CHAPTER 1

INTRODUCTION

Among the world’s largest growing and most common metabolic disorder is type 2 diabetes mellitus (T2DM). According to International Diabetes Federation, an estimated 381 million people suffer from diabetes globally with the prevalence of diabetes continuing to increase and by 2030 the diabetic population is estimated to become almost double (Wild et al 2004). Studies have shown insulin resistance to be a strong forecaster in the development of T2DM, which has been confirmed in a population study, based on subjects with T2DM (Shulman 2000; Ali & Fonseca 2012; Bonora et al 1998).

Insulin resistance and impaired fasting glucose are becoming common in developed countries and its prevalence is increasing rapidly. Insulin resistance is a pathophysiological condition in which the target cells fail to respond to ordinary levels of circulating insulin, resulting in an increase in blood glucose levels. Although, the biological mechanisms are intricate and complex, insulin resistance is believed to be manifested at the cellular level due to post receptor defects in insulin signaling. Despite promising findings in experimental animals with respect to insulin signaling defects, their significance to human insulin resistance is presently unclear.

Pathogenesis of insulin resistance is a highly complex phenomenon with various physiological factors involved, including hyperinsulinemia. Hyperinsulinemia is a common characteristic of several ethnic groups with a high prevalence of diabetes (Weyer et al 2000; Hannon et al 2008). Modan et al (1985)
and Rizza et al (1985) proposed that hyperinsulinemia, associated with peripheral insulin resistance, is linked to hypertension, obesity and glucose intolerance in humans. *In vivo* studies have observed insulin resistance to be linked to hyperinsulinemia (Martin 1983; Marban & Roth 1996). In the current study, the role of hyperinsulinemia in impaired insulin signaling and the molecular signaling events occurring during the process has been investigated.

1.1 **GLUCOSE HOMEOSTASIS**

The process of maintaining blood glucose at a steady state level is called “glucose homeostasis”, which is critical for normal physiological functioning (DeFronzo 1988). Hormone regulation of peripheral glucose uptake, hepatic glucose production and glucose uptake during carbohydrate ingestion maintains glucose homeostasis (Szablewski 2011). Glucose is a crucial metabolic substrate for energy production and many other anabolic process in all mammalian cells. Glucose is transported to various tissues by facilitated diffusion and stored as fatty acids, amino acids and glycogen by the organs or it is oxidized by the various catabolic pathways in cells.

1.1.1 **The Organs Regulating Glucose Homeostasis**

Among the various organs in the body, liver, kidney, skeletal muscle, adipose tissue and pancreas play a crucial role in glucose homeostasis (Dr. Brandt) (Figure 1.1).

The liver is the major metabolic regulatory organ. The liver is capable of producing glucose from substrates such as glycogen, lactate, amino acids and glycerol. About 90% of all circulating glucose is released from the liver. During low plasma glucose levels, the liver is the source of both glucose and the ketone bodies required by the brain.
Kidney also plays a major role in glucose homeostasis during prolonged starvation, wherein glucose is released into the blood by gluconeogenesis. The function of kidney is critical for glucose homeostasis since, plasma glucose continuously passes through the kidney and must be efficiently reabsorbed to prevent losses.

Skeletal muscle is a vital metabolic organ for glucose homeostasis, since it has the ability to rapidly increase its glucose uptake during high levels of plasma glucose levels. The skeletal muscle also maintains plasma glucose level by releasing free amino acids into circulation, which serve as substrates for liver gluconeogenesis.

The adipose tissue is a major site for glucose metabolism. In the adipose tissue the triacylglycerides are synthesized from glycerol phosphate and free fatty acids (FFA). During gluconeogenesis adipose tissue provides FFA and glycerol to the liver as substrate.

The pancreas is the source of insulin and glucagon, which are the most important metabolic regulatory hormones for maintaining glucose homeostasis. Insulin and glucagon hormones play a major role in avoiding postprandial and fasting hypoglycemia.

During periods of hyperglycemia, the β cells of the pancreatic islets of Langerhans secrete more insulin, which regulates glucose metabolism at many sites by stimulating glucose uptake in the peripheral tissue and reducing hepatic glucose output, via decreasing gluconeogenesis and glycogenolysis. Thus, insulin maintains appropriate plasma glucose levels. On the other hand, during periods of hypoglycemia, α cells of the pancreatic islets of Langerhans secrete more glucagon. This induces a catabolic effect, by activating the liver glycogenolysis and gluconeogenesis, which results in the release of glucose into the bloodstream, leading to an increase in blood glucose levels.
Figure 1.1  The organs that regulate plasma glucose levels (Dr. Brandt)

1.2  INSULIN

Insulin is coded on the short arm of chromosome 11 (Schroder & Zuhlke 1982). Initially, insulin mRNA translates as a single chain precursor pro-pre-pro-insulin, which enters into the endoplasmic reticulum (ER) as pro-insulin by removal of its signal peptide. Pro-insulin consists of three domains: an amino terminal B chain, a carboxy terminal A chain and a connecting peptide at the centre known as the C peptide. In the ER, the aqueous zinc and calcium rich environment favors formation of soluble zinc containing proinsulin hexamers.

Endopeptidases convert pro-insulin to mature form of insulin and C peptide. Insulin is the zinc containing hexamer (Figure 1.2), which is insoluble and can be precipitated as chemically stable crystals at pH 5.5 (Chang et al 1997). When β cells are stimulated appropriately, mature granules are secreted into
circulation by exocytosis wherein insulin and an equimolar ratio of C-peptide are released (Wilcox 2005).

**Figure 1.2  Structure of human insulin - (PDBID: 1AI0)**

Six insulin molecules assembled in a hexamer, the zinc ion holding it together (red sphere), and the histidine residues (pink sticks) involved in zinc binding.
1.2.1 Factors Influencing Insulin Biosynthesis and Release

The secretion of insulin is influenced by alterations in synthesis at the level of gene transcription, translation and post translational modification in the Golgi apparatus or the release of insulin from secretory granules. The longer term modification may occur via influence on \( \beta \) cell mass and differentiation. Glucose exhibits multiple influence on insulin biosynthesis as well as on secretion, since insulin is known to play a major role in glucose utilization and metabolism.

Other factors such as amino acids, fatty acids, acetylcholine, pituitary adenylate cyclase activating polypeptide (PACAP), glucose dependent insulinotropic polypeptide (GIP), glucagon like peptide-1 (GLP1), vasoactive intestinal peptide (VIP) and several other agonists, together in combination, also exhibit an influence.

1.2.2 Physiology of Insulin Secretion

Insulin is an essential hormone that regulates glucose homeostasis in blood and is secreted by the pancreatic \( \beta \) cells in response to appropriate stimulation, with glucose being the principal stimuli. Transcription of the insulin gene and translation of the insulin mRNA are stimulated by glucose. An acute increase in glucose level largely affects secretion of preformed insulin from secretory vesicles and mRNA translation and stability, while a chronic increase in glucose level increases transcription of insulin mRNA.

Entry of glucose into the \( \beta \) cell is through the GLUT 2 transporters, which is sensed by glucokinase leading to the phosphorylation of glucose to glucose 6 phosphate (G6P), generating ATP and leading to the closure of the \( K^+ \) ATP dependent channels.
This results in membrane depolarization and activation of voltage dependent calcium channels, leading to an increase in intracellular calcium concentration. An increase in cytosolic calcium (Ca\(^{+2}\)) leads to exocytosis of membrane docked granules from the readily releasable pool.

Thus, the first phase of insulin release occurs within the first few minutes of exposure to elevated glucose level. The slower second phase of secretion results of recruitment of granules from reserved pool to the readily releasable pool. The amplifying action of glucose elevates the cytosolic Ca\(^{+2}\), which serves to replenish the readily releasable pool with insulin secretory granules thereby inducing insulin secretion (Henquin et al 2006). In addition to K\(^+\) ATP dependent pathway G6P directly stimulates exocytosis of secretory granules (Figure 1.3).

Hormones such as VIP, GIP and GLP1 also stimulate insulin secretion. These mediators increases cyclic adenosine monophosphate (cAMP) levels in \(\beta\) cell, which stimulates insulin exocytosis through protein kinase A (PKA) activation. Muscarinic acetylcholine receptors expressed by pancreatic beta cells also plays an important role in insulin secretion.

Binding of acetylcholine to muscarinic acetylcholine receptors activates di-acyl glycerol (DAG). Increased levels of DAG stimulates protein kinase C (PKC) activity, which potentiate insulin secretion. These hormones play a major role in the second phase of glucose mediated insulin secretion (Bratanova-Tochkova et al 2002) (Figure 1.3).
Figure 1.3 Insulin secretion pathway (Wilcoxon 2005).
1.3 INSULIN SIGNALING PATHWAY

The insulin action is mediated by the insulin receptor (IR), a complex multi subunit cell surface glycoprotein consisting of two extracellular α subunits and two transmembrane β subunits. Binding of insulin to a subunit of IR activates the tyrosine phosphorylation of β subunit, which results in the catalytic activity of kinase. As a result of IR phosphorylation, a number of intracellular substrates such as insulin receptor substrates (IRSs), GRB2-associated-binding protein (Gab-1), casitas b-lineage lymphoma (Cbl), Shc isoforms and signal regulatory protein (SIRP) family members get activated (Saltiel & Pessin 2003; Chang et al 2004).

The IRS proteins interact with the p85 regulatory subunit of phosphoinositol 3-kinase (PI3K), leading to an increase in the catalytic activity of p110. This activation triggers the conversion of intracellular phosphatidylinositol 3, 4-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Shepherd 2005). This, in turn, activates the serine/threonine protein dependent kinase (PDK1), which further phosphorylates and activates protein kinase B (PKB/Akt) and PKC (Mora et al 2004), leading to the translocation of glucose transporter 4 (GLUT4) from an intracellular pool to the plasma membrane and facilitating glucose uptake (Huang & Czech 2007).

In addition to glucose transport, insulin stimulates glycogen, lipid and protein synthesis. It has been demonstrated that insulin dependent activation of Akt triggers glycogen synthesis. Akt inhibit glycogen synthase kinase 3 β (GSK3β) by phosphorylation, subsequently, leading to an activation of glycogen synthase which converts excess glucose to glycogen (Cross et al 1995). Insulin stimulated Akt effectively activates the mammalian target of rapamycin (mTOR), which regulates protein synthesis by phosphorylating the proteins P70 ribosomal
protein S6 kinase (P70S6K) and eukaryotic translation initiation factor 4E binding protein1 (4E-BP1) (Harris & Lawrence 2003).

In adipocytes insulin activated mTOR plays a critical role in inducing adipogenesis and lipogenesis by provoking the translation of peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer binding protein (C/EBP) mRNA, which are key components of the adipogenic process (Cho et al 2004). C/EBP-β and C/EBP-δ trigger the expression of C/EBP-α, which in turn induces the expression of PPARγ, a member of the nuclear receptor superfamily of ligand activated transcription factors. The activation of PPARγ leads to profound changes in gene expression that ultimately lead to the stimulation of lipogenesis. The mTOR promotes lipid synthesis by increasing cleavage and activation of transcription factor sterol regulatory element binding protein-1 (SREBP-1) (Laplante & Sabatini 2009) (Figure 1.4).

Insulin stimulated glucose transport also occurs by a CAP/Cbl pathway, which is essential along with PI3K dependent pathway (Baumann et al 2000). The IR activated by insulin binding, phosphorylates tyrosine residues of proto-oncogenes Cbl. The activated Cbl forms a complex with 2 adaptor proteins, the adapter protein with pleckstrin homology and Src homology 2 domains (APS) and Cbl associated protein (CAP), which is recruited to lipid raft (Liu et al 2002). Within the lipid raft, tyrosyl-phosphorylated Cbl recruits the adapter protein CrkII as a complex with C3G via an interaction between the SH2 domain in CrkII and a tyrosine phosphorylation site in Cbl. C3G, a guanine nucleotide exchange factor for small molecular weight GTPases, activates the small G protein TC10 (Prada et al 2006). These associations, direct the fusion of the GLUT4 vesicle with the plasma membrane and facilitate glucose uptake. It has been suggested that CAP/Cbl complex plays a crucial role in insulin stimulated glucose uptake through a PI3K independent pathway (Figure 1.4).
Figure 1.4  Insulin Signaling Pathway.
1.4 GLUCOSE TRANSPORTER PROTEINS

Glucose transporters (GLUT) are a group of membrane proteins that facilitates the transport of glucose across the plasma membrane in various cell types. These proteins are a family of sugar transporter proteins containing 12-transmembrane domains that mediate the passive (i.e. non energy dependent) transport of glucose. Currently, 13 members of GLUT have been identified, which have been named in their order of discovery.

Each glucose transporter isoform plays a specific role in glucose metabolism determined by its pattern of tissue expression, substrate specificity, transport kinetics and regulated expression in different physiological conditions (Thorens 1996). Based on sequence similarities and binding affinity, GLUTs have been classified into three classes (Cura & Carruthers 2012).

1.4.1 Class I Transporters

These comprise the high affinity binding proteins GLUT1, GLUT3, GLUT4 and the lower affinity transporter GLUT2. The principal glucose transporter protein that mediates glucose uptake is GLUT4. It is found primarily in muscle and adipose tissue, where it is normally sequestered in intracellular vesicles. In response to insulin, it translocate to the plasma membrane resulting in an enhanced glucose uptake. In addition to insulin other hormones like cortisol affects GLUT4 gene transcription and its localization. The transcription of GLUT4 gene is increased by high levels of glucocorticoids and inhibited by high levels of insulin. However, GLUT4 expression is also reduced by low insulin states, such as in muscle during fasting and in insulin resistant adipose tissue.
GLUT1 and GLUT3 are found in most tissues and are especially important in the transport of glucose into the brain. They have a high affinity for glucose and transport glucose efficiently throughout the normal range of plasma glucose concentration. GLUT2 is found primarily in the pancreas and liver. It has a low affinity for glucose and therefore mediates glucose transport only during high plasma glucose levels. GLUT2 is the transporter that is responsible for allowing the β cells to sense hyperglycemia and for transporting high glucose into the liver for storage.

**1.4.2 Class II Transporters**

This comprises GLUT5, GLUT7, GLUT9 and GLUT11. They have a very low affinity for glucose and preferentially transport fructose. GLUT5 is found in gut, liver and is thought to function primarily as a fructose transporter. GLUT7 is an intracellular liver protein responsible for G6P transport into the ER. GLUT9 is present in spleen, peripheral leukocytes and brain. GLUT11 is found in the heart, skeletal muscle, placenta and kidney.

**1.4.3 Class III Transporters**

This class comprises of four novel GLUTs, GLUT6, GLUT8, GLUT10, and GLUT12 which particularly transport glucose, similar to class I transporters and are mostly expressed in spleen, leukocytes and brain.

In kidney and intestine glucose transport occurs against a concentration gradient through the activation of active glucose pumps. In contrast to the passive transport mediated by GLUT gene products, these pumps are energy dependent and are responsible for the absorption of glucose from the diet and the absorption
of glucose in the kidney. Kidney pump becomes saturated at plasma glucose concentrations above 180 mg/dL and ends up in the urine.

1.5 ACTION OF INSULIN AT THE CELLULAR LEVEL

Insulin’s action at the cellular level encompass carbohydrate, lipid and amino acid metabolism and mRNA transcription and translation.

1.5.1 Carbohydrate Metabolism

Insulin regulates carbohydrate metabolism at multiple steps. In liver, muscle and adipose tissue, insulin facilitates glucose transport by GLUT4 transporter. It is involved in the control of the physiological balance between glucose absorption from the intestine, production by the liver and uptake and metabolism by peripheral tissues. Insulin stimulates glycogen synthesis and decreases glycogen breakdown, in addition to stimulation of glycolysis and inhibition of gluconeogenesis. Insulin enhances the irreversible conversion of pyruvate to acetyl Co-A by activation of the intra mitochondrial enzyme complex pyruvate dehydrogenase. Acetyl-CoA may then be directly oxidised via the Krebs’ cycle, or used for fatty acid synthesis (Denton & Tavare 1997; Wilcox 2005).

1.5.2 Lipid Metabolism

Insulin plays a vital role in lipid metabolism by stimulating lipogenesis and inhibiting lipolysis. Insulin induces storage of extra glucose in the form of triglycerides in adipose tissue and liver. It increases fatty acid synthesis by increasing phosphorylation of acetyl-CoA carboxylase and simultaneously suppresses fatty acid oxidation by inhibition of carnitine acetyltransferase. Insulin stimulates triglyceride synthesis by esterification of glycerol phosphate and
inhibits triglyceride breakdown by dephosphorylation of hormone sensitive lipase. Cholesterol synthesis and phospholipid metabolism are also regulated by insulin (Hunter & Garvey 1998; Wilcox 2005).

1.5.3 Protein Synthesis

Insulin promotes protein synthesis in a variety of tissues and has an effect on the transcription of specific mRNA, as well as translation of mRNA into proteins in the ribosomes. Insulin action enhances mRNA transcription of pyruvate carboxylase in the adipose tissue, glucokinase, fatty acid synthase and albumin in the liver, casein in the mammary gland and amylase in the pancreas (Hunter & Garvey 1998). Insulin action also decreases transcription of some proteins, such as liver enzymes carbamoyl phosphate synthetase, a key enzyme involved in the urea cycle. Effects on translation are widespread and influenced by insulin and various growth factors (Wilcox 2005).

1.6 Physiological Role of Insulin

Insulin is an important hormone involved in the regulation of cellular energy and macronutrient balance along with playing a significant role in the intracellular transport of glucose to insulin dependent tissues such as muscle and adipose tissue. In muscle cells, insulin stimulates to store glucose in the form of glycogen via activation of glycogen synthase and to be utilised as the immediately available energy source for muscle contraction. In adipose tissue insulin promotes synthesis of triglycerides and increases the uptake of triglycerides from the blood. In addition, insulin suppresses the rate of lipolysis and hence lowers plasma free fatty acid level. Therefore, insulin promotes glycogen and lipid synthesis, while suppressing lipolysis and gluconeogenesis.
1.7 INSULIN RESISTANCE

Insulin resistance is a pathophysiological condition in which the peripheral tissue fails to respond to physiological levels of insulin, resulting in an increased blood glucose level, which has been identified as a key factor in the development of T2DM (Mlinar et al 2007). The effect of insulin resistance on various tissues and organs vary depending on their function and their dependence on insulin for their metabolic processes. Insulin resistance results in an impaired carbohydrate and lipid metabolism leading to the progression of metabolic disorders. The function of insulin is influenced by an interplay with other hormones.

Insulin induced hypoglycemia is prevented by many other hormones, including the growth hormone, the counter regulatory hormones such as glucagon, glucocorticoids and catecholamines, which regulate glucose homeostasis during the fasting state. Excess secretion of these hormones also may contribute to insulin resistance in particular settings, but does not account for the vast majority of insulin resistant states. In spite of intensive research the mechanisms of development of insulin resistance is still elusive (Matthaei et al 2000).

1.7.1 Insulin Resistance Disrupts Insulin Secretion

Increasing evidence show a dynamic association between insulin resistance and a compensatory increase in β cell mass and β cell glucose metabolism. When the compensatory process is adequate, normal glucose tolerance is maintained. When β cell compensation fails, glucose levels rise, leading to either impaired glucose tolerance or is manifested as diabetes (Cavaghan et al 2000). It has been reported that mutation of the IRS2 gene results in impaired insulin secretion causing peripheral insulin resistance and diabetes.
The inflammatory cytokines secreted by adipocytes due to different metabolic stresses disrupt insulin signaling in organs such as pancreas, leading to systemic insulin resistance and disrupting insulin secretion (Shimizu et al 2013).

1.7.2 **Insulin Receptor (IR)**

Since, insulin stimulates glucose uptake and its metabolic functions through insulin downstream signaling, insulin resistance could be associated with defects in insulin signaling cascade at a cellular level (Shulman 2000). Insulin receptor is one of the key targets that enhances insulin signaling and restores glucose transport (Moller 2001). Any alterations in insulin receptor and post receptor signaling events could be a major cause of insulin resistance. The defect in the insulin receptor kinase activity is acquired due to obesity and metabolic changes such as hyperinsulinemia and hyperglycemia (Matthaei et al 2000).

Some studies have shown that individuals with rare genetic defects in the IR, exhibit an inhibition in ligand binding and tyrosine kinase activity leading to severe insulin resistance. Several reports have also shown T2DM patients to exhibit a reduced auto activation status of the IR in skeletal muscle and adipocytes (Thies et al 1990; Nolan et al 1994).

1.7.3 **Insulin Receptor Substrate (IRS)**

The IRS1 and IRS2 proteins have adaptor function between the IR and other cellular substrates such as the PI3K. Phosphorylation of IRS at tyrosine residues by insulin stimulated IR is an important event for insulin stimulated glucose transport. Therefore, any defect in IRS phosphorylation leads to an impaired glucose uptake. Adipocytes from diabetic and insulin resistant individuals have shown impaired insulin signaling via reduced expression of IRS1 at the gene and protein level (Smith 2002). Serine phosphorylation of IRS by
stress kinases and proinflammatory cytokines reduce its ability to interact with PI3K thereby inhibiting glucose uptake. Mice with IRS2 silencing have shown insulin resistance in both peripheral tissues and liver, along with impaired insulin secretion (Tamemoto et al 1994).

1.7.4 Phosphatidyl Inositol 3 Kinase (PI3K)

The insulin dependent tyrosine kinase activation of IRS proteins provides docking sites for many Src homology 2 (SH2) domain proteins like PI3K. IRS1 binds with p85 regulatory subunit of PI3K and translocate the catalytic p110 subunit to the plasma membrane, which catalyzes the phosphorylation of PIP2 to PIP3. Earlier studies using pharmacological inhibitors, microinjection of blocking antibodies and constitutively active mutants have shown the activation of PI3K to be necessary for insulin stimulated GLUT4 translocation (Czech & Corvera 1999). Some reports have also shown insulin stimulated glucose uptake to be possible in a PI3K independent pathway (Ribon et al 1998).

1.7.5 Protein Kinase B and Protein Kinase C (Akt and PKC)

The major target of PI3K is a serine threonine kinase Akt. PI3K phosphorylates PIP2 to PIP3, resulting in the recruitment of Akt/PKB and PDK1 to the plasma membrane. Activated Akt regulates several protein kinases involved in glucose transport, glycogen synthesis and protein synthesis (Marte & Downward 1997). The PKB activates PKC isoforms ζ and λ, which are involved in the translocation of the GLUT4 from cytoplasmic vesicles to the cell membrane. In type 2 diabetic patients, a decreased expression and phosphorylation level of early insulin signaling targets, the IRS, PI3K, and PKB have been demonstrated in insulin target tissues. Although, inhibition of PKB in
skeletal muscle of type 2 diabetic patients has been reported (Krook et al 1998), there have also been other reports which have described normal PKB/Akt activation in skeletal muscle of type 2 diabetic patients (Kim et al 1999). Matthaei et al (2000) has reported possible gene mutations of PI3K and Akt responsible for insulin resistance.

1.7.6 Glucose Transporter 4

Glucose enters into the cell through facilitated diffusion by means of glucose transporters (GLUT). Among the different GLUTs that have been identified, the principal glucose transporter protein that mediates glucose uptake in muscle and adipose tissues is GLUT4. The role of GLUT4 as a dominant regulator of glucose homeostasis has been well established (Huang & Czech 2007). In contrast to the other GLUT isoforms, which are primarily localized to the cell surface membrane, GLUT4 transporter proteins are sequestered into specialized storage vesicles (Pessin & Saltiel 2000).

As post prandial glucose levels rise, there is an increase in the circulating insulin, leading to the activation of intracellular signaling cascades and the translocation of GLUT4 storage compartments to the plasma membrane through a process of exocytosis. In T2DM, the decreased insulin sensitivity in adipocytes is due to a decreased expression of GLUT4 (Shepherd & Kahn 1999). Abel et al (2001) has shown GLUT4 genetically engineered mice, exhibiting insulin resistance and an overall impaired insulin sensitivity.

1.7.7 c-Cbl Associated Protein (CAP)

Insulin stimulated glucose uptake can be distinctly divided into two types: CAP/Cbl pathway (PI3K independent) and PI3K pathway (Michell 2006). CAP/Cbl complex is a necessary second signal that functions in parallel with the
activation of the PI3K dependent signaling pathway. Previous studies have shown that 3T3-L1 adipocytes with CAPΔH3 mutant decreased 50% insulin stimulated glucose uptake without affecting PI3K activity (Baumann et al 2000). The proto-oncogene, cbl has been implicated to play a role in insulin mediated glucose transport (Saltiel & Pessin 2003). These studies indicate that the CAP/Cbl complex plays a critical role in insulin stimulated glucose transport along with the PI3K dependent pathway.

1.7.8 Obesity Induced Insulin Resistance

One of the crucial factors in the development of insulin resistance is obesity, a chronic disease of appetite regulation and energy metabolism. At the cellular level, obesity is characterized by an excess of fat cell numbers, sizes, and/or fat accumulation in adipocytes (Cao et al 2008). An increasing body mass index, waist circumference and in particular waist-hip ratio implicates to insulin resistance (Aronne & Segal 2002). It has also been reported that lipid derivatives such as DAG and ceramides accumulated due to increased lipolysis impairs insulin signaling (Schenk et al 2008). During obese condition, the dysfunctional lipid metabolism increases free fatty acid turnover in the blood, leading to insulin resistance at a cellular level via cell surface pattern recognition receptors (PRRs) (Shi et al 2006). The increased FFA circulation in the blood also activate inflammatory pathways which exhibit a negative effect on insulin signaling (McArdle et al 2013).

Obesity induced inflammation increases the risk of developing T2DM and the metabolic syndrome. Inflammation is a critical mediator of insulin resistance, wherein, an influx of immune cells to the adipose tissue initiate a cascade of inflammatory events leading to impaired insulin signaling. Adipocytokines such as TNF-α (tumor necrosis factor alpha), IL (interleukin) -6,
PAI-1 (plasminogen activator inhibitor-1), angiotensinogen, leptin and resistin secreted by adipocytes during obesity are associated with the pathogenesis of the metabolic syndrome (Kusminski et al 2005). Reactive oxygen species (ROS) activated stress kinases also play a role in the development of obesity induced insulin resistance (Furukawa et al 2004). The mechanism involved in the pathogenesis of obesity induced insulin resistance has not been completely understood. Reports have suggested that anti-obesity drugs effectively decrease lipid accumulation, but does not exhibit any effect on improving insulin sensitivity. New therapeutic drugs developed that target multiple signaling pathways could be the best method for treatment.

1.7.9 Reactive Oxygen Species and Insulin Resistance

There is considerable evidence that an increased ROS level leads to oxidative stress, which is a key pathogenic factor responsible in the development of metabolic disorders. The sources of ROS are mitochondrial and extra mitochondrial. Oxidative stress has also been implicated in the pathophysiology of insulin resistance both in animals and in cultured cells (Samuel & Shulman 2012). An imbalance in the oxidative stress and antioxidant system leads to the activation of stress sensitive signaling pathways, such as nuclear factor kappa B (NFkB), p38 mitogen-activated protein kinases (p38 MAPK), c-Jun N-terminal kinases (JNK), PKC and sorbitol, causing cellular damage and ultimately responsible for the long-term complications of T2DM. Earlier reports have shown that hyperglycemia and elevated levels of FFA induced oxidative stress leads to insulin resistance through an upregulation of JNK and p38MAPK (Evans et al 2002). In vivo studies have also shown that hyperglycemia induced oxidative stress plays a crucial role in the pathogenesis of late diabetic complications (Nishikawa et al 2000).
Studies have shown the individual contributions of extracellular signal regulated kinase (ERK), p38 and JNK pathways activated by oxidative stress in inducing insulin resistance via distinctly different molecular mechanisms. A strong contribution of ERK was observed in inducing insulin resistance, than those of p38 and JNK by markedly suppressing the expression of IR, IRS and GLUT4 (Fujishiro et al 2003). Aguirre et al (2000) have observed that insulin stimulated Erk1/2 and JNK enhance the phosphorylation of IRS1 on serine 307 and inhibit its interaction with the insulin receptor along with the degradation of the IRS1 protein. Bard-Chapeau et al (2005) have shown that Erk1/2 attenuates insulin signaling via serine 612 phosphorylation of IRS1. The ROS resulting from ER stress activates pro-inflammatory pathways that include JNK and IKK, which suppress the insulin signaling (Hotamisligil 2010) (Figure 1.5).

Figure 1.5  Involvement of ROS in insulin resistance.
Although antioxidant therapy may prevent oxidative stress induced diabetic complications. The classical antioxidants, such as vitamins E and C, are not known to improve insulin sensitivity. Studies have shown that some antioxidants such as α-lipoic acid (LA), N-acetyl cysteine (NAC), super oxide dismutase and catalase inhibit the oxidative stress induced diabetic complications and improves insulin sensitivity (Henriksen 2000; Fulghesu et al 2002; Ceriello 2003). However, despite this strong evidence, the usefulness of antioxidants in preventing diabetic complications is still elusive (Ceriello & Testa 2009). Identification of antioxidants that improve insulin sensitivity along with controlling free radical production might lead to prevention, reversal or delay the onset of the resultant pathologies.

1.7.10 Inflammation and Insulin Resistance

Sedentary life style, genetically modified environmental factors, poor nutrition, obesity and infection trigger chronic inflammation is associated with the development of T2DM and its complications (King 2008). An increased production of proinflammatory cytokines like TNFα, IL-1 and resistin induce impaired glucose metabolism by activating JNK and NFκB signalling through a feed-forward mechanism. It has been reported that oxidative stress induced p53 activation causes NFκB dependent induction of inflammatory cytokines, which accelerates the development of insulin resistance (Minamino et al 2009).

Cytokines such as TNF α and IL-6 activates the phosphatase SH2-containing inositol 5-phosphatase 2 (SHIP2) and signal transducer and activator of transcription 3 (STAT3) causing an increased expression of suppressor of cytokine signalling 3 (SOCS3), which leads to insulin resistance by IRS1 serine phosphorylation (Kim et al 2004; Lebrun & Van Obberghen 2008). Obesity and
T2DM share the same metabolic situation which is characterized by insulin resistance and chronic inflammation.

Therefore, drugs that directly target inflammation may treat or prevent insulin resistance, T2DM and other metabolic conditions. These approaches may provide clinical benefits to a large population affected by the cluster of metabolic disorders (Shoelson et al 2006).

1.7.11 Inflammation Leading to Insulin Resistance in Liver, Muscle, and other Organs during Diabetes

Reports have related inflammatory response and the development of insulin resistance in 2 different ways. Primarily, inflammatory signaling intermediates may directly induce insulin resistance by activation of serine phosphorylation of IRS1. Alternatively, inflammatory cell infiltration within adipose tissue may be involved in altering lipid metabolism as well as affecting cytokine production by adipose tissue, which in turn alters insulin action in other metabolically important tissues (Hotamisligil 2003; Savage et al 2005) (Figure 1.6).

Inflammatory cytokines like TNFα, ILs secreted by adipocytes due to different metabolic stress conditions disrupt insulin signaling in organs such as skeletal muscle and the liver, leading to systemic insulin resistance and the onset of diabetes mellitus (Shimizu et al 2013).

Obesity induced inflammation is associated with development of hepatic insulin resistance (Lanthier et al 2010). Deregulation of adipokines and non-esterified fatty acids (NEFA) secreted by hyperinsulinemia induced human adipocytes have been observed to cause insulin resistance in myocytes
(Fernandez-Veledo et al 2008). Proinflammatory cytokines such as TNF-α and IL-1β induce insulin resistance by activating JNK and IKKβ/NFκB pathways. Concordantly, genetic or chemical inhibition of either JNK or IKKβ/NFκB can improve insulin sensitivity (Shoelsone et al 2006).

Figure 1.6 Critical role of adipose tissue inflammation in insulin resistance.

1.7.12 Molecular Targets Involved in Linking Diabetes, Inflammation and Insulin Resistance

1.7.12.1 Jun kinase

The cJun NH2-terminal kinases (JNKs) comprise of ten isoforms encoded by three genes: JNK1, JNK2, and JNK3 (Waetzig & Herdegen 2005).
The JNK1 which is activated by inflammatory cytokines and FFA inhibit insulin action and have been implicated in the development of T2DM (Hirosumi et al 2002). Stress activated JNK1 inhibits insulin signalling by phosphorylating serine and threonine residues of IRSs. The JNK signaling pathway contributes to inflammation and plays a key role in the metabolic response to obesity, including insulin resistance (Han et al 2013). The JNK pathway has been reported to play a central role in the progression of insulin resistance and also in the reduction of insulin biosynthesis due to β cell dysfunction (Kaneto 2005). Consequently, JNK could be a potential therapeutic target for diabetes. Inhibition of JNK by gene disruption or pharmacological inhibitors would be a healthier approach for protection against obesity induced insulin resistance (Hirosumi et al 2002; Kaneto 2005; Tuncman et al 2006).

1.7.12.2 IKK β

Inhibitor of nuclear factor kappa-B kinase subunit beta (IKK β) is essential for NFkB activation in response to proinflammatory stimuli. In response to various stress conditions, IKK β gets phosphorylated, and undergoes polyubiquitination which targets them for rapid degradation by the 26S proteasome. This results in the separation and translocation of NFκB to the nucleus to activate genes that mediate innate immune and inflammatory responses (Karin & Delhase 2000). Incubation of L6 and C(2)C(12) skeletal muscle cells with saturated fatty acid was observed to induce insulin resistance through NFκB activation (Hommelberg et al 2009). Shoelson et al (2003) have reported the involvement of IKKβ/NFκB pathway in the pathogenesis of insulin resistance in high fat diet fed mice.

It has also been demonstrated that TNFα-IKKβ-mediated inactivation of TSC1 resulted in an impaired insulin induced glucose uptake by increasing
serine phosphorylation of IRS1 (Lee et al 2008). Mice treated with salicylates and mice with gene deletion (Ikk β+/−) showed improved insulin sensitivity in insulin resistant rodent models (Shoelson et al 2003). Furthermore, high doses of salicylates (aspirin or salicylate) improved insulin sensitivity in patients with T2DM (Hundal et al 2002). Hence, IKKβ represents a new target for treating insulin resistance.

1.7.12.3 TNF α

A number of studies in humans, animals and *in vitro* systems have shown substantial evidence linking TNFα induced insulin resistance. Exposing 3T3-L1 adipocytes to TNFα for a prolonged time causes a substantial reduction in the mRNA and protein expression of IRS1 and GLUT4 (Stephens et al 1997). It has also been reported that TNFα secreted by adipose tissue is associated with obesity induced insulin resistance (Hotamisligil et al 1993). Various studies have suggested the involvement of some inflammatory factors such as cytokines TNF-α, IL-1 and IL-6 in the development of insulin resistance. These factors lead to the activation of signaling pathways that ultimately impair insulin signaling (Hirabara et al 2012).

1.8 HYPERINSULINEMIA

An excess level of circulating insulin in blood, termed as “hyperinsulinaemia”, is a hallmark of peripheral insulin resistance and has been linked with a variety of abnormalities and clinical syndromes, including T2DM, cardiovascular disease, hypertension, obesity, dyslipidemia and glucose intolerance (Dankner et al 2009; Chaoyang et al 2006). The metabolic changes that commence with hyperinsulinemia develops a condition of insulin resistance, preceding obesity and T2DM. However, the relationship between
hyperinsulinemia, insulin resistance, obesity, and T2DM remains unclear (Gray et al 2010; Le Stunff & Bougneres 1994; Shanik et al 2008; Zimmet et al 1991).

1.8.1 Origin of Hyperinsulinemia

Insulin at a physiological level regulates glucose homeostasis, but a persistently elevated level impairs glucose metabolism by desensitizing the target cells through multiple mechanisms. Consumption of high glycemic foods causes a rapid raise in the level of insulin to meet the demand of blood sugar levels. This sets the stage for hyperglycemia (high blood glucose) and hyperinsulinemia (high blood insulin) which leads to the consequent onset of insulin resistance. Genetic predisposition, dietary habits, rapidly changing lifestyle, physical inactivity and migration are contributory factors for the high prevalence of hyperinsulinemia and insulin resistance in Indians. Recently, it has been reported that dietary ω-6 PUFAs intake is significant independent predictors of fasting hyperinsulinaemia in young Asian Indians (Isharwal et al 2009). Studies have shown that in normoglycemia subjects with fasting hyperinsulinemia causes an increased risk for development of glucose dysmetabolism (Dankner et al 2009).

1.8.2 Hyperinsulinemia - Insulin Resistance

Hyperinsulinemia leads to generalized insulin resistance and is associated with disturbances in glucose metabolism, which appears to be part of the vicious cycle involved in the pathogenesis of T2DM (Shanik et al 2008). Several in vitro and in vivo studies have reported that hyperinsulinemia leads to a desensitization of insulin receptor to subsequent responses through multiple mechanisms (Gonzalez et al 2011; Fernandez-Veledo et al 2008; Marban & Roth 1996). In 1983, Martin et al have demonstrated that administration of high doses of NPH insulin in rats showed a defect at the insulin receptor and postreceptor
level, with a corresponding decrease in insulin sensitivity. Rizza et al (1985) have shown patients treated with increasing doses of insulin to exhibit both hyperinsulinemia and insulin resistance. Rat adipocytes exposed to continuous hyperinsulinemia showed an impaired glucose transport through loss of insulin receptor, as well as a marked postreceptor defect (Marshall & Olefsky 1980).

In the human adipocytic cell line, prolonged insulin treatment impaired insulin signaling at the IRS1/Akt level and GLUT4 translocation to the plasma membrane. Furthermore, decreased adiponectin and leptin levels, increased the expression of proinflammatory cytokines, such as MCP-1 and IL-6, as well as NEFA release during hyperinsulinemia (Fernandez-Veledo et al 2008). Mehran et al (2012) have confirmed using genetically modified animal models that circulating hyperinsulinemia drives diet induced obesity and its complications. In pancreatic β cell leptin receptor deficient mice hyperinsulinemia was observed to cause obesity associated insulin resistance (Gray et al 2010).

Insulin mediates its metabolic and mitogenic functions through its downstream signaling IRS/PI3K/Akt, and any impairment in the insulin downstream signaling is a hallmark of insulin resistance. In vitro studies have shown hyperinsulinemia induced insulin resistance to be associated with dysregulated PI3K/Akt and an impaired GLUT4 translocation (Ricort et al 1995; Maier & Gould, 2000). Interestingly, Minamino et al (2009) have observed that mice with an excessive calorie diet intake, led to glucose intolerance through increased expression of oxidative stress induced p53. Inhibition of p53 activity showed significantly better insulin sensitivity, glucose tolerance and normalization of cytokine, macrophage marker expression by adipose tissue. Shimizu et al (2012) have shown that adipocyte specific p53 knockout in mice improved insulin sensitivity and glucose tolerance. These studies indicate a
significant role played by adipose tissue p53, which could be a good strategy for the treatment of diabetes.

1.9 p53

p53 is a stress activated transcription factor, also known as protein 53 or tumor protein 53.

Figure 1.7 Stress induced p53 leads to cell death, cell cycle arrest, senescence, metabolic alterations and autophagy (Loughery & Meek 2013).
P53 play a crucial role in multicellular organisms, and are also termed as “guardian of the genome or tumor suppressor” as they are involved in the regulation of cell cycle by defending against tumor development (Rubbi & Milner 2003). p53, plays a significant role in cell cycle regulation, in response to various stresses such as DNA damage, hypoxia and oxidative stress in many cell types (Vogelstein et al 2000). Any mutations in p53 suppress its transcription activity, leading to tumourigenesis (Vousden & Lu 2002). The effect of p53 in altering metabolism has consequences not only in cancer, but in various aspects of metabolic disorders (Vousden & Ryan 2009) (Figure 1.7).

### 1.9.1 Biochemistry of p53

p53 is encoded by the TP53 gene in humans (Kern et al 1991; McBride et al 1986) and is a sequence specific DNA binding protein. p53 get activated upon stress and controls the expression of various genes involved in the regulation of elimination of damaged protein, DNA repair, ATP generation via oxidative phosphorylation, organelle functions that maintain autophagy signaling and mitochondrial function, the cell division cycle, and the programmed cell death (Vousden & Ryan 2009; Puzio-Kuter 2011). Thus, stress activated p53 maintains cell and tissue integrity and contributes to the health and viability of the organism. In unstressed cells, p53 is maintained at a basal level through ubiquitinylated proteolysis regulated by MDM2 (Ogawara et al 2002; Maclaine & Hupp 2009).

The modification of p53 by multisite phosphorylation has been suggested as an important link between stress signaling and regulation of p53 activity. Depending on the type of stress, selective changes in the phosphorylation mechanism occurs regulating its stabilization and function (Meek 1998). *In vitro* studies have shown the phosphorylation of serine and threonine residues at the N and C terminal regions of p53 (Prives 1998) with the serine15, 20 and threonine18
being the conserved regions (Dornan et al 2003). Phosphorylation of serine15 leads to activation of other residues and decreases the interaction with MDM2 (Loughery & Meek 2013) signifying its importance.

1.9.2 Response of p53 in Cellular Metabolism

Recent studies have shown that p53 plays a crucial role in the regulation of energy metabolism, oxidative stress and amino acid metabolism by glycolysis, autophagy and respiration (Zhang et al 2010). Since, tumorigenesis is associated with increased metabolism for their continued growth and survival, activation of p53 could alter metabolic changes and influence metabolic pathways. Studies have shown p53 to be involved in the modulation of cellular metabolic adaptation during nutritional starvation in cancer cells (Zhang et al 2010; Puzio-Kuter 2011). The regulation of IGF/Akt-1 and mTOR pathways by p53 plays a central role in the physiological and pathological conditions including cancer, diabetes, and aging (Levine et al 2006).

In normal cells, IGF-1/Akt and mTOR pathways play critical roles in the regulation of cell proliferation, survival and energy metabolism. Novel functions of DNA damage activated p53 revealed that a downregulation in the IGF/Akt and mTOR pathways (Zhang et al 2010; Levine et al 2006). Furthermore, p53 alters glucose metabolism through different mechanisms, including, inhibition of GLUT1 and GLUT4 gene transcription, a downregulation of insulin and IGF1 receptor transcription or an upregulation of the tumor suppressor PTEN, which leads to an impairment of the insulin/IGF1-Akt pathway (Erol 2010).

An up regulation of TP53 (the human gene that encodes p53) reduces glycolysis and fatty acid synthesis and enhances gluconeogenesis and β-oxidation of lipids (Bensaad & Vousden 2007; Matoba et al 2006). A decrease in the
ATP/ADP ratio directly modulates the ability of p53 to bind DNA (Okorokov & Milner 1999).

In addition to the extrinsic and intrinsic stress, metabolic stress like limited nutrient or O\textsubscript{2} availability can also activate p53. Nutritional deprivation activated AMPK leads to induction of p53 either by increasing transcription of TP53 (the human gene that encodes p53) or through direct phosphorylation that stabilizes p53 (Jones et al 2005; Vousden & Ryan 2009), in addition metabolic stress induced nucleocytoplasmic malate dehydrogenase activates p53 (Lee et al 2009).

### 1.9.3 Emerging Role of p53 in Insulin Resistance

Previous studies have shown the involvement of p53 in the development of insulin resistance in adipose tissues. Recent reports have shown a relation between shortening of telomeres, cellular senescence, p53 upregulation, pro-inflammation and origin of insulin resistance in 3T3-L1 adipocytes under oxidative stress (Monickaraj et al 2013). The association between p53 polymorphism and the risk for type 2 diabetes has been reported in Europeans and Chinese Han population (Burgdorf et al 2011; Qu et al 2011).

In \textit{vitro} and \textit{in vivo} studies have demonstrated that an upregulation of p53 in adipose tissue due to increased FFA, promotes the expression of proinflammatory adipokines via the NF\textsubscript{κ}B signaling pathway and cause systemic insulin resistance (Shimizu et al 2012). PAI-1 a fibrinolytic inhibitor, which mediates senescence via p53 activation is associated with metabolic disturbances such as obesity and insulin resistance (Erol 2010).
Figure 1.8  Schematic representation of p53 induced due to DNA damage leading defects in insulin signaling.

Genotoxic stress induced p53 stimulates the transcription of IGF binding protein-3, which suppresses the insulin and IGF1 signaling pathway. p53 has been observed to induce insulin resistance through suppression of the transcription of insulin receptor (Webster et al 1996) and by reducing the IRS1 tyrosine phosphorylation (Ohlsson et al 1998). Alternately, p53 impairs insulin signaling by direct inhibition of PI3K function by suppressing p110 alpha, a subunit of the PI3K complex and indirectly by upregulation of PTEN transcription, leading to an impairment in PI3K/Akt signalling (Donehower
p53 also inhibits glucose metabolism by repressing GLUT4 and GLUT1 gene transcription (Schwartzzenberg-Bar-Yoseph et al 2004). It has been reported that genotoxic stress activated p53, initiates senescence, leading to alterations in the insulin signaling pathway (Erol 2010) (Figure 1.8).

1.9.4 p53 and Cellular Senescence

Several stress conditions independent of DNA damage, such as chromatin damage, telomere shortening, oxidative stress and metabolic stress induce irreversible cell cycle arrest known as cellular senescence. p53 activated due to DNA damage also turns on the expression of genes that induce cell cycle regulation, DNA repair, senescence and cell death (Herbig et al 2006). Cellular stress sensor p53 is known to activate senescence by phospho-serine15-p53/p21 and p16 hypophosphorylated Rb pathway (Jeyapalan & Sedivy 2008).

The factors that lead to cell senescence through activation of p53 includes, telomere shortening and oxidative stress in the adipose tissue (Minamino et al 2009). Jiang et al (2007) have shown that p53 activated by metabolic stress transcriptionally represses the expression of malic enzymes and the associated NADPH production, which results in sustained p53 activation, cellular senescence and tumor suppression. Cellular senescence is known to increase inflammatory response, leading to an increased occurrence of metabolic disorders.

1.10 INSULIN RESISTANCE - TELOMERE LENGTH

There is increasing evidence that telomere dysfunction induces aging and increases the risk factor for T2DM (Zhao et al 2013; Mulder 2010) with a shortening of the telomeres associated with insulin resistance in young adults (Gardner et al 2005). Telomeres are chromosomal ends consisting of 5-15 kb of
(TTAGGG)\textsubscript{n} repeats and are important for chromosomal stability and integrity. The structure and function are maintained by telomerase and telomere repeat binding factor 2 (TRF2) (de Lange 2002; Blackburn 2001; van Steensel et al 1998).

Each round of cell division shortens the length of telomeres in human cells because of the “end replication problem” of DNA polymerase and suppression of telomerase expression. When the length of telomeres get critically shortened they are recognized as DNA damage and induce DNA damage checkpoints (Jiang et al 2007). Metabolically induced abnormalities like ROS, DNA damage and telomere shortening results in biologically accelerated aging, T2DM and atherosclerosis (Lu & Finkel 2008; Sampson et al 2006; Salpea et al 2010; Monickaraj et al 2012). Monickaraj et al (2013) have reported that ROS induced DNA damage and shortened telomeres impaired glucose uptake through senescent/pro-inflammatory phenotype in 3T3L-1 adipocytes.

1.11 ADIPOSE TISSUE

Adipose tissue stores fat, resulting from diet and liver metabolism or degrade the stored fat to supply fatty acids and glycerol depending on the body’s physiological conditions. The storage or degradation of fat in adipocytes is regulated by a number of hormones, including insulin, glucagon, epinephrine, etc. The location of adipose tissue determines its metabolic profile, with the fat located beneath the wall of abdominal muscle known as visceral fat and the fat located in the abdominal area, beneath the skin known as subcutaneous fat.

The, visceral fat is a significant producer of hormones and chemokines involved in the inflammatory tissue response and insulin resistance (Scherer 2006; Frayn 2001). Even though the precise mechanism by which an increased adiposity
contributes to T2DM is still being investigated, it has widely been reported that obesity induced inflammatory response contributes to an inflammatory state in distant cells, leading to insulin resistance which often progresses to diabetes (Salinas et al 2007; Bastard et al 2006).

There are two types of adipose tissue, the white adipose tissue (WAT) and the brown adipose tissue (BAT), which are known as white fat and brown fat, respectively. The function of WAT includes lipid metabolism. In adipocytes, energy is stored in a lipid droplet in the form of triglycerides. The catabolised triglycerides release glycerol and fatty acids that are involved in the metabolism of glucose in the liver and other tissues.

The adipocytes secrete adipokines, which include hormones, cytokines and other proteins with specific biological functions (Vazquez-Vela et al 2008). In addition WAT acts as an endocrine organ by expressing and secreting a variety of adipokines, including adiponectin, resistin, and leptin, and proinflammatory cytokines, such as IL-6, monocyte chemotactic protein (MCP)-1 and TNFα (Kershaw & Flier 2004; Tilg & Moschen 2006).

The BAT is comprised of multilocular adipose cells and utilizes its nutrient stores to generate heat in order to warm the body. This tissue is found in infants and is found to reduce in adulthood (Cannon & Nedergaard 2004). It has been observed that preadipocytes play a key role in the development of insulin resistance in adipocytes (Chung et al 2006).
1.11.1 **White Adipose Tissue - Insulin Resistance**

Resistance to insulin in adipose tissue is one of the earliest defects detected. The function of insulin in adipose tissue includes differentiation of preadipocytes to adipocytes, stimulation of glucose uptake, lipogenesis and inhibition of lipolysis. Further, insulin is also involved in the regulation of glucose homeostasis, inflammation, energy balance, lipid metabolism and the fibrinolytic system/vascular haemostasis (Arner 2003). Therefore, any alterations in adipocyte metabolism can modulate both the local and systemic environment which will affect insulin sensitivity.

Adipokines secreted by adipose tissue under different stress conditions have been identified to serve as an interface between inflammation, insulin resistance and cardiovascular diseases (Kershaw & Flier 2004; Shoelson et al 2006). An increased adipose tissue mass during obesity condition is a risk factor for the development of insulin resistance.

An increased accumulation of lipid and circulation of NEFAs in the blood during obesity, results in an altered secretion pattern, this causes an increase in proinflammatory cytokines and a decrease in anti-inflammatory factors, thus leading to insulin resistance (Trayhurn & Wood 2005; de Luca & Olefsky 2008; Abel et al 2001; Fernandez-Veledo et al 2008).

The visceral fat mass has been associated with peripheral and hepatic insulin resistance and removal of visceral fat mass, appeared to improve insulin sensitivity (Cases & Barzilai 2000; Einstein et al 2005). It has been demonstrated that the subcutaneous adipose tissue does not contribute to the metabolic abnormalities occurring during obesity.
An increase in abdominal fat mass, either visceral or subcutaneous, has been observed leads to the pathogenesis of insulin resistance and contributing to dyslipidemia, glucose intolerance, hypertension and cardiovascular risk (Smith et al 2001; Vazquez-Vela et al 2008). These studies signify that the functional integrity of adipose tissue is crucial in regulating insulin sensitivity and metabolism.

1.12 **IN VITRO CELL CULTURE MODEL**

1.12.1. **Importance of *In Vitro* Cell Culture Model for Research**

The cell lines as an *in vitro* models for drug screening and toxicity studies are being used widely in various research labs and large pharmaceutical companies. A ready access to cells provides the possibility of understanding the cellular mechanisms that may lead new potential drug targets or could be used in the evaluation of therapeutic agents for the treatment of a dysfunction (Allen et al 2005).

Much of the progress in understanding adipocyte biology has depended on experiments using immortal preadipocytic cell lines, 3T3-L1 and 3T3-F442A cells (Novakofski 2004). 3T3-L1 cells are mouse embryonic fibroblast cell line, isolated from nonclonal Swiss 3T3 cells and are of adipocytic lineage. 3T3-L1 cells represent an established and validated experimental system for diabetes and obesity research, since the cells can be chemically induced to differentiate into adipocytes (O’Shea Alvarez 1991) (Figure 1.9). These cells enable study of glucose uptake and the alterations in the cellular mechanisms under different pathological conditions of diabetes. Hence, the present study was conducted using 3T3-L1 adipocytic cells obtained from NCCS, Pune, India.
Figure 1.9  (A) 3T3-L1 Preadipocytes (B) Differentiated adipocytes.

1.13 PHARMACOLOGICAL TREATMENT OF T2DM

Currently, oral agents used in the treatment of T2DM include a wide range of chemically derived drugs, including \(\alpha\)-glucosidase inhibitors, sulfonylureas, thiazolidinediones and biguanides.

1.13.1 \(\alpha\)-Glucosidase Inhibitors

Acarbose, miglitol and voglibose are the currently available \(\alpha\)-glucosidase inhibitors. They act by decreasing glucose level in the blood by delaying digestion of complex carbohydrates and disaccharides (starch, dextrin, sucrose) to absorbable monosaccharides by reversibly inhibiting \(\alpha\)-glucosidases (glucoamylase, sucrase, maltase, and isomaltase) within the intestinal brush border (Bischoff 1994). This leads to a decrease in glucose absorption and the subsequent attenuation of post-prandial hyperglycemia.

Some reports have shown that \(\alpha\)-glucosidase inhibitors improved insulin sensitivity in subjects with IGT but have no effect on insulin sensitivity in
subjects with overt type 2 diabetes (Jenney et al 1993). This class of drugs is preferred as primary therapy in the stages of very high postprandial glucose and have also been found to be useful in combination with other drugs in the treatment of T2DM.

### 1.13.2 Sulphonylureas

Sulfonylureas were the first widely used oral hypoglycemic medications and are insulin secretagogues, wherein they trigger insulin release by closing the $K_{ATP}$ channel and opening the voltage-dependent $Ca^{2+}$ channel of pancreatic β cells. The other beneficial effects of the sulfonylurea class of drugs include suppression of glucose production in the liver and enhancement of the body's ability to dispose the excess glucose into fat and muscle tissue. The first generation drugs include tolbutamide, chlorpropamide and tolazamide.

The second generation of drugs belonging to this class include glyburide, glipizide and glimepiride act by increasing insulin secretion through voltage gated calcium channels (Riddle 1996). Since, they are long-acting insulin secretagogues they cause hypoglycemia and body weight gain and are insufficient to control postprandial hyperglycemia, although, they can be safely used in combination with metformin or glitazones.

### 1.13.3 Thiazolidinediones

These are a new class of hypoglycemic agents and are termed as insulin sensitizers as they improve insulin sensitivity in the absence of insulin. They enhance insulin sensitivity in muscle, fat and other tissues by enhancing the nuclear receptor, PPAR-γ. A few examples of this class include rosiglitazone, pioglitazone, troglitazone etc.
Animal and human studies have shown that the thiazolidinediones sensitize both muscle and liver tissues to the hypoglycemic action of insulin (Fulgencio et al 1996; Nolan et al 1994). But the thiazolidineones have also been reported to exhibit some adverse effects. The most commonly reported side effect due to rosiglitazone is upper respiratory tract infection. The other contraindications of PPAR gamma agonists include obesity and cardiovascular complications (Lincoff et al 2007). Due to these side effects, FDA banned troglitazone (Rezulin) from the market in March 2000 and rosiglitazone from the market in 2010.

1.13.4 Biguanides

Biguanides are a group of oral drugs for T2DM, which act by inhibiting the production of glucose in the liver thereby improving insulin sensitivity. They do not affect insulin secretion, so unlike the other sulfonylureas and meglitinides they don’t exhibit side effects like hypoglycemia. The widely used and currently available biguanide class of drug is metformin. Several reports based on clinical, pharmacological and basic cellular observations have observed the insulin sensitizing properties of the drug (Dunn & Peters 1995; Ramachandran et al 2006).

Although, the mechanistic action of metformin has not been clearly elucidated, although the cellular mechanisms have been described by many reports (Matthaei et al 2000). However, a single unifying site of action, such as a receptor, an enzyme, or a transcription factor, has yet to be identified. It has been reported that metformin suppress endogenous glucose production and increase insulin stimulated glucose disposal and enhance fatty acid oxidation with increased intestinal utilization of glucose. The risk of lactic acidosis and
gastrointestinal side effects are the major drawbacks of this class drugs (Misbin et al 1998).

1.14 **Herbal Based Drug for Diabetes**

Inflammatory and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel diseases, cancer, diabetes, multiple sclerosis, psoriasis and asthma, provide tremendous challenge to current drug discoverers. Although, the precise cause of these diseases is not known, they, in general, can be considered as ‘gene expression diseases’ in which the pro-inflammatory gene program of the organism is aberrantly activated (Baeuerle 1998). In the current drug discovery approaches, combination drugs and targeted molecules are preferable choices to control complex diseases like cancer, diabetes and inflammation (Zhang & Meier 2006).

Botanicals are thought to offer strong therapeutic efficacy with minimal side effects, particularly against autoimmune and metabolic disorders since most of their efficacies are from a mixture of active molecules acting at the same time (Muthusamy et al 2008). The rapid growth of robust biotechnological and analytical techniques provide a ray of hope for plant based drug discovery approach. Traditional medicines have been used for thousands of years and for most such drugs the active component or their molecular targets have not been identified. The complex nature of the extracts, indistinct mechanism of action, herbal drug interaction and quality assurance are the main disputes of herbal therapy. However, ethanopharmacological observation in the past have resulted in the discovery of several important drugs.
Although, numerous components are present in plants, only a few of them are therapeutically effective. Plants are able to synthesize secondary metabolites like, alkaloids, terpenoids, tannins, saponins and glycosides through highly specific reaction mechanisms that they use for defense and communication. These secondary metabolites exhibit therapeutic effect either as purified isolates or as mixture formulations (Rahul et al 2005).

The most widely used drug compounds from plants include, vincristine and vinblastine isolated from *Catharanthus roseus*, which is being used effectively in the treatment of cancer (Van der Heijden et al 2004). Topotecan from *Camptotheca acuminata* serves as a potent anti-cancer agent. Cyanarin, the chemical component of *Cynara scolymus* has been used for liver problems and hypertension (Wojcicki et al 1981). Metformin is a successful anti-diabetic drug isolated from *Galega officinalis*, which is an efficacious glucose-lowering agent.

The World Health Organization (WHO) expert committee on diabetes has recommended that traditional medicinal herbs be further investigated (Modak et al 2007). In India ethno pharmacological surveys indicate that more than 1200 plants have antidiabetic activity. The plant constituents such as polysaccharides, peptides, alkaloids, glycopeptides, triterpenoids, xanthones, flavonoids, lipids, phenolics, coumarins, and guanidines are reported to have antidiabetic activity (Mishra et al 2010).

In the modern world, herbal medicines, playing a significant role with an annual production of 83 billion dollars. In developing countries, 70-95% of the population depend on herbal medicines for primary care mainly due to cost imperatives or unavailability of conventional drugs (Chawla et al 2013). A rapid improvement in molecular biology over the last decade has led to the
identification of bio markers for many diseases, providing plenty of novel targets for therapeutic intervention.

Polygenic diseases such as cancer or multifactorial diseases that affect various tissues or cell types, such as diabetes and inflammatory disorders, are not completely controlled by the drugs devised to act on single molecular target (Effert et al 2007). The heterogeneity of the affected cells, tissues and organs as well as redundancy of defensive mechanisms favour the development of drugs that affect multiple pathways. Introducing modern drug discovery tools for the exploration of natural values can help to minimise the search efforts for multi-targeted molecules to nullify the so called flaws of herbal therapy. Since, T2DM is a multifactorial disease leading to several complications, a multiple therapeutic approach could be beneficial. Although several therapies including insulin sensitizers, α-glucosidase inhibitors are in use, they exhibit side effects like liver toxicity, hypoglycemia and obesity. Many of the conventional drugs in use today have been derived from prototypic molecules in medicinal plants.

The present study investigated the potential anti-diabetic compounds Methyl tetracosanoate (MT), Aloe emodin glycoside (AEG) and Chlorogenic acid (CGA) in reversing hyperinsulinemia induced insulin resistance. These compounds have been extensively studied in our lab for their potential anti-diabetic activity.

1.15 ALOE EMODIN GLYCOSIDE

Aloe emodin, was originally isolated from Aloe vera leaf (Hamman 2008) and root Rheum palmatum L. (Liang et al 1993). It has been reported for its antiproliferative effect on merkel carcinoma cells, Hep G2, Hep 3B, liver cancer cell lines, HL-60 and human promyelocytic leukaemic cells (Wasserman et al
2002; Fenig et al 2004; Chen et al 2004). It has also been reported for its anti-neuroectodermal tumor activity (Pecere et al 2000) and as a potent hypotensive agent (Saleem et al 2001).

We have observed AEG isolated from the flowers of *Cassia fistula* to exhibit anti-diabetic activity wherein the insulin sensitizing and insulin mimetic action of AEG was observed in L6 myotubes and 3T3-L1 adipocytes. In addition to insulin stimulated glucose disposal, it was also observed to enhance its transformation to glycogen (Anand et al 2010) (Figure 1.10).

![Figure 1.10 Flowers of *Cassia fistula* (A) and Structure of Aloe emodin glycoside (B)](image)

**Figure 1.10** Flowers of *Cassia fistula* (A) and Structure of Aloe emodin glycoside (B)

### 1.16 CHLOROGENIC ACID

Chlorogenic acid (CGA) is an abundantly available compound present in the human diet. It is a polyphenolic compound present in the plant species as a secondary metabolite. CGA is an ester formed from cinnamic acids and quinic acid and the most common form of CGA is 5-caffeylquinic acid (5-CQA) (Clifford 2000). The antibacterial, antioxidant and anticarcinogenic properties of CGA has been reported (Meng et al 2013). Accumulating evidence has also demonstrated that it is an effective insulin sensitizer, involved in the regulation of
glucose and lipid metabolism. *In vivo* studies have shown that CGA improves glucose tolerance, decreases various plasma and liver lipids and improves mineral pool distribution (Rodriguez de Sotillo & Hadley 2002; Hunyadi et al 2012). Van Dijk et al (2009) have reported that CGA ingestion significantly reduced fasting glucose level and insulin response in overweight men. Clinical trials have also demonstrated that CGA is able to lower the glycemic impact of foods and lower background blood glucose levels of T2DM (Ahrens & Thompson 2013). In addition to anti-diabetic activity clinical trials have shown that CGA is able to improve insulin secretion.

We have observed CGA, isolated from the leaves of *Cichorium intybus* to exhibit improved insulin sensitivity in 3T3-L1 adipocytes. The anti-hyperglycemic activity of CGA is aided by PTP-1B inhibition and insulin downstream signaling activation (Muthusamy et al 2010). Both in silico and in vitro studies predicted novel allosteric inhibition of PTP-1B by CGA (Baskaran et al 2012) (Figure 1.11).

![Image of leaves of Cichorium intybus](image_a.png)

![Structure of Chlorogenic acid](image_b.png)

**Figure 1.11** Leaves of *Cichorium intybus* (A) and Structure of Chlorogenic acid (B)
1.17 Methyl Tetracosanate

Methyl tetracosanoate (MT) also known as Methyl lignocerate, is a saturated fatty acid. MT isolated from the leaves of *Costus pictus* was observed to exhibit enhanced glucose uptake activity and improve insulin sensitivity in 3T3-L1 adipocytes. Shilpa et al (2009) has been reported that a methanolic extract of *Costus pictus* showed optimum anti-diabetic activity and along with anti-adipogenic activity. MT was identified to exhibit insulin sensitivity through upregulation of IRβ, PI3K, GLUT4 and inhibition of PTP-1B in 3T3-L1 adipocytes (Figure 1.12).

![Figure 1.12](image)

**Figure 1.12** Leaves of *Costus pictus* (A) and Structure of Methyl tetracosanoate (B)
1.18 OBJECTIVES OF THE STUDY

1. To evaluate the molecular mechanism of hyperinsulinemia induced insulin resistance in 3T3-L1 adipocytes.

2. To investigate the effect of hyperinsulinemia on inflammation and senescence in 3T3-L1 adipocytes.

3. To evaluate the effect of herbal compounds during hyperinsulinemia and to investigate the molecular mechanism of active herbal compound in reverting insulin resistance caused due to hyperinsulinemia.