenced by height which makes it a better predictor of some cardiovascular diseases and more correlated with levels of abdominal visceral adipose tissue (Wei *et al.*, 1997).

Traditionally, BMI and Waist-to-Hip Ratio (WHR) are the most cited indices in literature as they approximate adiposity and fat distribution. Opinions vary as to whether or not waist circumference is as good a predictor as other anthropometric parameters. However, studies have supported that waist circumference is a better correlation with visceral adipose tissue and is a better predictor of cardiovascular disease than are BMI and WHR.

A study demonstrated that waist circumference is significantly and independently associated with increased risk of hypertension and diabetes in men and women of three African populations in widely contrasting environments. In an ecological analysis, waist circumference was positively correlated with blood pressure and fasting blood glucose ($p=0.05$) and increasing waist quartiles were significantly associated with higher risks of hypertension. A highly elevated risk of type 2 diabetes, 10-fold for Jamaican men and 23-fold for African-American women, was observed (Okosun *et al.*, 1998).

The lipid profile of patients with type 2 diabetes includes decreased HDL cholesterol levels and increased serum LDL cholesterol and triglyceride levels. This combination can be directly referred to atherogenic dyslipidaemia, which are commonly observed in patients with metabolic syndrome and type 2 diabetes (Alexander *et al.*, 2003). A significant proportion of diabetic patients (40 to 60%) have co-existing hypertension or dyslipidaemia (Lombard *et al.*, 2002).

Studies have shown several ways in which increased insulin concentrations could lead to hypertension- stimulating sodium and water absorption in the kidney, increasing

intracellular sodium and calcium in the vascular smooth muscle, causing hypertrophy and hyper-reactivity of vascular smooth muscle and centrally enhancing sympathetic nervous system activities (Bjorntorp *et al.*, 2000).

Furthermore, microalbuminuria, a common and independent cardiovascular risk factor that is predominantly but not exclusively present in diabetes, is perhaps one of the key early indications of the beginning of systemic vasculopathy and associated target organ damage to the heart and the kidneys. Microalbuminuria is defined as persistent elevation of albumin in the urine, of 30–300 mg/day. If present, microalbuminuria identifies patients at risk for early cardiovascular death, as well as those who would indeed have a significantly increased risk of microvascular complications, such as retinopathy and neuropathy (Varughese *et al.*, 2005).

As mentioned before, the aetiology of type 2 diabetes is currently unknown; however extensive research in this area during the last decade has revealed some likely predictors and abnormalities leading to this ambiguous disease.

Current knowledge suggests that the development of impaired glucose tolerance is initiated by insulin resistance and is worsened by the compensatory hyperinsulinemia. Others suggest that the progression from normal glucose tolerance to type 2 diabetes is characterized by dual defects of insulin resistance and insulin secretory defect caused by beta-cell dysfunction. The progression to type 2 diabetes is further influenced by genetics and environmental or acquired factors such as a sedentary lifestyle and dietary habits that promote obesity (Figure 1.2).

The first glucose abnormality that is detected is a rise in the postprandial glucose levels because of reduced first-phase insulin secretion. Over time, further decline in beta-cell function leads to elevation of the fasting glucose levels. Eventually, diabetes occurs, with more insulin secretory loss.

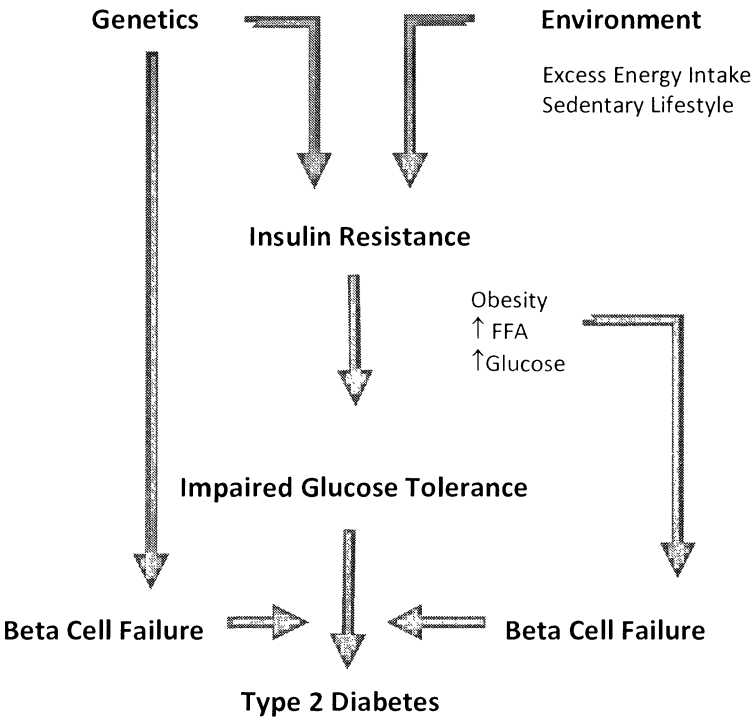


Figure 1.2 The progression to type 2 diabetes. Environmental factors and predisposition of genetic factors may lead to insulin resistance. This insulin resistant state over time causes the onset of impaired glucose tolerance which may further progress into type 2 diabetes. Beta cell dysfunction is also a common cause of diabetes which has been found to be exacerbated by genetics and insulin resistance.

Also, current data suggests that development of glucose intolerance or diabetes is initiated by insulin resistance and is worsened by the compensatory hyperinsulinemia.

The progression to type 2 diabetes is influenced by genetics and environmental or acquired factors such as a sedentary lifestyle and dietary habits that promote obesity.

1.2.3 Complications of Type 2 Diabetes

Chronic elevation of blood glucose level leads to damage of blood vessels. This is caused by endothelial cells lining the blood vessels taking in more glucose than normal, since they don't depend on insulin. This result in the onset of a large number of microvascular and macrovascular diseases in patients suffering from type 2 diabetes and other metabolic disorders.

Diabetes, especially type 2, remains the serious social and health problem related to the consequences of late complications. The initiation of microvascular diseases such as retinopathy, nephropathy and neuropathy, are all common to diabetic patients. On a more serious note, 85% of all cases of diabetes are patients with type 2 have been reported to be exposed mainly to macrovascular complications such as coronary heart disease, diabetic foot, and cerebrovascular disease (Strojek, 2003).

Microvascular Complications

Type 2 Diabetes is known to be associated with a high risk of developing vascular complications which can lead to premature death and/or disability mainly by increasing the risk of myocardial infarction, stroke and peripheral vascular disease. Patients with metabolic disorder are two to four times more likely to develop

cardiovascular disease than those in the general population and have two to five times greater risk of dying from these diseases (Al-Maskari, *et al.*, 2007).

Common microvascular complications include retinopathy, nephropathy and neuropathy. Non-proliferative retinopathy can be recognized by development of microaneurysms, venous loops, retinal haemorrhages, hard exudates and soft exudates. Proliferative retinopathy is defined as presence of new blood vessels with or without vitreous haemorrhage. Proliferative retinopathy ultimately represents a progression of non-proliferative retinopathy.

Diabetic nephropathy is defined as the presence of persistent proteinuria >0.5 gms/24 hours. Overt nephropathy is characterized by progressive decline in renal function resulting in end stage renal disease.

Neuropathy is a heterogeneous condition that is associated with nerve pathology. The condition is classified according to the nerves affected. The classification of neuropathy includes focal, diffuse, sensory, motor and autonomic neuropathy (Zimmerman, 2005).

To exemplify this problem studies have shown that type 2 diabetic patients taking insulin have a 40% prevalence of retinopathy at 5 years, while those on oral hypoglycaemic agents have 24% prevalence. By 15 -19 years of diabetes, the rates increase to 84% and 53% respectively (Klein, 1984).

Macrovasular Complications

Macrovascular complications are the major cause of morbidity and mortality in type 2 diabetes. Hyperglycaemia promotes the reaction of glucose with components of the arterial wall to form advanced glycation products. These products cross-link with collagen, thereby increasing arterial stiffness. In dyslipidaemia, increased levels of LDL cholesterol, consisting mostly of small dense particles, promote atherogenesis. Hypertension promotes the development and progression of vascular disease (Bate and Jerums, 2003).

Studies have revealed that diabetes increases the risk of myocardial infarction (MI) 2-fold in men and 4-fold in women, and many patients suffer other risk factors too. The risk of stroke in diabetic patients is double that of non-diabetic people, and the risk of peripheral vascular disease is 4 times that of people without diabetes. Subtle differences in the pathophysiology of atherosclerosis in patients with diabetes result in both earlier development and an added malignant course. Consequently, lipid abnormalities must be treated aggressively to lower the risk of serious atherosclerosis (Votey, 2007).

Coronary and cerebrovascular diseases are reported to be two to three times more common in those with type 2 diabetes, and their associated mortality is also increased. In the worldwide INTERHEART study of patients from 52 countries, diabetes accounted for 10% of the population attributable risk of MI. Transient ischaemic attacks are two to six times more common in patients and the risk of vascular dementia is also augmented. The risk of peripheral vascular disease (PVD) in

diabetics is four times higher and is known to increase the risk of lower limb amputation by 15 to 40 times compared to the general population. In Canada, 21% of diabetic patients were found to have heart disease and 25% of all cardiac surgery can be attributed to diabetes (Al-Maskari, *et al.*, 2007).

Peripheral neuropathy affects sensory, motor, and autonomic pathways. Sensory neuropathy deprives the patient of early warning signs of pain or pressure from footwear, from inadequate soft-tissue padding, or from infection. Furthermore, motor neuropathy leads to muscle weakness and intrinsic muscle atrophy in the hands and feet, causing diabetic foot syndrome. Patients with motor neuropathy can develop bunion, claw toe, and hammertoe deformities as a result of muscle imbalance and lose normal vascular tone and thermal regulation, often developing severe venous swelling. Severe tissue swelling ultimately leads to ulceration and infection (Pinzur, 2007).

1.3 Pathogenesis of Type 2 Diabetes

Research has identified that type 2 diabetes is a disease caused by both insulin resistance and an insulin secretory defect. This is the consequence of factors such as the impairment of glucose uptake in muscle and adipose tissue with endogenously secreted insulin, an increase in hepatic glucose production and an inadequate compensation of insulin secretion from pancreatic β-cells (Kahn and Mauvais-Jarvis, 2000).

Furthermore, many factors, of both predisposition and life style-related, have been found to increase the occurrence and possibility of type 2 diabetes.

Studies into the pathogenesis of this disease strongly suggest that genetic defects may cause the onset of this disease at the pre-receptor (abnormal insulin molecules, insulin antibodies), receptor (abnormal insulin receptor) or post-receptor (abnormal signal transduction leading to decreased GLUT4 translocation) levels.

Studies over the years has identified that defects in receptor function, insulin receptor-signal transduction pathway, glucose transport and phosphorylation, glycogen synthesis, glucose oxidation and the inhibition of adipose tissue lipolysis all strongly contribute to the progression to type 2 diabetes (DeFronzo, 2004).

In recent years it has been clinically observed and scientifically hypothesized that insulin resistance precedes the development of type 2 diabetes. There is often an association of high insulin levels, central obesity, cholesterol abnormalities (high LDL blood cholesterol levels and low HDL levels), high levels of TG, high blood pressure, and hypertension. This constellation of disease processes are commonly referred to as metabolic syndrome.

Insulin is released in the body to remove and use glucose from the blood and ultimately maintain glucose homeostasis. This is one way in which insulin controls the level of glucose in blood. Insulin binds to insulin receptors on the surface of the cells.

The resistance of the cells continues to increase over time. As long as the pancreas is able to produce enough insulin to overcome this resistance, blood glucose levels remain normal. When the pancreas can no longer produce enough insulin, the blood glucose levels begin to rise, initially after meals when glucose levels are at their

highest and more insulin is needed, but eventually in the fasting state too. At this point, type 2 diabetes is present. The insulin resistant state is also commonly referred to as the pre-diabetic state (Meigs *et al.*, 2003). This indicates that it is assumed that insulin resistance and type 2 diabetes are two metabolic illnesses that are closely linked, and there is increasing evidence that insulin resistance may form the foundation for type 2 diabetes to situate.

Current theories in the development of type 2 diabetes indicate that glucose and fasting insulin levels may be normal for a number of years before the development or progression to the disease. There is an increased risk of this illness in the presence of obesity and a family history of diabetes, insulin resistance (Cefalu, 2001).

In addition, the dramatic change in modern lifestyle of the world population, the easy accessibility and availability of foods, lack of exercise, stress causes weight gain, which thereby significantly increases the risk and susceptibility of incurring the disease (Lombard *et al.*, 2002).

Furthermore, obesity has been recognised to be a strong precursor for the onset of type 2 diabetes. Visceral fat mass, both mesenteric and abdominal fat deposits are closely related to obesity-associated pathogenesis. Probable mechanisms underlying this chief clinical feature include uncontrolled lipolysis with elevated circulation of FFA, with a specific increase in portal delivery of FFA. This results in an undesirable FFA flux to the liver from the portal vein causing an overall increase in hepatic FFA (Boden, 2006).

A decrease in adiponectin levels has been found to couple obesity and type 2 diabetes. In a previous study, adiponectin increased the ability of insulin to suppress glucose production in hepatocytes (Sun *et al.*, 2006). Also another study revealed that decrease in leptin, a protein secreted from adipose tissue which has been found cause obesity, was found to reduce glucose uptake and augment visceral adiposity (Barzilai *et al.*, 1997).

There is also growing evidence of the relationships between inflammation, insulin resistance and type 2 diabetes. Adipose tissue produces numerous pro-inflammatory molecules, such as tnf-α and IL-6 and as a result, the presence of these adipokines is increased.

Adipose-derived pro-inflammatory molecules are believed to induce systemic insulin resistance and to contribute to type 2 diabetes. Tnf-α has been found to directly mediate insulin resistance whereby multiple mechanisms have been suggested to account for these metabolic effects. Interleukin- 6 (IL-6) has been found to increase lipolysis and mitogen-activated protein 1 (MCP-1) was shown to impair adipocyte insulin sensitivity. With the onset of obesity, secretion of low levels of tnf-α by adipocytes is believed to promote MCP-1 production (Lau *et al.*, 2005).

With the prevalence of this relatively new disease rapidly escalating, the need to fully understand this illness has been reached, in order for optimal strategies to be created in relation to both treatment and prevention of this illness. This has lead to the establishment and facilitation of wide spread research at a global level, in an attempt to identify the causes, pathogenesis, and progression of the disease.

Studies into significant metabolic systems, bio-molecular structures, the actions of glucose and insulin on a cellular and metabolic level, has greatly contributed the knowledge of type 2 diabetes. Some of these include the biochemically pathways of insulin signalling, glucose and lipid metabolism.

1.3.1 Biochemical Pathways of Insulin Signalling

Insulin is a hormone released by pancreatic β-cells in response to elevated levels of nutrients in the blood. Insulin triggers the uptake of glucose, fatty acids and amino acids into adipose tissue, muscle and the liver and promotes the storage of these nutrients in the form of glycogen, lipids and protein respectively. Type 2 diabetes occurs when the body becomes resistant to the effects of insulin seemingly due to defects in the insulin signalling pathway (Saltiel and Pessin, 2000).

Insulin is an essential peptide hormone (Levine *et al.*, 1981), which binds to its receptor leading to the auto-phosphorylation of the β-subunits and the tyrosine phosphorylation of insulin receptor substrates (IRS). Activation of the insulin signalling pathway results in the translocation of the glucose transporter 4 (GLUT4) from cytoplasmic vesicles to the cell membrane (Bevan *et al.*, 2001). It has been identified that normal signalling through the insulin pathway is critical for the regulation of intracellular and blood glucose levels.

A key action of insulin is to stimulate glucose uptake into cells by inducing translocation of the glucose transporter, GLUT4, from intracellular storage to the

plasma membrane. Phosphoinositide 3-kinases (PI3-K) and AKT have been recognized to play a role in GLUT4 translocation (Lizcano *et al.*, 2002).

GLUT4 is highly expressed in adipose tissue and striated muscle with significantly lower levels of the GLUT1 isoform. In the basal state, GLUT4 cycles slowly between the plasma membrane and one or more intracellular compartments, with the vast majority of the transporter residing in vesicular compartments within the cell interior (Rea *et al.*, 1997).

Activation of the insulin receptor triggers a large increase in the rate of GLUT4 vesicle exocytosis which stimulates GLUT4. In contrast to GLUT4, GLUT1 is localized both to the plasma membrane and intracellular storage sites in the basal state but only displays a modest insulin-stimulated redistribution to the plasma membrane. Thus, the overall insulin-dependent shift in the cellular dynamics of GLUT4 vesicle trafficking results in a net increase of GLUT4 on the cell surface, thereby increasing the rate of glucose uptake.

Defects at the level of glucose uptake and phosphorylation characterize insulin resistance in skeletal muscle of type 2 diabetic patients.

Insulin normally increases uptake, storage, and oxidation of glucose in skeletal muscle. Insulin-stimulated glucose disposal is significantly reduced in insulin-resistant subjects, representing a defect in glucose transport, phosphorylation, and utilization or storage (Brady and Saltiel, 1999).

In patients with type 2 diabetes, defects in insulin-stimulated glucose metabolism in skeletal muscle have been attributed to impaired glucose transport, glycogen synthesis, and glycogen synthase activation. These defects may result from impaired insulin signal transduction. The molecular signalling mechanisms by which insulin regulates glucose uptake and storage is initiated by the binding of insulin to its specific cell-surface receptor, which results in receptor auto-phosphorylation and activation of insulin receptor tyrosine kinase. It has been suggested that IRS-1 is the major post-receptor component of the signalling machinery involved in mediating GLUT4 translocation, glucose transport, and glucose metabolism in human skeletal muscle (Storgaard *et al.*, 2001).

Furthermore, in insulin-resistant rodent skeletal muscle, the decrease in total PI3-K activity closely parallels the reduction in IRS-1 activity. Binding of IRS-1 to the regulatory subunit of PI3-K results in activation of the kinase and initiation of a phosphorylation cascade involving phosphoinositide-dependent protein kinase-1 and the downstream kinase AKT (Storgaard *et al.*, 2001).

A study using nuclear magnetic resonance in patients with type 2 diabetes, quantified glucose metabolism, pinpointing glucose transport as the primary site at which insulin action fails. As glucose uptake and metabolism are critical for the control of glycogen synthase activity by insulin, impaired glucose transport was identified to lead to a secondary reduction in glucose storage in muscle (Cline *et al.*,1999). When patients with Type 2 diabetes have been compared to control subjects in clinical experiments, such as the ones mentioned above, glucose transport has been found to be significantly impaired per unit of insulin.

Other studies reveal that agents that induce insulin resistance exploit phosphorylation-based negative-feedback control mechanisms, otherwise utilized by insulin itself, to uncouple the insulin receptor from its downstream effectors and thereby terminate insulin signal transduction (Zick, 2001).

1.3.2 Insulin Resistance and Glucose Metabolism

The major characteristic of insulin resistance that has been recognised is the inefficient glucose uptake and utilization in response to insulin stimulation in insulin-sensitive tissues. Under *in vivo* conditions, this is represented by a reduction in the insulin-stimulated storage of glucose as glycogen in both muscle and liver. It has been described that the primary mechanism in muscle appears to be a block in the glucose transport and phosphorylation step, and both genetic and environmental factors appear to induce this defect (Cefalu, 2001).

Insulin allows the transport of amino acids into cells, providing basic building blocks that are essential for cell functions and survival. Insulin has anabolic functions on cells, where it stimulates glucose oxidation, protein synthesis, RNA synthesis, fatty acid synthesis, and glycosaminoglycan synthesis. It also inhibits the manufacture of glucose by cells which can thereby prevent the breakdown of protein and amino acids.

When present in the blood stream, insulin prevents the mobilization of fat from stores, lowers blood sugar levels, and increases glycogen levels in muscles and liver. Insulin activates fat formation and helps to improve nitrogen retention (i.e., protein and

amino acid synthesis). Insulin is released by pancreatic β-cells into the blood stream when blood levels of glucose, amino acids or other hormones increase. Insulin is relatively short-lived in the blood stream and is broken down within minutes to hours.

Insulin forms a hormonal partnership with glucagon which generally has opposite effects on metabolism than on insulin. Insulin attaches to specific receptors on cell membranes, activating a series of events that require calcium, manganese, chromium, zinc and cyclic nucleotides. Cascades of events then occur inside the cells to support glucose and amino acid transport, and biosynthetic activities. Cells have the ability to change numbers and activity of insulin receptors, thus modulating how cells respond to insulin (Cefalu, 2001).

When insulin function is impaired, glucose cannot be transported into the cells fast enough to support regular cellular metabolism. The result is a form of cellular starvation in which fat is mobilized, and amino acids are broken down to manufacture glucose intra-cellularly. This results in ketone body formation, small organic acids, in addition to more anaerobic metabolism forming excess lactate which can acidify cells and the blood to harmful levels. Diabetes mellitus is the name given to a lack of insulin function (Cefalu, 2001).

Disordered glucose metabolism is commonly observed in obesity. Kinetic studies of glucose utilization in obese patients have demonstrated a reduction in the disappearance rate and uptake of glucose from the blood. There is now considerable evidence in support of the concept that the disordered glucose metabolism of obesity may be related to the presence of insulin resistance (Salans and Dougherty, 1971).

Several recent studies have focused attention on the role of the expanded adipose tissue in the insulin resistance of obese humans and experimental animals. Glucose metabolism and insulin sensitivity of adipose tissue have been shown to be influenced by the cellular character of the tissue. Basal glucose metabolism, in the absence of added insulin, was reported to be related to the number of fat cells in the tissue fragment; small and large adipose cells oxidize glucose to carbon dioxide at similar rates. In contrast, the ability of insulin to stimulate glucose oxidation in adipose tissue was found to be inversely related to the size of the fat cells in the tissue (lipid content per cell) (Salans and Dougherty, 1971).

A study also found that the inhibition of the PI3-K pathway with non-specific inhibitors has been seen to moderate insulin-stimulated glucose uptake in rat adipocytes, suggesting a potential role in translocation of GLUT4 – an insulin-sensitive transporter (Okada *et al.*, 1994).

Type 2 diabetes and obesity are common metabolic disorders characterized by resistance to the actions of insulin to stimulate skeletal muscle glucose disposal. Insulin-resistant muscle has defects at several steps of the insulin-signalling pathway, including decreases in insulin-stimulated insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation, and PI3-K activation.

Insulin resistance of type 2 diabetes appears to be caused in part by the presence of high levels of lipids in cells such as skeletal muscle where this would not normally be found. The existence of excess lipid storage in skeletal muscle cells interferes with

energy metabolism, thus impairing glucose oxidation and insulin response. Skeletal muscle is one of the primary glucose-consuming tissues, making it a key facet in insulin resistance. The increased risk of diabetes associated with obesity may be caused by increased lipid deposits in skeletal muscle and liver, creating insulin resistance.

Leptin is a peptide hormone secreted by adipose tissue that has been associated with numerous processes. One of the target tissues of leptin is the hypothalamus where it is able to regulate and control appetite, food intake and metabolism. Another leptin target is skeletal muscle, where activation of leptin signalling in skeletal muscle further activates the AMP-activated protein kinase (AMPK). This is known to play a key role in signalling in response to nutrients (Minokoshi *et al.*, 2002).

AMPK phosphorylates and inactivates the enzyme ACC, acetyl-CoA carboxylase. ACC catalyzes the production of malonyl-CoA from acetyl-CoA. Malonyl-CoA in turn is an inhibitor of the import of fatty acids into mitochondria by carnitine palmitoyl-transferase I for oxidation and energy production. In the presence of leptin, AMPK is activated, ACC is inhibited, and malonyl-CoA levels fall, increasing the oxidation of fatty acids and reducing the lipid content of cells. The reduced lipid content in skeletal muscle allows insulin signalling and glucose consumption to return to their normal levels, reducing insulin resistance (Winder, 2001).

Subjects with type 2 diabetes had the highest ratio of glycolytic to oxidative enzyme activities, with obese and lean non-diabetic subjects manifesting stepwise decrements in these ratios. This pattern for ratios of glycolytic to oxidative enzyme activities

emerged so clearly because there were oppositely directed patterns for glycolytic and oxidative enzyme activities. Type 2 diabetic subjects had the highest mean value for glycolytic activity and the lowest mean value for oxidative capacity, with obese non-diabetic subjects manifesting intermediate values for each pathway (Simoneau and Kelley, 1997).

The ratio between glycolytic and oxidative enzyme activities reflects proportionality between cytosolic and mitochondrial capacities for ATP resynthesis. During insulin-stimulated conditions, replenishment of ATP in skeletal muscle is nearly entirely derived from oxidative phosphorylation. A hindrance within this pathway, or an increased reliance on cytosolic ATP resynthesis, negatively influences the steps that require ATP, such as glycogen formation or trapping of transported glucose via its phosphorylation (Simoneau and Kelley, 1997).

Thus it seems plausible to assume that adjustments within glycolytic and oxidative pathways defects in insulin regulation of substrate transport and metabolism. Alterations in the glycolytic-to-oxidative ratio may dispose skeletal muscle toward lipid accumulation both in and around muscle fibres, thereby creating an environment for substrate antagonism, which in turn contributes to insulin resistance (Simoneau and Kelley, 1997).

An increase in glucose uptake in adipose tissue translates into increased re-esterification of free fatty acids (FFA) through augmented generation of glycerolphosphate from glycolysis. Increased FFA re-esterification reduces net lipolysis and FFA delivery to the circulation; in turn, a reduced supply of FFA to the

liver may be coupled with increased glucose uptake. Hepatic FFA oxidation promotes gluconeogenesis, thereby feeding indirect glycogen synthesis, and it might also directly regulate glycogen turnover, whereas circulating glucose provides the substrate for direct glycogen formation (Iozzo *et al.*, 2003).

According to a recent study, the ability of insulin to stimulate hepatic glucose uptake (HGU) was found to be impaired in patients with type 2 diabetes, which ultimately contributes to the development of hyperglycaemia. The defect appeared to involve the initial steps of glucose uptake and metabolism in the liver, eventually leading to a decrease in glycogen synthesis. These findings are in line with the notion that glucokinase is rate limiting for glucose entry into the liver and that genetic defects of glucokinase activity associated with human maturity onset diabetes of young lead to decreased HGU and glycogen synthesis (Iozzo *et al.*, 2003).

Interestingly, in diabetic animal models, defects of glucokinase activity and glycogen synthesis were partially reversed by normalization of glycemia, implicating glucose toxicity as a mechanism. Its relative contribution to the impairment of insulin-mediated HGU in human type 2 diabetes remains to be determined (Iozzo *et al.*, 2003).

A further study revealed that the suppression of insulin-stimulated glycolysis and a compensatory increase in glycogen synthesis (presumably arising from the glucose-fatty acid cycle) preceded decreases in insulin-stimulated glucose uptake in skeletal muscle during high-fat feeding. These findings suggest that the insulin resistance may develop as a secondary response to impaired intracellular glucose metabolism (Kim *et al.*, 1997).

More recently suggestions that local accumulation of fat metabolites inside skeletal muscle may activate a serine kinase cascade involving protein kinase C–θ (PKC-θ), leading to defects in insulin signalling and glucose transport in skeletal muscle, have been made. Studies have found increased expression of PKC in the skeletal muscles of insulin resistant animal models (Donnelly *et al.*, 1995).

Previous studies have shown that local accumulation of fat metabolites inside skeletal muscle may activate a serine kinase cascade, involving serine phosphorylation of IRS-1, leading to defects in insulin-mediated IRS-1–associated PI3K and glucose transport in skeletal muscle (Kim *et al.*, 2004).

In addition, TNF-α, a protein which has been originally demonstrated to be involved in various immune and inflammatory responses, has been also found to be over-expressed in adipose tissues of obese and insulin resistant subjects (Kern *et al.*, 1995). Animal studies have revealed that administration of TNF-α leads to insulin resistance (Lang *et al.*, 1992), and neutralisation of with a TNF receptor IgG restores insulin-stimulated glucose uptake in obese rats (Homamisligil *et al.*, 1994).

1.3.3 Insulin Resistance and Lipid Metabolism

Lipids are biological molecules that are insoluble in aqueous solutions and soluble in organic solvents. They are of physiological importance for humans due to their four major functions. They serve as structural components of biological membranes, supply energy reserves mainly in the form of triacylglycerols, lipids and lipid

derivatives serve as vitamins, hormones and lipophilic bile acids, aid in lipid solubilisation.

Lipid metabolism mainly involves fatty acid oxidation to produce energy and lipogenesis. Lipid metabolism is closely connected to the metabolism of carbohydrates which may be converted to fats. The impairment in insulin-mediated glucose metabolism affects both hepatic and peripheral tissues, primarily muscle, however, on the other hand, the effect of insulin on FFA metabolism in type 2 diabetes remains controversial (Groop *et al.*, 1989).

Abnormalities of triglyceride storage and lipolysis in insulin-sensitive tissues are early manifestations of insulin resistance and are detectable before the development of postprandial or fasting hyperglycemia. Increased FFA flux from adipose tissue to non-adipose tissue, resulting from abnormalities of fat metabolism, contributes to and amplifies many of the fundamental metabolic features that are characteristic of the insulin resistance syndrome and type 2 diabetes.

Adipose tissue storage, release of fatty acids, and its control by insulin are grossly abnormal in insulin resistant states. In the post-absorptive period, basal lipolysis is elevated and suppression by insulin diminished. In the postprandial period, there is likely to be a net diversion of fat away from adipose tissue depots and toward non-adipose tissues. FFA efflux from an enlarged and lipolytically active visceral fat depot plays a major role in the elevation of fatty acids, which are then free to exert their biological effects in non-adipose tissues (Katayama *et al.*, 2006).

Studies have suggested that impaired lipid metabolism plays an important role in the progression from normal glucose tolerance to fasting hyperglycemia and conversion to type 2 diabetes in insulin resistant individuals. Adverse metabolic consequences of increased FFA flux include dyslipidemia, hepatic steatosis, impaired glucose metabolism and insulin sensitivity in muscle and liver, diminished insulin clearance, aggravating peripheral tissue hyperinsulinemia, and impaired pancreatic ß-cell function (Katayama *et al.*, 2006).

There is evidence that postprandial FFA levels may also be higher in obese, insulin-resistant individuals and in subjects with type 2 diabetes (Lewis *et al.*, 1991).

Also prospective epidemiological studies have suggested that elevated plasma FFA is an independent predictor of progression to type 2 diabetes in Caucasians and Pima Indians (Knowler *et al.*, 1990). Fasting plasma FFAs have generally been found to be elevated when examined in large, well-characterized populations of individuals with insulin resistance (Baldeweg *et al.*, 2000).

A study also found that subjects displayed a number of disturbances in FFA and total lipid metabolism. At physiologic insulin concentrations there was an impaired suppression of both plasma FFA concentration and turnover in the diabetic subjects compared with the control subjects. Also suppression of plasma FFA concentrations and total FFA flux were found to be extremely sensitive to small changes in plasma insulin (Groop *et al.*, 1989).

Excessive FFA oxidation leads to the intracellular accumulation of acetyl CoA which is a potent inhibitor of pyruvate dehydrogenase (PDH), increases the NADH/NAD ratio causing a slowing of the Krebs cycle and further results in the accumulation of citrate, a powerful inhibitor of phosphofructokinase (PFK). Inhibition of PFK leads to the accumulation of glucose-6-phosphate which in turn inhibits hexokinase II. The block in glucose phosphorylation causes a build up of intracellular free glucose which restrains glucose transport into the cell via the GLUT4 transporter. The decrease in glucose transport is suggested to account for the impairment in glycogen synthesis, although a direct inhibitory effect of fatty acyl CoAs on glycogen synthase also has been demonstrated (DeFronzo *et al.*, 2007).

Research found that defect in insulin suppression of FFAs in subjects with IGT and type 2 diabetes compared with subjects with normal glucose tolerance. This supports findings from earlier studies that insulin resistance with IGT encompasses both resistance to insulin-stimulated glucose uptake and FFA suppression (Reaven and Greenfield, 1981).

Insulin clamp studies investigating mechanisms for impaired FFA suppression among persons with type 2 diabetes have shown defects in several areas of FFA metabolism: FFA turnover, non-oxidative FFA disposal (an estimate of FFA re-esterification), and FFA oxidation (Laws *et al.*, 1997).

In one study, absolute non-oxidative and oxidative FFA metabolism were actually higher in type 2 diabetic patients than control subjects and were strongly correlated with plasma FFA concentrations (Groop *et al.*, 1989). This suggests that the major

defect in FFA metabolism in type 2 diabetes is impaired insulin suppression of lipolysis (production) rather than impaired disposal (Laws *et al.*, 1997).

Another major quantitative change associated with the insulin resistance and type 2 diabetes is an elevation in triglyceride-rich lipoproteins, often accompanied by a decreased HDL cholesterol level. Thus, dyslipidemia (by its association with insulin resistance) may precede the diagnosis of type 2 diabetes. Although LDL cholesterol levels may be comparable to those seen in the general population, LDL compositional differences may make these particles more atherogenic (Cefalu, 2001).

Insulin resistance has also been associated with this predominance of small dense LDL particles. It is the small dense LDL particle that has been suggested to be the more atherogenic LDL. Further studies have suggested that insulin sensitizers (e.g., thiazolidinediones) may favourably improve LDL size. Though it has been made known that the ratio of LDL to HDL cholesterol may not alter with treatment with insulin sensitizers, the qualitative properties of LDL may change with their use: large LDL is increased and small dense LDL is decreased (Tack *et al.*, 1998).

1.3.4 Beta Cell Dysfunction

Insulin resistance and β-cell dysfunction are two interrelated metabolic abnormalities that are central to the aetiology of type 2 diabetes. It results from an inadequate mass of functional beta cells and such inadequacy could result from loss of β-cell due to immune assault or the inability to compensate for insulin resistance (Georgia, 2006). The ability to maintain normoglycaemia is primarily dependant on two factors, the

capacity of the pancreatic β-cell to secrete insulin (β-cell function) and the sensitivity of glucose utilising tissues to the prevailing insulin concentration (insulin sensitivity).

Studies have revealed that rising blood glucose levels were mirrored closely by an ongoing fall in β-cell function and a number of have been implicated in driving β-cell dysfunction, such as amyloidosis, defects in the leptin signalling pathway, elevated levels of blood glucose and free fatty acids. Thus, by studying the mechanisms that regulate β-cell, may be the key to understanding both the pathogenesis of type 2 diabetes and for developing new therapies.

Initiation of the insulin response depends upon the trans-membranous transport of glucose and coupling of glucose to the glucose sensor. The glucose/glucose sensor complex then induces an increase in glucokinase by stabilizing the protein and impairing its degradation. The induction of glucokinase serves as the first step in linking intermediary metabolism with the insulin secretory apparatus. Glucose transport in ß-cells of type 2 diabetes patients appears to be greatly reduced, thus shifting the control point for insulin secretion from glucokinase to the glucose transport system (Leahy, 1991).

As the disease progress to its later stage, the second phase release of newly synthesized insulin is impaired, an effect that can be reversed, in part at least in some patients, by restoring strict control of glycemia. This secondary phenomenon, termed desensitization or ß-cell glucotoxicity, is the result of a paradoxical inhibitory effect of glucose upon insulin release and may be attributable to the accumulation of glycogen within the ß-cell as a result of sustained hyperglycemia (Malaisse, 1996). The

transcription factor Pax6 has been identified to be an important regulator of α and β cell function. These cell types produce and release glucagon and insulin, which are equally regulated in response to glucose. Gel shifts and chromatin immuno-precipitations were used to find that at low concentrations of glucose and enhanced amounts of H2O2 Pax6 acts as a negative regulator of insulin gene expression (Wolf, 2000).

An animal study has found that β-cell damage occurred in nutritionally-induced type 2 diabetic sand rats. This animal model develops into hyperglycaemia, initially hyperinsulinaemia and later hypoinsulinaemia accompanied by insulin resistance when placed for three weeks in a high rate diet. The study also found that mechanisms leading to β-cell damage during the development of hyperglycaemia in the animal model were not mediated by induction of pro-inflammatory cytokines and a resulting up-regulation of iNOS induced by IL-1B (Jorns, 2001).

Another animal study has shown that cell cycle regulator p27, is crucial in establishing β-cell. It found that disabling p27 allows terminally differentiated β-cell to re-enter the cell cycle and proliferate, and as a consequence excess beta cells are generated. The expanded β-cell mass was accompanied by increased insulin secretion; however the mice were glucose intolerant as these mice were insulin insensitive (Georgia, 2006).

Also, a cell study has recognised that lipid perturbation and accumulation of TG in pancreatic β-cell are associated with impaired insulin secretion. Hormone-sensitive lipase (HSL) is expressed and active in beta cells and may be involved in regulating

the turn-over of the TG pool. Through the analysis of palmitate oxidation and triglyceride content in cells, the β-cell line over-expressing HSL was found to show resistance to conditions of high glucose, which manifested as a higher maintained proliferation rate and a retained glucose-stimulated insulin secretion function after long time exposure to high levels of glucose (Klint, 1999).

1.3.5 Glucose Transport System

The insulin resistance of type 2 diabetes mellitus has been found to be due to both receptor and post-receptor defects of *in vivo* insulin action, with the post-receptor defect being the predominant abnormality. Diminished glucose transport has been further found in adipocytes from patients with type 2 diabetes, suggesting that decreased cellular glucose transport activity may be responsible in part for the *in vivo* post receptor defect observed in these patients (Scarlett *et al.*, 1983).

Glucose transporter 1 (GLUT1) is most plentiful in the blood brain barrier micro-vessels of rodents and ruminants, and in humans, it is a major protein of the erythrocyte membrane.

Glucose transporter 2 (GLUT2) is a gene product expressed in the tissues specialized in glucose export to circulation, such as the liver, kidney, and small intestine, and is also present in the pancreatic β-cell, seemingly fulfilling the role of being a component of the glucose sensor. Glucose transporter 3 (GLUT3) is present in neuronal cells of the rat and in low levels in several tissues in the human. Finally, Glucose transporter 4 (GLUT4), primarily expressed in the muscle and fat, has been named the insulin-responsive glucose transporter.

GLUT4

Glucose uptake, which is mediated by the insulin-responsive glucose GLUT4, is the rate-limiting step in glucose utilization in adipocytes. It is the major facilitative glucose transporter isoform in skeletal muscle, which is redistributed from intracellular stores to the sarcolemma and transverse tubule system on stimulation by insulin, ischemia or exercise (Kotani, 2004).

Glucose transport into skeletal muscle occurs by two membrane proteins, the GLUT1 and GLUT4 gene products. It has been identified that because glucose transport across the cell membrane is a key step in glucose utilisation, it has been surmised that alterations in transmembrane glucose transport may be an essential part of the defect in glucose utilisation seen in diabetic states.

Previous studies have demonstrated that insulin-stimulated GLUT4 vesicle translocation to the plasma membrane is critical for homoeostasis (Cushman and Wardzala, 1980). Expression and localization of GLUT4 are greatly regulated in insulin-responsive tissues, such as skeletal muscle and adipose tissue. Insulin-stimulated glucose uptake requires the activation of several signalling pathways to mediate the translocation of GLUT4 vesicles to the plasma membrane. The binding of insulin to its receptor activates the tyrosine kinase activity of the receptor and results in auto-phosphorylation and subsequent phosphorylation of IRS proteins (White, 1997).

The IRS proteins instigate a cascade of events that results in the translocation of GLUT4 vesicles to the plasma membrane and thus glucose uptake into the cell. Research has found that one of the critical targets of IRS proteins is PI3-K, which activates downstream targets, such as the serine/threonine protein kinase Akt/protein kinase B (Taha and Klip, 1999).

Early studies have revealed by subcellular fractionation and Western blotting with isoform-specific antibodies, that isolated plasma membranes contain GLUT4 and GLUT1 proteins and found an intracellular fraction different from sarcoplasmic reticulum contains only GLUT4 transporters (Dimitrakoudis, 1992).

Yki-Jarvinen and Rossetti *et al.* have previously demonstrated that in type 2 diabetic muscle, the insulin resistant state is accompanied by decrease in glucose-6-phospate and in glucose-derived glycogen, suggesting that before glucose transport and hexokinase activity is responsible for the defect. Thus suggestions were raised that the glucose transport system may not only be a key regulated step, but also be a step defective in diabetes.

Studies have shown that resistance to insulin-stimulated glucose transport in adipose tissue and skeletal muscle is one of the earliest defects detected in insulin-resistant states. With the development of insulin resistance, GLUT4 expression is down-regulated selectively in adipose tissue but not in skeletal muscle. Down-regulation of GLUT4 expression in adipose tissue is an almost universal feature of insulin-resistant states, including obesity, type 2 diabetes and the metabolic syndrome (Yang *et al.*, 2005).

An *in vivo* study into the translocation of GLUT4 found that insulin induced translocation of the protein to the plasma membrane in 3T3-L1 adipocyte cell line. The maximum level of surface GLUT4 was reached after 6 min of 200 nM insulin stimulation, and ultimately represented a five-fold increase above the observed in non-stimulated cells. The study also strongly suggested that insulin regulates GLUT4 trafficking via a secretory or release mechanism rather than by mobilizing the entire intracellular pool at discrete kinetic rates. At different doses of insulin, there was a progressive increase in the total amount of GLUT4 introduced into the cell surface recycling pathway rather than a change in the rate of movement of a defined GLUT4 pool (Govers *et al.*, 2004).

Also, the hypothesis that over expression of GLUT4 in transgenic mice improves whole-body insulin action has been well supported by a number of recent studies (Kotani *et al.*, 2004). For instance, a study with the use of myosin light chain (MLC) promoter- driven MLC-GLUT4 transgene to achieve selective over expression of GLUT4 in muscle increased 2.5 fold in *in vivo* insulin- stimulated 2-deoxyglucose uptake (Kotani *et al.*, 2004).

It has been previously hypothesised that altered regulation of GLUT4 is responsible for the decrease in glucose uptake in the adipose tissue and muscles of diabetic animals and humans. Under insulin-stimulated conditions, the glucose uptake in the soleus and extensor digitorum muscles of hyperinsulinemic GLUT4 +/- mice was decreased 38% and 34% respectively. Coinciding with this increase in glucose uptake

there was also a 46% and 26% decrease in GLUT4 content of soleus and extensor digitorum longus muscles (Stenbit *et al.*, 1997).

The deletion of both alleles did not produce a diabetic phenotype, although many abnormalities including growth retardation, severely reduced adipose tissue, hypertrophic hearts, and decreased insulin sensitivity were measured by insulin tolerance test. However they further discovered that the deletion of one allele resulted in the development of an overt diabetic phenotype. GLUT4+/- mice became hyperinsulinemic and eventually became hyperglycaemic (Stenbit *et al.*, 1997).

Defects in skeletal muscle GLUT4 is a major regulator of muscle glucose metabolism, and further research in this area to find a new mode of therapy based on increased GLUT4 expression or activity may be effective for type 2 diabetes (Kotani *et al.*, 2004).

1.4 Current Therapies for Type 2 Diabetes

In response to the size of the growing problem, efforts to identify and develop new pharmaceutical agents for type 2 diabetes have become crucial in the recent years. This has resulted in the successful establishment of conventional therapies and treatment options for patients globally. Currently, there are five main classes of oral agents: sulfonylureas, meglitinides, metformin, thiazolidinediones, and alpha-glucosidase inhibitors.

The actions of sulfonylureas and meglitinides involve the stimulation of insulin secretion; metformin suppresses hepatic glucose production; thiazolidinediones target peripheral tissue insulin resistance; the alpha- glucosidase inhibitors that inhibit complex carbohydrate breakdown in the gut (Evans and Rushakoff, 2007).

1.5 Oral Anti-Diabetic Drugs

1.5.1 Current Oral Anti-Diabetic Drugs

Sulfonylureas

The first class of drugs against diabetes type 2 developed, and which are still in use, are the sulfonylureas drugs like Glucotrol® and Micronase®. Sulfonylureas stimulate the pancreas to make more insulin. They promote insulin release by binding to adenosine triphosphate sensitive potassium channels in pancreatic cells, thereby keeping these channels closed.

In a retrospective study, mortality risk showed a dose-dependent rise, which further suggests a causal link to adverse cardiac events for sulfonylureas. Dose-response relation was found between sulfonylurea drugs and mortality in type 2 diabetes mellitus: a population-based cohort study (Simpson *et al.*, 2006).

A follow up study showed 1,503 deaths during the study period, of which 372 (about 25%) were attributable to an acute ischemic event. First-generation sulfonylurea users had the highest mortality (67.6 deaths per 1,000 person-years), compared with Diabeta users (61.4 deaths per 1,000 person-years) and metformin users (39.6 deaths per 1,000 person-years). Compared with the low-dose patients, a greater risk of death

was found in the high-dose patients receiving first-generation sulfonylureas and Diabeta (an oral blood-glucose-lowering drug of the sulfonylurea class) but not metformin (Simpson *et al.*, 2006).

The study also found an increased risk of death from ischemic event in the high-dose patients receiving first-generation sulfonylureas, although it was not statistically significant. A significant association was found for Diabeta and a slight and non-significant association was found for metformin. One likely mechanism to explain the link to cardiovascular risk is that this class of drugs also binds to the same channels in cardiac myocytes and smooth-muscle cells, interfering with these cells' ability to withstand brief periods of ischemia (Simpson *et al.*, 2006).

Biguanides

Biguanides, such as Metformin, has been utilized in the treatment of type 2 diabetes for nearly 50 years. It acts as an insulin-sensitising agent, lowering fasting plasma insulin concentrations by inducing better peripheral uptake of glucose, as well as decreasing hepatic glucose output.

More recently, the use of metformin has broadened, with evidence for its benefit in other insulin-resistant states. In polycystic ovary syndrome, it has been found to decreases insulin resistance, restore menstruation, facilitate conception, and reduce the rate of first-trimester spontaneous abortion (Glueck *et al.*, 2002).

It is a drug that acts on the liver to suppress gluconeogenesis by potentiating the effect of insulin or by reducing hepatic extraction of substrates and glycogenolysis, and it decreases the activity of glucose-6-phosphatase. It has been found to also delay the progression to type 2 diabetes in people with impaired glucose tolerance. In vitro experiments have shown that it also enhances insulin binding to hepatocytes of insulin resistant mice, and stimulates tyrosine kinase activity. Glucose transport and glycogen synthesis represent post-receptor targets of this drug (Iozzo *et al.*, 2003).

Thiazolidinediones

Rosiglitazone, one of the thiazolidinedione (TZD) class of antidiabetic drugs, is an agonist for peroxisome proliferator activated receptor-γ (PPARγ), a nuclear receptor predominantly expressed in adipose tissue. It increases insulin sensitivity both in the liver and peripheral tissues (Yki-Jarvinen, 2004).

TZDs act by binding to PPARs which are group of receptor molecules inside the cell nucleus- PPARγ. PPARγ activation stimulates a decrease in insulin resistance, modification of adipocyte differentiation, inhibition of vascular endothelial growth factor-induced angiogenesis, decrease in leptin levels (leading to increased appetite) and an increase in adiponectin levels.

A study published in 2006 found that Rosiglitazone altered expression of genes involved in fatty acid synthesis and storage, structural proteins, macrophage-related and inflammation-related genes and genes involved in glucose transport and insulin sensitivity. TDZ-induced changes in expression of genes involved in fatty acid uptake,

metabolism, and triacylglycerol synthesis in rodents and cell lines matched results they and others attained from human studies (Kolak *et al.*, 2006).

Alpha-Glucosidase Inhibitors

The ingestion of Alpha-glucosidase inhibitors (AGIs) results in delayed glucose absorption and lowers blood sugars following meals, and is therefore used for patients with Type 2 diabetes. The group of drugs blocks enzymes in the digestive tract from breaking down complex carbohydrates from food into glucose. This slows the absorption of sugars into the small intestine and bloodstream, keeping glucose levels in normal range.

Acarbose, an AGI has been shown to competitively inhibit the intestinal digestion of starch as well as sucrose. Addition of Acarbose to a starch or sucrose load reduces the postprandial glucose and insulin responses in dose dependent manner. The lower postprandial insulin levels are considered to form the basis for its potential use in the treatment of carbohydrate-dependent lipid disorders, diabetes and obesity. Numerous studies in animals have demonstrated the efficacy of AGIs in such conditions (O'Dea and Turton, 1985).

However, the AGIs, when compared to the other diabetic medications, have the least overall effect on glycosylated haemoglobin (HbA1c) levels when used alone. The primary use for the AGIs is to lower blood sugar levels after eating. The AGIs have only a minimal effect on fasting blood sugar levels. Compared to sulphonylurea, AGIs have less favourable effects with respect to glycemic control and adverse effects but

they lower fasting and post-load insulin levels compared to Sulfonylureas (Van de Laar *et al.*, 2005).

An advantage to this drug group is that they are typically not associated with weight gain (some of the other diabetes medications are associated with weight gain). Studies have shown that by lowering after meal blood sugar levels, the AGIs may be useful in preventing development of diabetic complications and death due to heart-related disorders that have been associated with elevations in blood sugars after meals.

1.5.2 Side Effects of Oral Anti-Diabetic Drugs

Over the years, systemic reviews and studies of current medications used to treat type 2 diabetes have revealed undesirable and life-threatening side effects such as weight gain, water retention, acidosis and cardiovascular complications.

Metformin, a commonly used biguanide derivative, has been used as an oral anti-diabetic drug for almost 50 years. It has been widely used as an insulin-sensitizing agent, lowering fasting plasma concentrations by inducing greater peripheral uptake of glucose, as well as decreasing hepatic glucose uptake. However in the recent years, possible side effects form this drug class such as nausea, appetite loss, diarrhoea, abdominal gas and metallic taste and more importantly, warnings of life-threatening lactic acidosis have been reported. Traditionally, this complication has been suspected as secondary to an accumulation of the drug. Further investigation has confirmed that

lactic acidosis occurs in one to five cases per 100 000 subjects, with mortality reported in up to 50% of these cases (Brown *et al.*, 2003).

Also, research into the drug has revealed that not only life-threatening lactic acidosis may occur, but patients may be at risk from renal impairment, with doses over 2 g per day. The estimated prevalence of lactic acidosis has been shown to be one to five cases per 100 000, with mortality in reported cases up to 50% (Brown *et al.*, 2003).

In 1998, the United Kingdom Prospective Diabetes Study reported that, in overweight patients with type 2 diabetes, treatment with metformin compared with di*et al*one resulted in statistically significant absolute risk reductions (ARRs) in all-cause mortality (ARR, 7%), diabetes-related deaths (ARR, 5%), any diabetes-related endpoint (ARR, 10%), and macrovascular disease (myocardial infarction, sudden death, angina, stroke, peripheral vascular disease) (UKPDS Group, 1998).

Between 1985 and 2001, there were 48 reported cases of lactic acidosis with the use of metformin by the Australian Adverse Drug Reactions Advisory Committee (ADRAC). To add the severity of this side effect, 15 of these cases, resulted in death. In 35 of the 48 cases, known risk factors were identified. Over the past 4 years, the average number of cases reported to ADRAC has been six per annum. In Australia in 2002–2003, about 200 000 patients were prescribed metformin, giving a reported frequency of lactic acidosis of one in 30 000 (Brown *et al.*, 1998).

Metformin has been found to be excreted unchanged in urine, with the half-life prolonged and renal clearance decreased in proportion to decrease in creatinine

clearance. There is a high risk that this may occur in chronic renal impairment, dehydration, shock, and intravascular administration of iodinated contrast agents, all of which have the potential to alter renal function. Tissue hypoxia may trigger this and sepsis, acute MI, pulmonary embolism, cardiac failure and chronic liver disease are conditions that may predispose to altered renal function. (Nisbet *et al.*, 2004) A large number of efficacy studies on thiazolidinediones (TZD) like Avandia® and Actos®, have shown various side effects too. They include anaemia, headaches, muscle aches, tooth aches, sore throat, increased upper respiratory tract infection rate, water retention, oedema, weight gain, heart problems and liver injury.

Weight gain has been widely observed in clinical trials involving TDZs, and the magnitude of weight gain is generally correlated with the degree of improvement in glycemic control (Einhorn *et al.*, 2000).

In addition to the weight gain associated with improved glycemic control, several other mechanisms may also contribute to weight gain, including fluid retention and an increase in overall adipogenesis. Stimulation of PPAR receptors on adipocytes initiates cell division, leading to an increase in the number of small fat cells in subcutaneous fat depots. In addition, activation of PPAR receptors induces numerous genes involved in lipogenesis and the inhibition of lipolysis. These effects lead to an increase in subcutaneous fat mass and are responsible, in part, for the reduction in plasma FFA concentration. Hence, a reduction in plasma FFA concentration and an increase in body weight reflect improved insulin action, which correlates with improvements in glycemic control (Kendall *et al.*, 2006).

Fluid retention leading to the development of peripheral oedema is a well-known effect of current PPAR activators. Oedema has been reported in up to 16% of patients treated with TZD monotherapy. PPAR_activators can cause fluid retention by promoting solute and water retention in the renal collecting duct. PPAR agonists also possess calcium channel, blocking activity and stimulate the release of nitric oxide, both of which are associated with oedema formation in 5–10% of individuals. PPAR agonists have also been shown to cause vascular leak in animals. Finally, it has been suggested that PPAR agonists enhance insulin-mediated sodium re-absorption by the kidney (Kendall *et al.*, 2006).

A meta-analysis was performed to assess the overall risk for developing oedema secondary to TZD and results demonstrated at least a two-fold increase in the risk for developing oedema with a TZD agent. In addition it was noted that factors such as the presence of hypertension, left ventricular hypertrophy or coronary artery disease may lead to higher rates of TZD induced oedema reported in this study. These conditions are commonly seen in patients with long-term or advanced diabetes and are more likely to be started on a TZD agent (Singh, 2008).

Gastro-intestinal side effects often limit the use of AGI. These include stomach pain, diarrhoea, and flatulence or gas.

Systemic reviews and other studies have revealed the side effects of sulfonylureas too. These include low blood sugar levels, water retention, oedema, weight gain, heart complications and allergic reactions. Low blood sugar levels have has been found to

more common if the drug is administered with the presence of alcohol. Insulin secretagogues, such as sulfonylurea has been identified to have the ability to make normoglycaemia in the early stages of diabetes (Raskin, 2007). However, most of these drugs stimulate insulin secretion with and without glucose challenge by closing ATP-sensitive potassium channels in β-cells. As a result, they increase secretion without glucose loading, resulting in exhausting and damaging β-cells and frequent hypoglycaemia. Eventually, they have been found to exacerbate the symptoms of diabetes (Ko *et al.*, 2005).

1.5.3 Novel Treatments and New Approaches

The development of new oral therapeutic agents for type 2 diabetes is being encouraged and much required in our present society. The need for continuous expansion, intensive research and developmental efforts has been emphasized due to the increasing prevalence of the disease and related co-morbidities.

There is no doubt that our present society is in need for an effective treatment of type 2 diabetes, by both pharmacologic and non-pharmacologic means, and in addition, in the hope that type 2 diabetes can be prevented or delayed (Cefalu, 2001).

A growing understanding of the pathophysiology of the disease needs to be coupled with identification and validation of new pharmacological targets. These targets include receptors and enzymes that: enhance glucose-stimulated insulin secretion, suppress hepatic glucose production, increase skeletal muscle glucose transport and

utilization, increase insulin sensitivity and intracellular insulin signalling, and reduce circulating and intracellular lipids (Evans and Rushakoff, 2007).

1.5.4 Chinese Herbal Medicines (CHM)

Type 2 diabetes has reached epidemic proportions in the US and worldwide, and by studying current lifestyle and dietary trends, is projected to increase dramatically in the fore-coming years. In addition, the prevalence of insulin resistance, a major causative factor in the early development of type 2 diabetes and an independent risk factor for cardiovascular disease and the metabolic syndrome as outlined above, is becoming even more widespread. This situation is further exacerbated by obesity, a major risk for type 2 diabetes.

Although pharmacological options for the management of type 2 diabetes has been increasing, and will continue to do so in the future, not all have been found to be beneficial to patients. In efficacy studies and reviews of common anti-diabetic drugs, many side effects were observed.

In addition, the cost of prescription medications may exceed the financial capacity of an increasing number of older citizens and people with inadequate health insurance, and those living in poverty. Moreover, through studies of certain ethnic groups who are at risk for developing diabetes such as Asians, Hispanics, and Native Americans), come from cultures with who highly regard, have a long history of the use of herbal medicines and have better access to natural medications than prescription drugs.

Furthermore, a preponderant number of diabetic patients who are being treated with prescription drugs, are unable to achieve the current American Diabetes Association-recommended goal of HbA1c <7%. Thus there is a need to identify and evaluate adjunct therapies that are safe, efficacious and cost effective through research (Evans, 2007).

In Asia, there have been records of the use of traditional herbs in lowering blood glucose levels for thousands of years. More recently, especially in China, products are being sold commercially, in numerous dosage forms such as tablets, capsules, granules, injectables, oral liquids, sprays and dripping pills. In view of the increasing interest in the use and modernization of herbal products and the large markets in Asian countries, there is a need to study the chemistry, pharmacology, pharmacokinetics, and clinical efficacy of these herbs (Zhou *et al.*, 2005).

1.5.5 Implementation of CHM in Conventional Medication

The use of natural products for metabolic illnesses has yet to be explored in depth, despite the fact that a number of modern oral hyperglycaemia reducing agents being derived from natural plant products.

This emphasises the importance of traditional herbs in the treatment of metabolic illnesses such as insulin resistance and type 2 diabetes.

Research has suggested that the use of Galega officinalis, a natural herb that is contained in metformin, can enlarge the Islets of Langerhans in the pancreas, which

are responsible for the production on insulin. Its active constituents galegine (isoamyleneguanidine), 4-hydroxyderivative and peganine Flavonoids and Saponins and substance guanidine has been found to reduce blood sugar levels (Sterne, 1969).

Although several traditional medicines have been reported to have anti-diabetic effects, the molecular targets of such compounds have not been revealed, and a careful analysis of their actions *in vitro* and *in vivo* has not been undertaken.

For example, recent study showed that in vivo administration of berberine has insulin sensitizing, and weight and lipid-lowering properties in both db/db mice and high-fat fed rats. They also found that berberine acutely stimulated AMPK activity in both myotubes and adipocytes *in vivo*, contributing to enhance GLUT4 translocation in myotubes and reduced lipid mass in adipocytes (Lee, 2006).

Based on the data presented, berberine and metformin share a number of features in common. Metformin causes weight reduction, improved insulin sensitivity, and lipid loweing in both human and animal models of insulin resistance. A major mode of action of metformin is activation of AMPK, particularly in liver. The latter effect is particularly of interest to the present studies as one of the major disadvantages of TZDs is that while they lead to improved insulin sensitivity and lipid lowering, they also lead to increased adiposity due to their stimulatory effects on adipocyte differentiation.

1.5.6 Monotherapy of Chinese Herbal Medicines

Salvia miltiorrhiza - Dan Shen

Dan Shen, with the pharmacological name Radix Salvia Miltiorrhizae, has been widely used in China, Japan, US and other European countries for treating cardiovascular and cerebrovascular diseases. Chemical constituents of the herb have been studied from as early as 1930s, with the main focus on lipophilic compounds. More than 30 diterpene compounds have been separated and identified from Dan Shen. Some examples of these are diterpene chinone compounds of Tanshinone group such as tanshinone I, IIA< IIB and cryptotanshinone (Lee, 2006).

In vitro and *in vivo* studies have suggested than Dan Shen has the chemical properties to improve microcirculation, dilate coronary arteries, inhibit platelet aggregation, oxidative modification of LDL, leading to the prevention of the uptake of LDL by cultured macrophages.

In studies, a greater reduction of cholesterol and triglycerides was found following Fufang Danshen Dripping Pill treatment of angina pectoris compared to isosorbide dinitrate (ISDN). The study also mentions that at least 4 other studies also showed that total cholesterol, TG and LDL cholesterol levels were significantly reduced by 28.3%, 34.3% and 29.9% respectively. In addition HDL cholesterol level was raised by 33.2% (Zhou, L *et al.*, 2005).

In another study comparing the Dan Shen injection with ISDN injection, the level of soluble intercellular adhesion molecule-1 and interleukin-6 was found to be lower in the Dan Shen group (Lee *et al.*, 2006).

Rhizoma Coptidis - Huang Lian

Huang Lian (Rhizoma Coptidis), is widely used in traditional Chinese medicine as an anti-microbial and anti-tumour agent. It has been recently reported to alleviate cardiovascular disease by decreasing LDL and may relieve metabolic diseases such as type 2 diabetes by altering cell signalling pathways (Ko *et al.*, 2005).

Recent research, such as the one mentioned above, has suggested that berberine, its active compound, is a highly effective insulin-sensitizing agent. Berberine was reported to reduce body weight and improve glucose metabolism in animal models of metabolic syndrome (Yin *et al.*, 2007). An *in vitro* study into berberine has found that berberine, the active compound, significantly reduces body weight with unaltered food intake. It also significantly lowers plasma triglycerides of the high-fat-fed rats with no adverse pathology or inflammation in major organs (Lee *et al.*, 2006).

The study also shows that berberine increases AMPK activity in multiple tissues and highlights the beneficial effects of berberine which may involve changes in gene expression to lessen insulin resistance, and the likelihood that berberine may contribute to reduced fat cell differentiation (Lee *et al.*, 2006). Another study has revealed that berberine stimulates glucose uptake and to a greater magnitude than insulin, by the activation of AMPK (Cheng *et al.*, 2006).

Significant insulin sensitizing activity was observed in 3T3-L1 adipocytes which were given 50 μM berberine plus 0.2 nM insulin to reach a glucose uptake level increased by 10 nM of insulin alone. This was found to be associated with increased glucose transporter-4 translocation into the plasma membrane *via* enhanced insulin signalling pathways and the IRS-1 PI3-K-Akt (Kong *et al.*, 2004).

Berberine also increased glucose-stimulated insulin secretion and proliferation in Min6 cells *via* an enhanced insulin/insulin-like growth factor-1 signalling cascade. Similarly to the data above, this result suggests that the herb may be used as an effective insulin sensitising herb and optimally treat type 2 diabetes. Furthermore, berberine was recently reported to alleviate cardiovascular disease by decreasing LDL *via* activating p44/p42 mitogen-activated protein kinase (Kong *et al.*, 2004).

Research has shown that berberine works not only as insulin secretagogue but also as an insulinotropic agent. The mechanism was closely associated to IRS-2 induction in Min6 cells. Berberine activated cAMP response element binding (CREB) phosphorylation in Min6 cells, resulting in enhancing insulin/ IGF-1 signalling cascade by the induction of IRS2 expression. (Jhala *et al.*, 2003).

Another study found that berberine increases insulin stimulated glucose uptake as much as pioglitazone, a TZD with hypoglycaemic action, in a cell-based glucose uptake screening assay. Also associations with increases GLUT4 contents in the membrane *via* potentiating the IRS1 PI3-K Akt signalling cascade in 3T3-L1 adipocytes, were established. Unlike pioglitazone, berberine decreased triglyceride accumulation and enhanced glucose-stimulated insulin secretion (Ko *et al.*, 2005).

The study also found that berberine remarkably decreased triglyceride accumulation in 3T3-L1 cells in a dose dependent manner. Thus the study established that increased glucose uptake with berberine with 0.2nM insulin was associated with the enhancement of the IRS-1 PI3-K Akt-GLUT4 translocation pathway.

More recently, research into dietary obese rats found that berberine increased insulin sensitivity after five week administration. Fasting insulin and HOMA-IR were decreased by 46% and 48% in the rats, respectively. Also, in multiple cell lines including 3T3-L1 adipocytes, berberine was found to increase glucose consumption, 2-deoxy-glucose uptake and to a less degree 3-O-methyl-glucose uptake independently of insulin. The long-lasting phosphorylation of AMPK was found to be associated with persistent elevation in AMP/ATP ratio and the reduction in oxygen consumption and an increase in glycolysis was observed with a rise in lactic acid production. It exhibited no cytotoxicity, and protected plasma membrane in L6 myotubes. These results suggest that berberine enhances glucose metabolism by stimulation of glycolysis, which is related to inhibition of glucose oxidation in mitochondria (Yin *et al.*, 2007).

Panax Notoginseng - San Qi

Recent studies have shown the effects of Notoginsenoside R1, the main ingredient of Panax Notoginseng in relation to cardiovascular activity. A study highlighted that notoginsenoside R1 significantly decreased TNF-α-induced plasminogen activator inhibitor-1 (PAI-1) mRNA, protein level and secretion in human aortic smooth

muscle cells (HASMCs) in a dose-dependent manner (Zhang and Wang, 2006). There is also evidence that notoginsenosides has anti-cancerous effects (Wang *et al*, 2006).

It has been recognized as a potent antioxidant, and has been shown to have the ability to counteract the free radical damage associated with cardiovascular disease. It also strengthens resistance to illness and acts as an anti-inflammatory.

These results indicate that the herb may have the potential to either treat or assist in the treatment of metabolic disorders, such as type 2 diabetes, insulin resistance, and glucose intolerance. Despite the considerable evidence and knowledge of the effectiveness of the herb in treating these diseases in clinical settings for a large number of years, there has been no study to reveal the chemical composition, properties and mechanisms of the active substances in the herb in relation to its effect on glucose and lipid metabolism, to date.

1.5.7 Combined Therapy

It has been reported recently that a large number of Chinese herbs have been found to possess hypoglycemic properties and have the potential to be used to treat patients with type 2 diabetes. The concept of treating complex, multi-factorial metabolic diseases, such as diabetes, using multi-component therapeutics, including single herbs and combination of herbs, has been highly regarded. Combining the conventional approach with Chinese herbs to increase the therapeutic action in lowering blood glucose levels, and hence treating type 2 diabetes has been suggested in the recent years (Tao *et al*.,2007).

Current trends indicate that many insulin resistant and diabetic patients, especially in China and Korea, are seeking Chinese herbal treatment in adjunction to the administration of oral anti-diabetic medication. There have been a large number of recent reports on the effectiveness of combined therapy.

However, extensive research is needed in this area to not only evaluate the efficacy of this therapy, but ensure it is safe. Pharmacological evaluation of the both conventional drugs and herbal medicines, their chemical structures, compositions and mechanism of action, and drug interaction are some areas that need to be examined.

1.6 Cellular Systems for Evaluating Anti-Diabetic Agents

1.6.1 3T3-L1 Cells

3T3-L1 cells are a useful model commonly used *in vitro* studies of metabolic syndrome and insulin resistance.

Insulin resistance manifests itself in two pathophysiological disease states, non-insulin-dependent diabetes and obesity. In both of these conditions, adipocytes become desensitized to the biological effects of insulin, which is reflected in a reduction of the efficacy of insulin to stimulate glucose transport. This cell type contains two isoforms of a family of proteins that facilitate transport of glucose across

the plasma membrane: GLUT1, the constitutive glucose transporter and GLUT4, the insulin-sensitive glucose transporter (Thompson *et al.*, 1997).

Derived from mouse embryonic tissue, 3T3-L1 cells differentiate in culture from a cell which exhibits a fibroblast phenotype to that of an adipocytes phenotype under the appropriate conditions. GLUT1 is present in both phenotypes while GLUT4 is expressed only in the adipocytes phenotype. In the adipocytes, GLUT1 is distributed between the plasma membrane and an intracellular vesicular storage site. GLUT4 under basal conditions resides almost exclusively intracellular but translocates to the plasma membrane when cells are acutely stimulated with insulin (Thompson *et al.*, 1997).

Recently, these cells have been used to perform studies to evaluate the properties and mechanisms of natural products in relation to their therapeutic effects on type 2 diabetes and insulin resistant states. A study has shown to support earlier hypotheses that 3T3-L1 adipocytes can serve as a model for studying the development of insulin resistance, a major factor contributing to the onset of type 2 diabetes. The study revealed that the cells develop insulin resistance in response to physiological relevant concentrations of insulin (Thompson *et al.*, 1997).

Also, glucose deprivation, which prevents the development of insulin-resistant glucose transport, also prevents the loss in GLUT4. Together, these data suggest that the loss of GLUT4 protein underlies the inability of 3T3-L1 adipocytes to respond to insulin after chronic exposure. This mimics the clinical manifestation of human

obesity and non-insulin-dependent diabetes where loss of GLUT4 protein has been observed in adipose tissue (Thompson *et al.*, 1997).

3T3- L1 cells have been reported to have increasing activity of enzymes on pathway of triacylglcerol synthesis during adipose conversion (Kuri-Harcuch *et al.*, 1977), and among the changes that take place during the adipose conversion are increases, in the activity of lipogenic enzymes and increases in the responsiveness of the cells to hormones affecting lipogenesis and lipolysis (Pairault and Green, 1979).

Recently, these cells have been used to perform studies to evaluate the properties and mechanisms of natural products in relation to their therapeutic effects on type 2 diabetes and insulin resistant states.

1.6.2 L6 Cells

The L6 cell line of skeletal muscle has been used widely in cellular experiments. The myoblast-like cell line retains many characteristics of skeletal muscle including differentiation into myotubes, display of electrical and contractile activity and production of muscle-specific proteins. L6 myoblasts have been found to fuse to form multi-nucleated and striated muscle fibres, whereby the fusion declines following serial propagation.

Insulin stimulation of glucose transport in L6 muscle cells results predominantly from the translocation to the cell surface of the glucose transporter GLUT4. L6 cells, originally derived from rat skeletal muscle, propagate as mononucleated myoblasts

but differentiate by spontaneous cellular fusion into multinucleated primary myotubes (Mitsumoto *et al.*, 1991).

The myotubes express several proteins typical of skeletal muscle including the GLUT4 glucose transporter. Insulin stimulates glucose uptake with high sensitivity and maximal responsiveness only in differentiated L6 myotubes; GLUT4 expression parallels the acquisition of these characteristics as the L6 cells differentiate (Mitsumoto and Klip, 1992).

These features of L6 myotubes are important in regards that GLUT4 is responsible for insulin-dependent glucose uptake in mature skeletal muscle. In the myotube stage, GLUT4 coexists along with the housekeeping glucose transporter GLUT1 and the fetal muscle transporter GLUT3. These cells have a fully functional insulin-signalling cascade including robust activation of Akt.

The detrimental effects of exposing cells to high glucose concentrations (so-called glucose toxicity) include impaired glucose metabolism and insulin resistance. This is recognized in part to increased metabolism of glucose via the hexosamine pathway. Although this pathway generally consumes only a small proportion (<3%) of glucose entering cells, it provides an over-spill route for excess glucose that is not utilized via glycolysis, glycogenesis or the pentose phosphate pathway (Marshall *et al.*, 1991).

They also found that increased activity of the hexosamine pathway appears to serve as an intracellular fuel sensor by generating signals within the cell to decrease glucose

uptake, particularly in insulin-sensitive cells such as skeletal muscle (Marshall S *et al.*, 1991).

1.6.3 HepG2 cells

The human hepatoma cell line HepG2 was isolated in 1979 by Aden *et al.* It was established from human liver tumour biopsies and was shown to have morphological characteristics and the epithelial cell shape compatible with those of liver parenchymal cells (Knowles *et al.*, 1980). The HepG2 cell line was also been shown to synthesise and secrete various human plasma proteins into the medium (Rash *et al.*, 1981).

Various studies have reported that this cell line, unlike most adipocytes and muscle cells is sensitive to insulin with respect to glucose uptake (Mueckler *et al.*, 1985). In hepatocytes, glucose transporters are mostly located in the plasma membrane. A study showed that hepatocytes express GLUT1 and GLUT2 glucose transports and that GLUT2 is stored in intracellular organelles but lack the insulin-responsive glucose transporter translocation mechanism (Hah *et al.*, 1992).

1.7 Aims of This Thesis

The global aim of this study was to discover new Chinese medicine approaches to treat type 2 diabetes and other metabolic disorders.

In recent years, this escalating prevalence of metabolic diseases has resulted in the successful establishment of conventional therapies and treatment options for patients with these metabolic illnesses. However, to couple with this, reviews have identified that there is an increasing prevalence of obesity combined with low efficacy and reports of side effects of these conventional treatments.

Research in the past decade in Chinese herbal medicines have shown to have positive effects on insulin resistance, obesity, type 2 diabetes and other metabolic disorders. Through profound review of literature and research in areas of both mainstream medicine and Traditional Chinese Medicine, our research group has recognised that Chinese herbal medicines will not only initiate and establish new treatment approaches for type 2 diabetes, but may also help to prevent or delay the risk factors and complications involved with the disease.

Saponins of *Panax notoginseng* (SPN), the active ingredient of the herb *Panax notoginseng*, has been used clinically to treat a variety of cardiovascular disorders. However, to date, there has been no studies designed to evaluate its metabolic effects.

The specific aims of my study were:

To evaluate effects and mechanisms of saponins of *Panax notoginseng* on the glucose metabolism in 3T3-L1 adipocytes.

To evaluate effects and mechanisms of saponins of *Panax notoginseng* on the lipid metabolism in 3T3-L1 adipocytes.

Carry out dose-response assessment on different concentrations of saponins of *Panax notoginseng* to establish a therapeutic window for further animal studies and clinical trials in patients with type 2 diabetes.

This study has utilised 3T3-L1 cells to identify and evaluate the effects and mechanisms of SPN on glucose and lipid metabolism. The results from this study will be useful to further evaluate SPN in animal models of metabolic syndrome and type 2 diabetes, as well as further clinical studies in diabetic patients.

The overall aim of this project is to develop a natural product to control type 2 diabetes and prevent its cardiovascular complications.

Chapter 2

The Effects and Mechanisms of Saponins of *Panax Notoginseng* on Glucose Metabolism

CHAPTER 2: THE EFFECTS AND MECHANISMS OF SAPONINS OF *PANAX NOTOGINSENG* ON GLUCOSE METABOLISM IN 3T3-L1 CELLS

2.1 Introduction

Type 2 diabetes is a metabolic disorder that has been increasingly impacting our society for the past decade. Clinical evidence and research has raised concern that type 2 diabetes may cause the onset of cardiovascular complications that often cause disability and mortality, if left neglected or ineffectively treated. Current oral anti-diabetic drugs targets lowering of glucose and lipid levels to treat type 2 diabetes but has not demonstrated to reduce micro- and macro-vascular disease. Some medications have been found to even trigger undesirable side effects such as weight gain - that aggravate other risk factors to promote the onset of diabetic cardiovascular complications (Moller, 2006).

This study was carried out in search of a new agent for type 2 diabetes and to minimise the associated cardiovascular complications by employing traditional knowledge of clinical benefits of Chinese herbal medicine. Herbal medicine has been used throughout China and East Asia for thousands of years and records and traditional texts have shown that they are highly beneficial in the treatment of a broad range of diseases. Particular attention has been drawn on the development of a novel therapy for the treatment of type 2 diabetes with Chinese herbal medicine in recent years. Promising results have been attained so far as they have been found to minimize frequent side effects associated with the disease.

It is essential and may be highly beneficial to elucidate the active compounds, properties and synergy these herbs comprise, so that these agents may be widely utilized in both the treatment of type 2 diabetes and in the prevention of diabetic complications.

The root of *Panax notoginseng* has been traditionally used to break down blood clots and improve blood circulation over a thousand years. In modern medical terminology, the herb has been found to have beneficial effects on thrombosis, coronary heart disease, hypertension, stroke and arteriosclerosis.

Saponins of *Panax notoginseng* (SPN), the major active compounds of *Panax notoginseng,* have been used to treat coronary heart disease, stroke and diabetic macroangiopathy (Yao and Li, 2002, Liu *et al.*, 2004) throughout hospitals of China. In this chapter, we have evaluated the effects and mechanisms of SPN on glucose metabolism in 3T3-L1 adipocytes.

2.2 Materials and Methods

2.2.1 Materials

3T3-L1 adipocytes were supplied by American Type Culture Collection (ATCC) (VA, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin were supplied by GIBCO (Aukland, NZ). Medical injectable saponins of Panax Notoginseng (brand name *Xue Sai Tong*) was supplied by

Helongjian Zhenbaodao Pharmaceutical Co. (Helongjian , China) and 0.9% saline (sodium chloride injection BP) was purchased from Pfizer Pty Ltd (WA, Australia). Insulin was supplied by Eli Lilly Pty Ltd (NSW, Australia).

Trypsin and Methylthiotetrazole (MTT) were purchased from Sigma-Aldrich (Irvine, Ayrshire, UK). DMSO was supplied by Amiresco (Solon, USA) and the chemicals NaCl, KCl, $NaHCO_3$, $MgSO_4$ and KH_2PO_4 were provided by BDH Chemicals (Kilsyth, Vic). BSA was supplied by FSA Laboratory Supplies (Loughborough, UK). 2-DOG and [3H]2Deoxyglucose were suppled by Sigma Chemicals Co, MO,USA). Optiphase 'Hisafe' 3 Scintillant (Fisher Chemicals, UK) and scintillation counter used was Beckman LS 7800 supplied by Beckman Scientific Instruments (Irvine, CA, USA). Triton X-100 was supplied by FISON Chemicals (Loughborough, UK), goat polyclonal IgG GLUT4 and Donkey anti-goat IgG was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein Streptavidin was from Fluorescein Streptavidin Kit SA-1200 (Vector Laboratories, Inc. Buringame, CA 94010). Vectashield Mounting Medium for Fluorescence H-1000 was provided by Vector Laboratories, Inc. (Buringame, CA, USA). The Olympus BX51 DP70 microscope and DP controller program 1.2.1.108 (Olympus Optical, Tokyo, Japan) was used for this study.

2.2.2 Cell Culture

3T3-L1 mouse adipocytes were maintained in complete medium (CM) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin in incubator (37°C and 10% CO_2). CM was replaced every second day until confluent.

2.2.3 Cell Treatment

4 x 10^3 cells were seeded to 24-well plate. When confluent, CM was discarded and 1ml starving medium (SM) containing 0.5% FBS and 1% penicillin was added and incubated overnight. 20 µl of 100 nM of insulin was added in each well. After incubation for 1 h at 37°C, concentrations of 10, 50, 100, and 200 µg/ml of SPN were added to the wells and incubated for 24 h at 37°C. This treatment protocol was used for all the following experiments.

2.2.4 Cell Viability Assay

Methylthiotetrazole assay was carried out to determine the viability of SPN treated 3T3-L1 cells. 3T3-L1 cells were treated according to the method above. Following treatment, 5 mg/ml methylthiotetrazole (MTT) in DMEM was added to the wells and incubated for 4 h at 37°C. The MTT medium was replaced with dimethyl sulfoxide (DMSO) and the optical density was measured at 570 nm with a micro-plate reader.

2.2.5 Glucose Uptake Measurement

2-deoxyglucose uptake was measured in cultured 3T3-L1 mouse adipocytes as described by Zhou *et al.* (Zhou *et al.*, 2007).

After treatment as described above, cells were washed with Krebs buffer (114 mM NaCl, 5 mM KCL, 25 mM $NaHCO_3$, 1.18 mM $MgSO_4$ and 1.17 mM KH_2PO_4, pH

7.4) at room temperature. 1ml radioactive Krebs solution containing [3H]2Deoxyglucose (0.2 μCi, specific activity 10 Ci/mmol) and 1 μM 2-deoxyglucose (Sigma Chemical Co., MO, USA) was added to the wells and incubated for 10 min at room temperature. The hot Krebs solution was then discarded and washed with ice cold Krebs buffer at 4°C.

Incorporated radioactivity was determined by the following procedure. Cells were solubilised in 500 μl of 1 M NaOH. After 2 h, cells were then transferred to scintillation vials and 4.5 ml Optiphase 'Hisafe' 3 Scintillant (Fisher Chemicals, United Kingdom) was added to each vial. Vials were vortexed thoroughly to ensure all test samples were mixed evenly. The amount of radioactive 2-deoxyglucose taken up by cells was determined by a scintillation counter (Beckman LS 7800, Beckman Scientific Instruments, Irvine, CA, USA). Data was expressed as a percentage of insulin-stimulated control.

2.2.6 GLUT4 Immunoflourescence

Translocation of GLUT4 in 3T3-L1 cells was determined by immunofluorescence. Cells were rinsed twice with warm PBS and fixed with 4% Paraformaldehyde for 30 min. 0.5% of Triton X-100 was added for 10 min in room temperature then washed with 0.02% PBS-T for 5 min. 1 ml of 1% BSA in PBS was applied to coverslips for 30 min to block cells.

The primary antibody, goat polyclonal IgG GLUT4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted to 1:50 and the plate was placed in a water bath for 45 min at 25°C to prevent coverslips from drying up. Coverslips were washed with

0.02% PBS/1% BSA for 5 min. The secondary antibody Donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) coupled with Fluorescein Streptavidin from Fluorescein Streptavidin Kit SA-1200 (Vector Laboratories, Inc. Buringame, CA, USA), was diluted to 1:100 with PBS and incubated for 45 min in a water bath. Coverslips were washed with 0.02% PBS-T for 5 min and PBS for a further 5 min.

Coverslips were air dried, mounted onto glass slides with Vectashield Mounting Medium for Fluorescence H-1000 (Vector Laboratories, Inc. Buringame, CA 94010) and examined under a fluorescent microscope. All images were captured with a 40x lens. (Olympus DP70 microscope and the DP controller program 1.2.1.108 Olympus Optical Co, Tokyo, Japan).

All sample images were taken at same positions and obtained on the same day. All positions were imaged at least three times to assure that consistency was maintained. Quantitation was carried out using Image-Pro Plus software (Media Cybernetics, Inc. Bethesda, MD, USA).

2.2.7 Glycogen Synthesis Assay

SPN mediated glycogen synthesis was determined by a modified method by Hess *et al* (Hess *et al.*, 1991). Following treatment of cells, wells were washed twice with Krebs-Ringer buffer (ph 7.4 - 30mM HEPES, 134 mM NaCl, 3.5 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$ and 5 mM $NaHCO_3$). Cells were incubated for 2 h at 37°C in the above buffer containing 11 mM D-[U-^{14}C]glucose (1 μCi/ml), glucose (5 mM) and insulin (10 nM). The reaction

was stopped by 0.7 ml 30% KOH. Cells were transferred into capped test tubes, and heated in a dry bath for 20 min at 100°C. 2.2 ml of 95% ethanol was added to the tubes, boil, then immediately remove and place in an ice bath for 20 min to allow precipitation of glycogen. Samples were centrifuged at 2,200 rpm for 10 min at 0°C, precipitate dissolved in 1 ml distilled water.

Total sample count was measured by rate of incorporation of D-[U-^{14}C]glucose into glycogen. Glycogen synthesis of the treated cell groups were expressed as a percentage of the control group.

2.2.8 Statistical Analysis

Data from all the experiments were calculated and expressed as a percentage of the insulin-stimulated control cells. Unpaired student t-test was used to compare insulin-stimulated control to SPN treated cells and a p-value of <0.05 was considered statistically significant.

2.3 Results

2.3.1 Viability of 3T3-L1 Cells with SPN Treatment

MTT assay was carried out to determine the cell viability of SPN concentrations of 10, 50, 100 and 200 μg/ml. This was carried out to assess whether treatment of SPN exert toxicity or cause death of 3T3-L1 cells. Figure 2.2 illustrates that concentrations of 10,

50, 100 and 200 μg/ml did not affect viability of 3T3-L1 cells. No significant difference was found between the insulin-control and the four SPN concentrations.

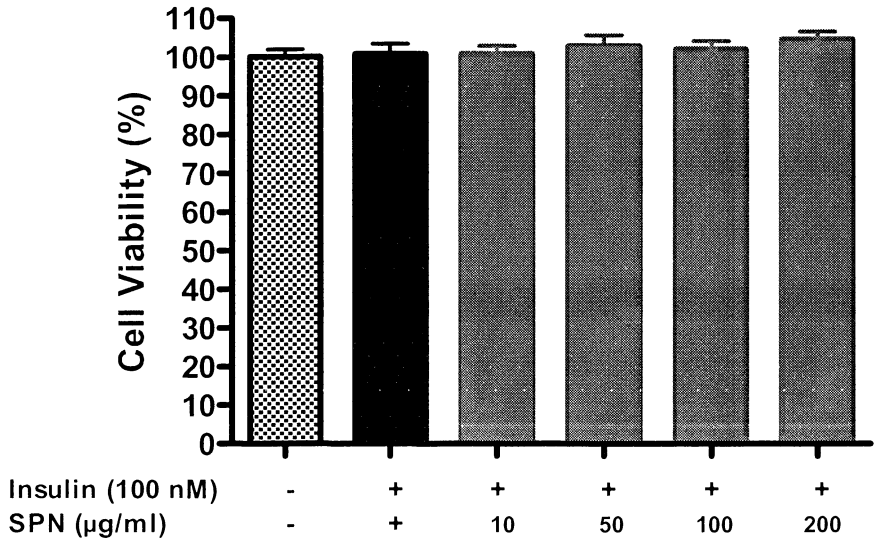


Figure 2.1 MTT assay of SPN-treated 3T3-L1 cells. Methylthiotetrazole assay was carried out on SPN treated 3T3-L1 cells. Following treatment, 5 mg/ml methylthiotetrazole (MTT) in DMEM was added to the wells, incubated for 4 h at 37°C and replaced with DMSO. Optical density was measured at 570 nm and results showed that there was no significant difference in cell viability between all control cells and SPN treated cells. Data was collected from 5 separate experiments.

2.3.2 Effect of SPN on Glucose Uptake

SPN significantly increased insulin-stimulated glucose uptake compared with insulin alone. Furthermore, SPN enhanced glucose uptake in a dose-dependent manner (Fig. 2.1). 50 μg/ml of SPN increased glucose uptake by 64% (100 vs 163.96 ± 12.08, $p<0.001$) whereas a positive control, 50 μg/ml of berberine, only increased glucose uptake by 20%. However, increasing concentration of SPN to 200 μg/ml reduced

glucose uptake, indicating that 50-100 μg/ml are optimal concentrations to achieve maximal glucose uptake *in vitro*.

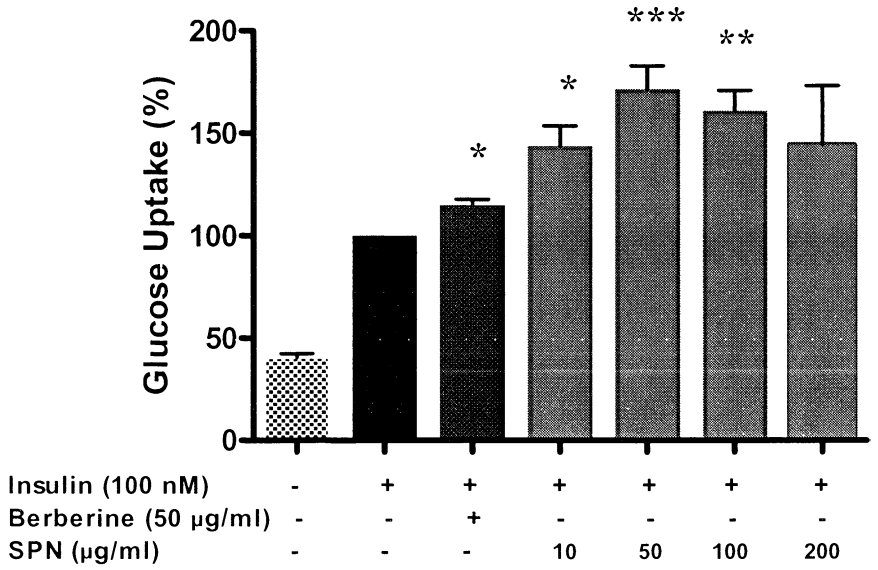


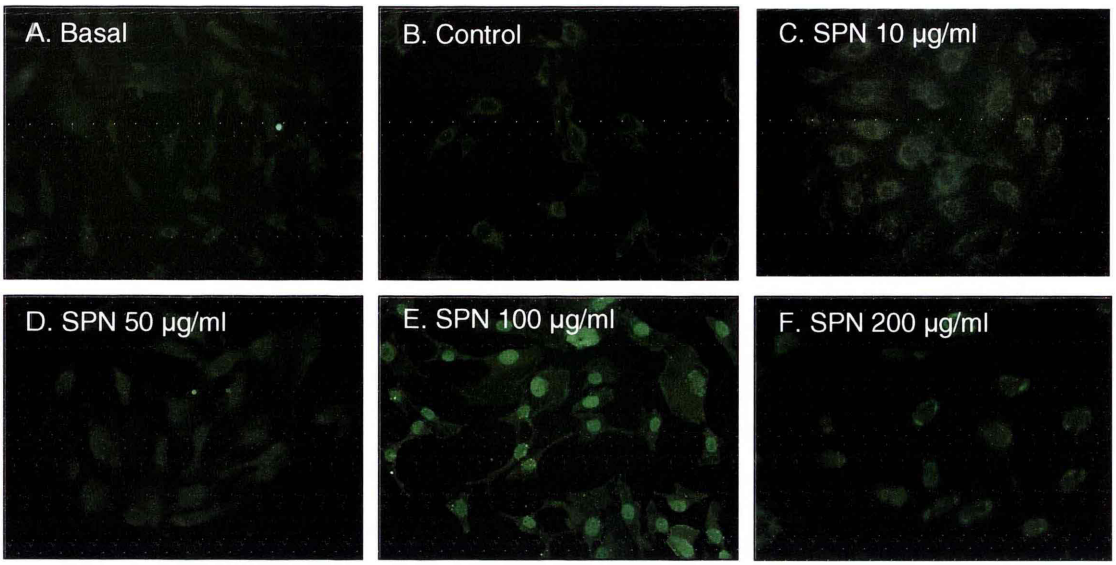
Figure 2.2 Measurement of 2-deoxyglucose uptake in SPN-treated 3T3-L1 cells. [^{3}H]2-deoxyglucose uptake was measured under basal conditions and after incubation of 100 nM insulin without and with SPN treatment (10, 50, 100, and 200 μg/ml) for 24 h. 100 nM with 50 μg/ml berberine was as a positive control. The values were calculated as percentage of insulin-stimulated glucose uptake from five separate experiments. ($^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared with insulin control). Data were from 5 separate experiments.

2.3.3 GLUT4 Translocation of 3T3-L1 Cells

Results indicate that SPN significantly increased GLUT4 translocation intracellular storage sites to the plasma membrane. Immunoflourescence imaging was carried out to examine the effect of SPN on GLUT4 content in the 3T3-L1 adipocytes. Immunoflourescence imaging in Figure 2.3 (A) visibly demonstrated an increase in the net content of GLUT4 in the SPN treated 3T3-L1 adipocytes compared with basal

and insulin stimulation only. The quantitative analysis (B) demonstrated that 50 and 100 μg/ml SPN significantly enhanced GLUT4 translocation and therefore increased overall GLUT4 content in the plasma membrane by 3-fold and 6-fold respectively (100 vs 323.08 ± 30.91 and 602.56 ± 60.53 respectively, $p<0.001$).

A



B

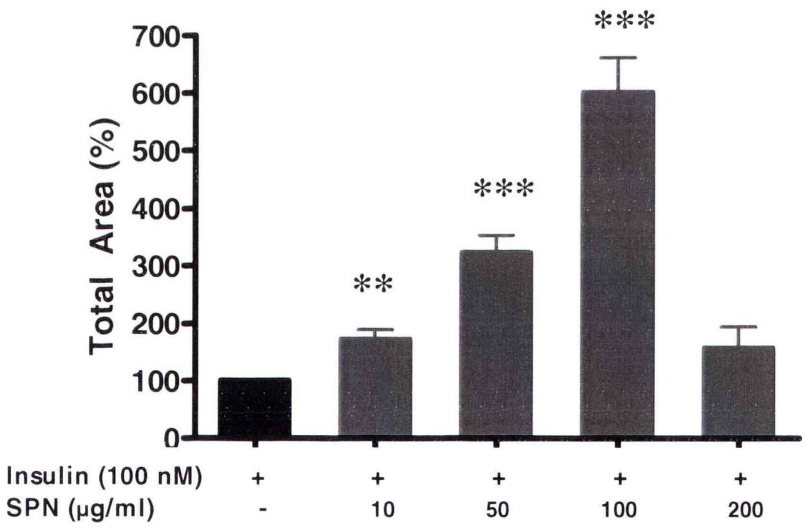


Figure 2.3 GLUT4 Immunofluorescence imaging of SPN-treated 3T3-L1 cells. (A) Serum-starved 3T3-L1 adipocytes was utilised to determine GLUT4 translocation at basal conditions and after 100 nM insulin stimulation with or without various concentrations of SPN (10, 50, 100, and 200 µg/ml) for 24 h before preparation of plasma membrane and immunofluorescence reaction with ant-GLUT4 antibody and fluorescein streptavidin. (B) Quantification of data from each experiment (n=5), utilizing 50 fields for each condition, was carried out using a fluorescent microscope. 50 and 100 µg/ml SPN significantly enhanced GLUT4 content in the plasma membrane by 3-fold and 6-fold respectively ($^{**}p<0.01$ and $^{***}p<0.001$).

2.3.4 Glycogen Synthesis Assay

Glycogen synthesis assay was carried out to assess the incorporation of D-[U-^{14}C] glucose into glycogen in 3T3-L1 cells. Results showed that 100μg/ml SPN significantly increased synthesis of glycogen in 3T3-L1 cells ($p<0.01$). Figure 2.5 illustrates that this group augmented glycogen synthesis by 53%, in comparison to the basal - non-insulin stimulated cells.

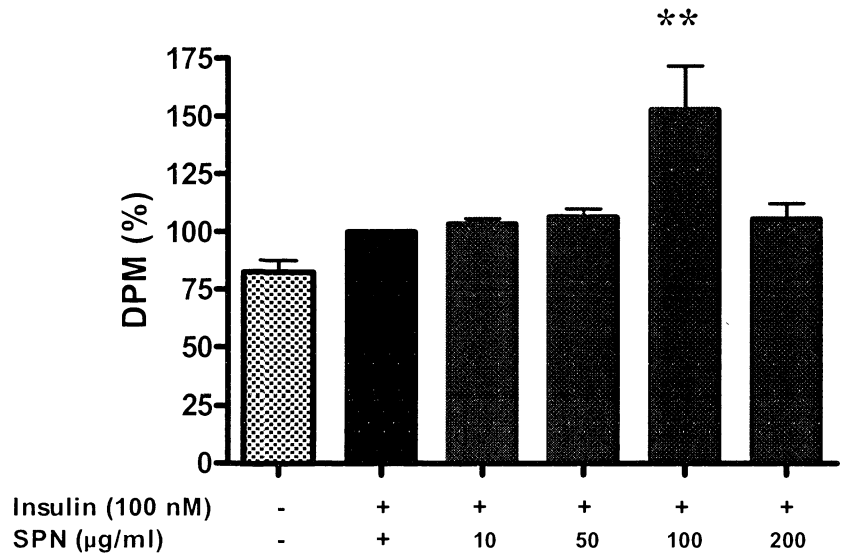


Figure 2.5 Glycogen synthesis assay of SPN-treated 3T3-L1 cells. Following treatment, cells were incubated for 2 h at 37°C in the buffer containing 11 mM D-[U-^{14}C] glucose and 5 mM glucose. The glycogen synthesis was determined by measuring the rate of incorporation of D-[U-^{14}C] glucose into glycogen. Results indicate that 100μg/ml SPN significantly increased synthesis of glycogen in 3T3-L1 cells ($^{**}p<0.01$).

2.4 Discussion

The results attained from this study suggest that saponins of notoginsenosides (SPN) enhances insulin-stimulated glucose uptake in adipose tissue and may potentially increase adipose tissue glucose metabolism in diabetic states. Previous studies have established that notoginsenosides comprise of properties that lower cholesterol levels, prevent and treat cardio-cerebral vascular disease (Zhang *et al.*, 1997), strengthen liver function (Chen *et al.*, 2008), lower blood pressure and strengthen immunity (Qin *et al.*, 2006).

States of type 2 diabetes or increased adiposity in animals and humans have been found to be associated with resistance to insulin-stimulated glucose uptake (Houseknecht and Kahn, 1997). Insulin stimulates glucose uptake via a complex cascade of signalling events. These signalling pathways are responsible for the metabolic regulation of carbohydrate, lipid, and protein utilization. These pathways are logical targets for defects associated with insulin resistance and type 2 diabetes (Li *et al.*, 2000).

Insulin resistance and type 2 diabetes amount to the major and rapidly increasing health problems in today's society. Recent research has both distinguished and support that the stimulation of insulin-mediated glucose uptake may further promote regulation of glucose metabolism (Iozzo *et al.*, 2003).

This study found that in 3T3-L1 adiopcytes, although 100 nM insulin increased glucose uptake considerably, the treatment of SPN brought about a further increase in glucose uptake, indicating that SPN possesses the insulin sensitising action.

Glucose uptake is mediated by the insulin-responsive glucose transporter 4 (GLUT4) and is also the rate-limiting step in glucose utilization in adipocytes. Earlier studies have demonstrated that insulin-stimulated GLUT4 vesicle translocation to the plasma membrane (PM) is critical for glucose homoeostasis (Paul *et al.*, 2003). Development of insulin resistance, GLUT4 expression is down-regulated selectively in adipose tissue but not in skeletal muscle. Down-regulation of GLUT4 expression in adipose tissue is an almost universal feature of insulin-resistant states, including obesity, type 2 diabetes and the metabolic syndrome (Yang *et al.*, 2005).

This study found that SPN significantly enhanced insulin-mediated glucose uptake in a dose-dependent manner in 3T3-L1 adipocytes. This study first confirmed that stimulation with 100 nM insulin alone brought about a slight increase in glucose uptake in 3T3-L1 adipocytes. However, SPN increased insulin-stimulated glucose uptake further compared to insulin alone. This significant increase in insulin-dependent glucose uptake was seen with SPN concentrations of 10, 50, 100 and 200 μg/ml. This result indicates that SPN may have synergic effect to insulin action. It may ameliorate insulin resistance in target tissues.

Resistance to insulin-stimulated glucose transport in adipose tissue and skeletal muscle is one of the earliest defects detected in insulin-resistant states.

To identify the mechanism of increased insulin-stimulated glucose uptake by SPN, GLUT4 translocation of 3T3-L1 cells was studied. Results from immunofluorescence imaging and quantitation demonstrated that SPN treatment of concentrations between 10 and 100μg/ml sourced a significant increase in GLUT4 content in the plasma membrane in a dose-dependent manner. A likely explanation is that SPN augments insulin-stimulated glucose uptake dependent of GLUT4 translocation.

Glycogen synthesis is a key step of glucose haemostasis and is regulated by the insulin-sensitive and rate-limiting glycogen synthase kinase-3. Recent studies using magnetic resonance spectroscopy have shown that decreased insulin-stimulated muscle glycogen synthesis due to a defect in insulin-stimulated GLUT4 activity is a major factor in the pathogenesis of type 2 diabetes (Morino *et al.*, 2006). Results of this study showed that SPN treatment encouraged the storage of glucose as glycogen in adipocytes through the coordinate increase in glucose uptake.

This result, together with results from glucose uptake and GLUT4 translocation, suggests that SPN is synergistically involved with insulin in several key steps of glucose metabolism.

Studies by Reavan and Laws support that type 2 diabetes is characterised by insulin resistance that is a result of reduced insulin-stimulated non-oxidative glucose metabolism primarily caused by impaired muscle glycogen synthesis. The defect responsible for the reduced flux through the glycogen synthetic pathway resides at the level of glucose transport or phosphorylation (Reaven and Laws, 1999).

Studies in cases of early-onset insulin resistance revealed that the first detectable abnormality in insulin action lies in the storage of glucose as glycogen. Although it has been identified that these observations were reflective of decreased glucose uptake in insulin-resistant subjects, glycogen synthesis is found to be typically diminished in patients with type 2 diabetes and may represent a critical event in the pathophysiology of the disease (Crosson *et al*, 2003).

Results attained from this study display an increase in glycogen synthesis with SPN treatment in 3T3-L1 adipocytes. 100 μg/ml SPN treatment ultimately increased the storage of glucose as glycogen in the cells through the coordinate increase in glucose uptake. A likely explanation is insulin binds to its receptor in peripheral tissues and initiates several signalling cascades to increase glucose uptake via translocation of the GLUT4 containing vesicles to the plasma membrane (Brady, 2003).

Also, 200 μg/ml of SPN reduced glucose uptake and glycogen synthesis. MTT assay indicated that this concentration did not affect the viability of 3T3-L1 cells, however, may have disturbed the physiological function of the cells due to increased osmolarity. Thus, 100 μg/ml SPN would be the optimal concentration to be used in further studies.

In summary, coordinate increase in glucose uptake and glycogen synthesis by SPN promotes the storage of glucose as glycogen. The probable mechanism behind the increase in glucose uptake of cells with SPN treatment is that stimulation of glucose uptake causes a significant increase in GLUT4 translocation from intracellular storage sites to the plasma membrane in 3T3-L1 adipocytes.

Chapter 3

The Effects and Mechanisms of Saponins of *Panax Notoginseng* on Lipid Metabolism

CHAPTER 3: THE EFFECTS AND MECHANISMS OF SAPONINS OF *PANAX NOTOGINSENG* ON LIPID METABOLISM

3.1 Introduction

It has been demonstrated that elevated non-esterified fatty acid (NEFA) levels, a decrease in lipolysis in addition to insulin-resistant glucose uptake and intracellular glucose metabolism (such as glucose phosphorylation and glycogen synthesis) are early hallmarks of the insulin resistance that proceeds to the onset of type 2 diabetes (Eriksson *et al.*, 1999).

The main features of lipid metabolism are involved with fatty acid oxidation to produce energy and the synthesis of lipids called lipogenesis. Lipid metabolism is also directly associated with the metabolism of carbohydrates which may be converted to fats. The metabolism of both is deregulated by type 2 diabetes and obesity.

Obesity, characterized by the storage of excess triglyceride in adipose tissue, coupled with type 2 diabetes, is a complex and chronic disorder that has become a global epidemic in recent years (Hu *et al.*, 2006). Studies have identified that obesity, particularly visceral fat, is often associated with insulin resistance, dyslipidemia, and hypertension, an increased risk of accelerated atherosclerosis, and other diabetic complications (He *et al.*, 2003).

Type 2 diabetes is a disease that has been identified to be commonly accompanied with an inapt increase and acceleration of lipolysis in adipose tissue. This may occur in the presence of a defect in the regular action of insulin, thereby causing an increase in serum free fatty acid (FFA) level and subsequent gluconeogenesis in the liver. In addition, increased FFA itself induces further insulin resistance directly in insulin sensitive tissues, which leads to the onset of hyperglycemic state (Solini *et al.*, 2001).

Thus, investigation into new therapeutic agents and treatments that target the inhibition of FFA released from adipose tissue will result in the treatment and prevention of obesity and type 2 diabetes.

This section of the study has been carried out to evaluate the effects of SPN on lipid metabolism in 3T3-L1 cells. The staining of lipids in 3T3-L1 cells was carried out to identify the effect of SPN treatment on total lipid content and lipolysis assay to further distinguish the effect of SPN on lipolytic activity.

3.2 Materials and Methods

3.2.1 Materials

3T3-L1 adipocytes were supplied by American Type Culture Collection (ATCC) (VA, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin were supplied by GIBCO (Aukland, NZ). Medical injectable saponins of Panax Notoginseng (brand name *Xue Sai Tong*) was supplied by Helongjian Zhenbaodao Pharmaceutical Co. (Helongjian , China) and 0.9% saline

(sodium chloride injection BP) was purchased from Pfizer Pty Ltd (WA, Australia). Insulin was supplied by Eli Lilly Pty Ltd (NSW, Australia). Lipolysis assay kit for 3T3-L1 cells was purchased from Zenbio (CAT # LIP-1-NCL1, NC, USA).

3.2.2 Cell Culture

3T3-L1 mouse adipocytes were maintained in complete medium (CM) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin in incubator (37°C and 10% CO_2). CM was replaced every second day until confluent.

3.2.3 Cell Treatment

Cell treatment for lipid staining was carried out as described in Chapter 2. 4 x 10^3 cells were seeded to 24-well plate. When confluent, CM was discarded and 1ml starving medium (SM) containing 0.5% FBS and 1% penicillin was added and incubated overnight. 20 µl of 100 nM of insulin was added in each well. After incubation for 1 h at 37°C, concentrations of 10, 50, 100, and 200 µg/ml of SPN were added to the wells and incubated for 24 h at 37°C. A commercially available kit was used for the lipolysis assay of 3T3-L1 cells. The treatment procedure was performed as described below.

3.2.4 Lipid Staining

Following treatment of 3T3-L1 cells, the cell media was removed from the wells with a pipette and coverslips were gently rinsed with PBS. 1 ml of 10% formalin was

added to each well for 10 min to fix the cell. Each well was then rinsed with 60% isopropanol for 30 sec and 1 ml oil-red-o stain was added to each well for 10 min and removed. The wells were rinsed with 60% isopropanol for 5 sec followed by distilled water for a further 1 min. 1 ml of Mayer's haematoxylin was added for 20 min and then washed with distilled water. 1 ml of sodium phosphate solution was added for 5 min to intensify the staining of the nuclei and thoroughly rinsed with distilled water for 30 sec. The wash process was repeated then coverslips were air-dried for 20 min.

Coverslips were air dried, mounted onto glass slides with Vectashield Mounting Medium for Fluorescence H-1000 (Vector Laboratories, Inc. Buringame, CA, USA) and examined under a microscope. All images were captured with a 40x lens. (Olympus DP70 microscope, Olympus Optical Tokyo, Japan).

All sample images were taken at same positions and obtained on the same day. All positions were imaged at least three times to assure that consistency was maintained. Quantitation was carried out using Image-Pro Plus software (Media Cybernetics, Inc. Bethesda, MD, USA).

3.2.5 Lipolysis Assay

Lipolysis assay was carried out using the commercially available adipocytes lipolysis assay kit for 3T3-L1 cells (Zenbio, Inc. NC). 3T3-L1 cells were passaged to a 96-well plate and grown until confluence was reached. 140 µl of complete media (CM) was removed from each well. 200 µl of wash buffer was gently added to each well. The wash buffer was then discarded. The treatment of cells followed whereby SPN

concentrations of 10, 50, 100 and 200 μg/ml were mixed with the assay buffer. 150 μl of test sample were added to each well.

0.1% DMSO was used as a negative control and 1 μM Isoproterenol was used as the positive control. 1 mM of glycerol standard was used to make up 8 standards (from 0 to 200 μM glyercol). 100 μl of glycerol standard was added to each well and cells were incubated in 37°C for 24 h. 100 μl of each cell sample was transferred to a fresh microplate and 100 μl of glycerol reagent A was added.

The plate was incubated for 15 min at room temperature and samples were measured with micro-plate reader at 540 nm. Data was expressed as μM glycerol released.

3.2.6 Statistics

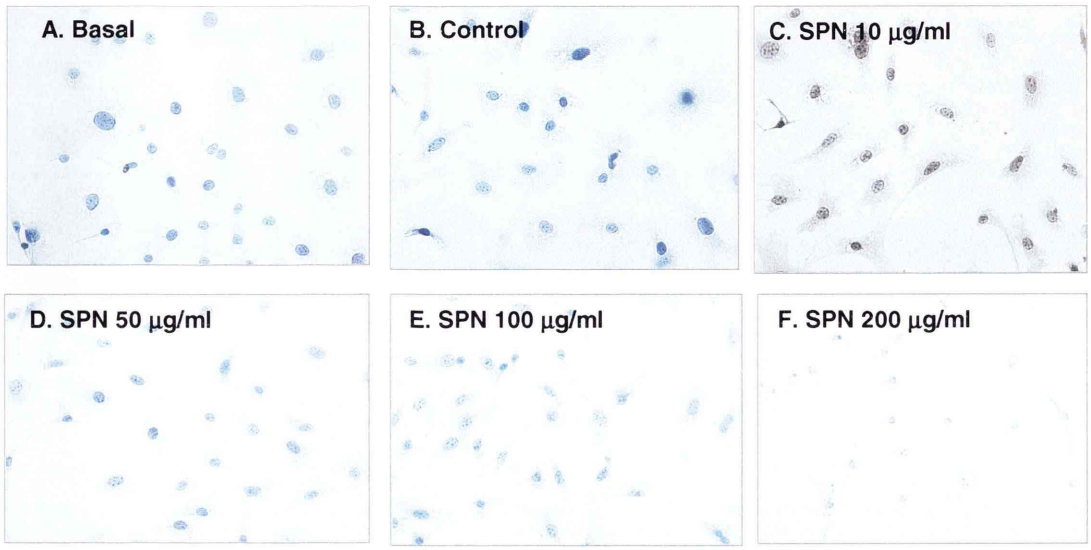
Data from all the experiments were calculated and expressed as a percentage of the insulin-stimulated control cells. Unpaired student t-test was used to compare insulin-stimulated control to SPN treated cells and a p-value of <0.05 was considered statistically significant.

3.3 Results

3.3.1 Effect of SPN on Total Lipid Content

Staining of 3T3-L1 cells was carried out to determine the effect of SPN on total lipid content. Results indicated that treatment of SPN significantly reduced lipid content compared to insulin-stimulated cells. Figure 3.1A show that lipid droplets in the control cells appear to be comparatively larger than the lipids of the SPN treated cells. Quantitative analysis of the stained images was carried out. Statistical analysis showed that 50, 100 and 200 μg/ml of SPN depicted a significant decrease in lipid levels compared to the control insulin stimulated cells (100 vs 92.26 ± 1.70, 93.17 ± 0.88 and 87.71 ± 0.53, $p<0.05$, $p<0.01$ and $p<0.001$ respectively).

A



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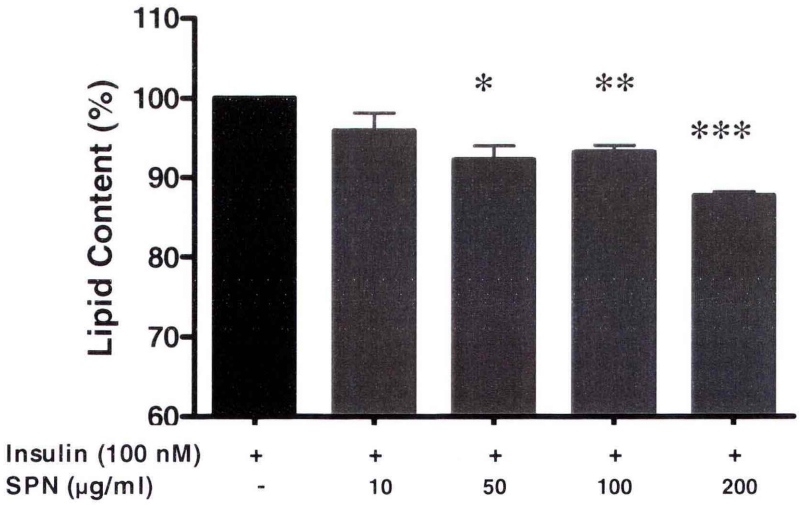

Figure 3.1 Lipid staining of SPN-treated 3T3-L1 cells. (A) Serum-starved 3T3-L1 adipocytes was utilised to determine total lipid content at basal conditions and after 100 nM insulin stimulation with or without various concentrations of SPN (10, 50, 100, and 200 µg/ml) for 24 h. 10% formalin was used to fix cells to coverslips. Oil-red-o staining solution was added for 10 min followed by Mayer's haematoxylin for 20 min. Sodium phosphate solution was added to intensify the staining of the nuclei. (B) Quantitative analysis showed SPN (10, 50, 100, and 200 µg/ml) significantly decreased in lipid content compared to the control insulin-stimulated cells (* $p< 0.05$ $^{**}p<0.01$ *** $p<0.001$ respectively).

3.2.2 Effect of SPN on Lipolysis

Lipolysis assay results showed that SPN treatment ultimately promoted lowering in the level of glycerol released in 3T3-L1 significantly, compared to the control insulin-stimulated cells. Data are expressed as μM glycerol released. In Figure 3.3 it can be seen that SPN concentrations of 10, 50, 100 and 200 μg/ml induced a significant reduction in lipolysis (100 vs 65.00 ± 6.98, 65.25 ± 9.85 and 60.82 ± 8.04 respectively, $p<0.05$).

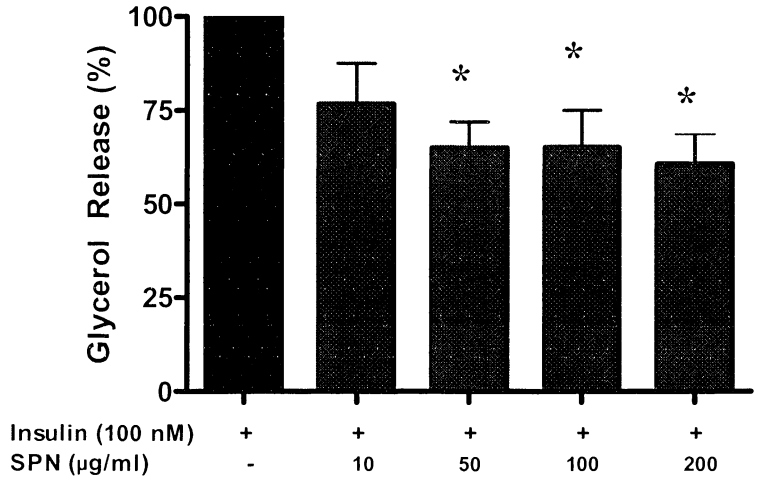

Figure 3.3 Lipolysis assay of SPN-treated 3T3-L1 cells. 3T3-L1 lipolysis assay kit was used to determine the amount of glycerol released from cells treated with SPN concentrations of 10, 50, 100 and 200 μg/ml. Test cells were incubated for 15 min at room temperature and were measured with micro-plate reader at 540 nm. Data was expressed as μM glycerol released and further converted as a percentage of the insulin-stimulated control. Results indicate that SPN-treated cells significantly decreased lipolysis ($^{*}p<0.05$) compared to insulin-stimulated control cells.

3.4 Discussion

The results of this study propose that SPN has a lipid lowering effect in 3T3-L1 cells. These results are consistent with previous animal studies in which the administration of SPN produced a moderate, non-dose related decrease in plasma levels of total cholesterol and triglycerides in rats fed with high-fat diet (Cicero *et al.*, 2003). Also, triglyceride levels in notoginsenosides-treated rats showed a significantly dose-dependent decrease in hepatic triglycerides compared to the control group (Ji and Gong, 2007).

More recently, a study into SPN and the treatment of atherosclerosis in rats found that the active compounds of this herb exhibited a considerable decrease in blood lipids and regulation of blood vessel activity. The precise mechanism was not determined; however, they believe the anti-inflammatory actions of SPN contribute to decrease in TG levels (Zhang *et al.*, 2008).

In this study, I found that SPN significantly inhibited lipolysis in 3T3-L1 cells, which in turn may reduce FFA release from adipocytes.

Research has suggested that the role of FFAs in type 2 diabetes has been most evident in obese patients who have several abnormalities in FFA metabolism, when the release of FFAs from the total adipose tissue depot to the blood stream is increased and the high concentration of circulating FFAs impairs muscle uptake of glucose. As a result, this ultimately causes a decline in glucose uptake (Arner, 2002).

Also, in type 2 diabetes, the ability of insulin to inhibit lipolysis and reduce plasma FFA concentrations is markedly reduced. It has been recognized that a chronic elevation of plasma FFA concentrations may increase the risk of insulin resistance in muscle and liver, and cause impairment in insulin secretion (Groop *et al.*, 1989).

3T3-L1 cells were utilized in this study to determine the effect of SPN on lipid metabolism and have found significant reduction in total lipid levels with SPN concentrations of 50 μg/ml ($p<0.05$), 100 μg/ml ($p<0.001$) and 200 μg/ml ($p<0.0001$). Upon examination of the lipid-stained images of SPN treated cells, it was revealed that insulin-stimulated SPN treated cells revealed a significant decrease in lipid deposits compared to the insulin-stimulated control cells.

Furthermore, a reduction in lipolysis was found with SPN. This indicates that SPN may reverse or ameliorate this pathogenesis and potentially be used for the treatment of diabetes and vascular complications.

TG undergoes lipolysis and is broken down into glycerol and fatty acids. Lipolysis of stored lipids takes place in adipose tisuue to release FFA. FFA flux and glycerol from adipose tissue to the blood stream primarily depends on the lipolysis of triacylglycerols in adipocytes. Hence, lipolytic activity is assessed by the measurement of glycerol released into the medium from TG breakdown in *in vivo* models.

Lipolysis has been found to be regulated by a number Of hormones, such as epinephrine, norepinephrine and glucagon. Catecholamine-induced lipolysis is well

characterized, initiated by stimulation of β-adrenergic receptors, which are coupled to activation of adenylyl cyclase by the heterotrimeric Gs protein, which in turn converts ATP to cAMP. cAMP- dependent protein kinase A (PKA) then phosphorylates two main targets, hormone-sensitive lipase (HSL), the primary lipase responsible for hydrolysis of triglycerides, as well as perilipin A, the coating protein of lipid droplets (Stich and Berlan, 2004).

Under physiological conditions, adipocyte lipolysis is stimulated by catecholamine hormones by elevating cellular cAMP content and activating cAMP-dependent protein kinase A (Londos *et al.*, 1999).

Lipolysis assay was carried out to determine the mechanism behind the reduction of lipid content in SPN treated cells. We found that cells treated with SPN concentrations of 50, 100 and 200 μg/ml were subject to a significant reduction in lipolysis. ($p<0.05$) Results indicate that insulin-stimulated SPN treatment embrace lipolytic inhibiting action upon 3T3-L1 adipocytes. Treatment of adipocytes with SPN attenuated lipolytic action, supporting that SPN may be used as an agent to decrease the circulating FFA levels and thus increase insulin sensitivity.

In summary, the results that have been attained from this study implicate that SPN plays an important role in the regulation of lipid metabolism. This further supports that SPN is a potential agent for the treatment and prevention of type 2 diabetes.

Chapter 4

Final Discussion Future Directions & Conclusion

CHAPTER 4: FINAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSION

4.1 Discussion

In recent years, particular interest has been targeted on the use of natural derivatives in an attempt to discover new therapeutic agents to treat type 2 diabetes and other metabolic disorders. Chinese herbal medicines have been identified to be a plausible candidate as groundwork studies into various herbal compounds have achieved promising results. They not only contain active compounds to lower glucose and lipid levels but possess minimal side effects unlike many of conventional oral medications that are being utilised in today's society.

Studies have revealed that most oral anti-diabetic agents that are currently administered to patients, trigger undesirable side effects such as, promoting weight gain- alleviating one symptom of type 2 diabetes while simultaneously aggravating other risk factors that may promote the onset of diabetic complications (Zhou *et al*, 2007). Current oral anti-hyperglycemic agents used to treat diabetes include sulfonylureas (eg glyburide, glipizide, glimepiride), alpha-glucosidase inhibitors (eg acarbose, miglitol), biguanides (eg metformin), thiazolidinediones (eg pioglitazone, rosiglitazone) and glinides (eg repaglinide, nateglinide) (Hall *et al.*, 2006).

Panax notoginseng, the herb employed in this study, has been used as a therapeutic agent in China and other parts of Asia to treat disorders analogous to the

complications associated with type 2 diabetes and other metabolic disorders for thousands of years.

There is diverse evidence that the active compound of the herb has a sanguine effect upon the treatment of diabetic complications in mainstream hospitals in China. SPN has been seen clinically to treat cardiovascular disorders such as coronary heart disease, stroke and atherosclerosis and also possess haemostatic, detumescent, anti-inflammatory and analgesic properties (Zang and Wang, 2006).

This has become a predominant concern as research has identified that patients with type 2 diabetes are at a high risk of myocardial infarction and stroke. The overall risk of cardiovascular disease is much higher than that of individuals without the disease. Therefore, it is crucial to develop new treatment strategies and options to treat these risk factors (Betteridge, 2007).

The aim of this study was to discover and evaluate the effects and mechanisms of SPN on glucose and lipid metabolism in 3T3-L1 cells. Results attained from this study show that SPN has a significant effect on insulin-stimulated adipocytes, whereby SPN significantly increased insulin-stimulated glucose uptake, GLUT4 translocation, glycogen synthesis, and significantly decreased lipid content and lipolysis in 3T3-L1 cells.

A study has explained that an increase in fat tissue and insulin resistance of adipocytes leads to increased lipolysis and release of by products such as FFA and cytokines from adipose tissue, eventually resulting in reduced glucose disposal and increased

hepatic glucose production. This stimulates the secretion of insulin to compensate for insulin resistance. Hyperinsulinemia further promotes insulin resistance at target sites through receptor desensitization and, indirectly, through its effects on lipogenesis. This will result in chronic hyperglycemia, down-regulated glucose-stimulated insulin secretion, ß-cell pathologies and development of type 2 diabetes (Uysal *et al.*, 2000).

Results in Chapter 2 show that SPN significantly enhanced insulin-mediated glucose uptake in 3T3-L1 adipocytes. SPN brought about an increase in glucose uptake, the rate-limiting step mediated by the insulin-responsive GLUT4 in adipocytes. SPN also up-regulated GLUT4 expression, whereby results from immunofluorescence imaging confirmed that SPN treatment significantly increased in GLUT4 content in the plasma membrane. This suggests that enhanced insulin-stimulated glucose uptake may be promoted through enhancing GLUT4 expression and translocation in 3T3-L1 cells. SPN treatment also enhanced the storage of glucose as glycogen in through the coordinate increase in glucose uptake. This result, together with results from glucose uptake and GLUT4 translocation, substantiate that SPN improves glucose metabolism.

Furthermore, the results in Chapter 3 confirm that SPN has a direct effect on lowering lipid levels and inhibiting lipolysis. The corresponding results attained from lipid staining and lipolysis assay of 3T3-L1 cells indicate the ability of SPN to regulate lipid metabolism. Lipid metabolism in adipocytes has great influence on the occurrence of type 2 diabetes and obesity. Hence, discovery of new agents, such as SPN, that is able to emulate insulin in lipid metabolism is strongly required for the development of novel anti-diabetic drugs.

4.2 Future Directions

This thesis has explored the effects of SPN on glucose and lipid metabolism *in vitro*. Promising results have been attained throughout the study, however, further study is needed; to elucidate and verify these preliminary results.

Studies have identified that fat accumulation is determined by the balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis). Lipogenesis encompasses the processes of fatty acid synthesis and subsequent TG synthesis, and takes place in both the liver and adipose tissue (Kerston, 2001). Lipid turnover in adipose tissue is determined by the relative rates of lipolysis and lipogenesis. Therefore, a lipogenesis study of SPN treatment in 3T3-L1 cells is required.

This study was carried out without utilising a conventional drug such as metformin or rosiglitizone, as a positive control. An additional *in vitro* study utilising a positive drug and multiple cellular models, will be useful in revising and confirming the results attained from insulin-stimulated glucose uptake, GLUT4 translocation, glycogen synthesis, lipid content and lipolysis.

Recent studies have shown that human adipose tissue is a major site for IL-6 secretion. IL-6 is increased in insulin resist states such as type 2 diabetes and obesity.

TNF-α is a mediator of insulin resistance in sepsis, obesity, and type 2 diabetes and is known to impair insulin signalling in adipocytes. Akt (protein kinase B) is a crucial signalling mediator for insulin. It has been shown to increase plasma TG and VLDL

concentrations, and increase lipolysis. Overexpression of TNF-α in fat cells from obese models has been discovered with a direct correlation to BMI and hyperinsulinemia (Rotter *et al*, 2003). TNF-α reduces insulin-stimulated receptor tyrosine kinase activity at low concentrations and at high concentrations, decreases IRS-1 and GLUT4 expression.

4.3 Conclusion

In conclusion, results attained in this thesis support that saponins of *Panax notoginseng* (SPN), may be used for regulating glucose and lipid metabolism in type 2 diabetic patients. This is evident in this *in vitro* study as significant improvement in insulin-mediated glucose and lipid metabolism in adipocytes has been discovered. The results also indicate that SPN is capable of enhancing insulin sensitivity, reducing blood glucose and lipid levels. SPN appears to be a potential agent for preventing the development of diabetic vascular complications, and upon further investigation may be used as therapeutic agent for type 2 diabetes.

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