3.0 REVIEW OF LITERATURE

3.1 DIABETES DEFINITION AND CLASSIFICATION

3.1.1 Historical Background

Diabetes mellitus is clinically and genetically a heterogeneous group of disorders characterized by abnormally high levels of glucose in the blood. The hyperglycaemia is due to the deficiency of insulin secretion, or to resistance of body's cells to the action of insulin, or to a combination of these. There are also disturbances of carbohydrate and protein metabolism.

The word ‘diabetes’ is derived from the Greek word 'Diabsinein' which means to pass through. Earliest reference about a disease with polyuria was made in “Ebers Papyrus” (Egypt), a document outlining about clinical symptoms of the disease (1550 B.C.). Indian physicians Charak and Susruta (600 B.C.) were the first to recognize that the disorder existed in two forms. But descriptions in most of the classic literatures relate to what we know today as type 1 insulin dependent diabetes. Greek physician Areteaus in 130 A.D. referred it to the “melting down of the flesh into urine” implying muscle wasting and polyuria. The presence of sugar in urine was first detected by Thomas Wills in 1664 and it was confirmed by laboratory test in 1776 by Mathew Dobson. Harley, a British physician commented in 1866 that there are at least two distinct forms of the disease requiring diametrically opposite forms of treatment. French physician Lancereaux is generally credited with making distinction between thin and fat diabetes: diabete gras and diabete maigre in 1880 (Gale, 2001). In 1869 German medical student, Paul Langerhans, noted that the pancreas contains two distinct groups of cells, the acinar cells which secrete digestive enzymes and second group of cells which are clustered in islands, or islets which suggested a second function. In 1889, Oskar Minkowski and Joseph Von Mering demonstrated that pancreatectomized dogs exhibit a syndrome similar to diabetes mellitus in man. Insulin was discovered and isolated in 1921 by Banting and Best and it became clear that insufficient insulin delivery was the cause of diabetes mellitus.

Insulin resistance was described in the 1930s when Himsworth reported diabetes patients who did not respond to insulin treatment. He developed a glucose challenge test in which glucose was given by mouth while insulin was injected intravenously. He found that lean young patients had insulin sensitivity equivalent to that of non-diabetic individuals, whereas older overweight patients were markedly insensitive to insulin. On the basis of this study, he proposed that there were at least two clinical types of diabetes mellitus, insulin sensitive and insulin insensitive, the former due to insulin deficiency. Bornstein and Lawrence confirmed the clinical observations of Himsworth with the development of bioassay of insulin. In recent decades, research has led to the recognition that diabetes mellitus is a syndrome and comprises a heterogeneous collection of disorders and that the different types of diabetes have different etiologies, although their pathologic effects after onset of disease may be similar.

3.1.2 Classification

NDDG/WHO Classification:

In 1979, a classification for diabetes mellitus and other categories of glucose intolerance, based on scientific research on this heterogeneous syndrome was developed by
an international workgroup sponsored by the National Diabetes Data Group (NDDG) of the National Institutes of Health (NIH) of United States of America (NDDG, 1979). This group recognized diabetes as being a syndrome, a collection of disorders that have hyperglycaemia and glucose intolerance as their hallmark characteristics, due either to insulin deficiency or impaired effectiveness of insulin's action or to a combination of these. The World Health Organization (WHO) expert committee on Diabetes in 1980 endorsed the substantive recommendations of the NDDG (WHO, 1980). These groups distinguished between the two major forms of diabetes, which they termed insulin dependent diabetes mellitus (type 1) and noninsulin dependent diabetes mellitus (type 2). The older terms 'juvenile onset', 'maturity-onset' and 'adult-onset' were recommended to be abolished.

American Diabetes Association Classification:

In 1996 and 1997, an expert committee of the American Diabetes Association considered the research findings of the last 20 years and proposed some changes to the NDDG/WHO classification scheme (ADAEC, 1997). The new classification is in Table 1. Changes to diagnostic criteria were also proposed. The main features of new classifications are elimination of the terms insulin-dependent diabetes mellitus and non-insulin dependent diabetes mellitus. However the terms type 1 and Type 2 were retained. Forms of diabetes involving pancreatic β-cell destruction, including those cases of autoimmune cause and those cases with unknown etiology were also included in type 1 diabetes mellitus. It also included more precise definition of type 2 diabetes with insulin resistance and insulin secretory defects. Each class in Table 1 may be heterogeneous in etiology and pathogenesis, and further research might provide more precise definitions of different types of diabetes mellitus.

The most common form of diabetes is type 2 diabetes that accounts for 85-90% of all diabetes. Globally, a doubling of the prevalence of type 2 diabetes can be expected in the next 10 years, with the largest increase of type 2 diabetes incidence in the developing countries due to changes towards a 'western life-style' (Zimmet, 2000). According to WHO calculations, in the year 2025 there will be 300-350 million adults with diabetes in the world and 80% of them will be found in the developing countries (Zimmet et al., 1997).

Table 1: ADA classification of diabetes mellitus

<table>
<thead>
<tr>
<th>Type 1 Diabetes mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caused by β-cell destruction, often immune mediated that leads to loss of insulin secretion and absolute insulin deficiency. The etiologic agents that cause the autoimmune process and β-cell destruction are not well established. Also includes cases which causes of the β-cell destruction are not understood. Comprises approximately 5% to 10% of cases in diabetes syndrome.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type 2 Diabetes mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caused by a combination of genetic and non-genetic factors that result in insulin resistance and insulin deficiency. The specific genes are not known but are under intense investigation. Non-genetic factors include increasing age, high caloric intake, overweight, central adiposity, sedentary lifestyle and low birth weight. Comprises of 90% to 95% of the diabetes syndrome.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other specific types of diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other types of diabetes comprise a heterogeneous etiologic group that includes those cases of diabetes in which causes are established or at least partially known. The causes include known genetic defects affecting β-cell function or insulin action, diseases of exocrine pancreas, endocrinopathies, drug or chemical induced pancreatic changes. Comprises approximately in 1% to 2% cases in the diabetic syndrome.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gestational diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caused by insulin resistance and relative insulin deficiency associated with pregnancy. Occurs approximately 3% to 5% of all pregnancies.</td>
</tr>
</tbody>
</table>
3.2 TYPES OF DIABETES

3.2.1 Type 1 Diabetes Mellitus

The onset is usually in childhood. It is characterized by absolute insulin deficiency (insulinopenia). β-cells in the pancreas are gradually destroyed. Autoimmune mechanism has been found to be involved in the destruction of β-cells. As insulin is required to move glucose into the cells, insulinopenia causes glucose to accumulate in blood, resulting in hyperglycaemia. When the blood glucose level crosses 180 mg/dl, (which the kidney threshold for glucose) glucose is excreted in urine. Weakness, weight loss and excessive thirst are the classic symptoms of diabetes. Patients become dependent on exogenous insulin for survival.

Two subclasses are discriminated, an autoimmune class (Type 1A) and idiopathic class (Type 1B). The autoimmune type is a chronic disease with a subclinical prodromal period characterized by cellular-mediated autoimmune destruction of the insulin producing β-cells in the pancreatic islets. The markers of autoimmune disease include antibodies to the islets and insulin, glutamic acid decarboxylase (GAD) and tyrosine phosphatases IA-2 and IA2β (Bingley et al., 1997).

3.2.1.1 Pathophysiology of Type 1 diabetes mellitus

The trigger for the process of destruction of β-cells is poorly characterized but is external factor (viral, chemical) or an internal stimulus (cytokines, free radical) that damages the proportion of the β-cells leading to the release of specific β-cell proteins, which can be taken up by antigen-presenting cells and processed to antigenic peptides. The process involves the transcription of cytokine genes including interferon-γ, which can feed back onto antigen-presenting cells to increase expression of IL-1β and TNF-α. Helper T-cells also activate B-lymphocytes, which produce islet cell auto-antibodies, and this is followed by cytotoxicity by killer cell activation.

Autoimmune etiology

The available data indicates that type 1A diabetes of human, mice and rats is of immune etiology and the major effector cells are T-lymphocytes (Bergman and Haskins, 1994). The hallmark of type 1A diabetes is lymphocytic invasion of islets, resulting in insulitis. This lymphocytic infiltration of islets is remarkable in that only islets containing insulin-secreting cells (β-cells) are infiltrated. Islets containing only glucagon, somatostatin and pancreatic polypeptide secreting cells are termed as pseudoatrophic islets and these are free of infiltration. Thus, insulitis is a remarkably β-cell specific process.

Development of Non-obese diabetic (NOD) mouse lead to the detailed study of many immunologic events associated with the pathogenesis of type 1A diabetes. The class I and class II genes of major histocompatibility complex (MHC) and a polymorphism affecting glycosylation of interleukin-2 are the only identified susceptibility genes of animal models (Ikegami et al., 1995).
Table 2: Major β-cell autoantigens associated with immune related type 1A Diabetes Mellitus

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>Inversely related to age of onset</td>
</tr>
<tr>
<td>GAD65</td>
<td>Age independent</td>
</tr>
<tr>
<td>ICA512/IA-2</td>
<td>Islet protein tyrosine phosphatase</td>
</tr>
<tr>
<td>Phogrin/IA-2β</td>
<td>Subset of ICA512/IA-2</td>
</tr>
<tr>
<td>Carboxypeptidase-H</td>
<td>Low sensitivity</td>
</tr>
</tbody>
</table>

There are some proteins identified which are targeted by the immune system; insulin, a neuroendocrine enzyme GAD, membrane granule proteins with homology to tyrosine phosphatase (termed as islet cell antibody (ICA)512/H-2) and a related molecule phogrin (ICA 512β/IA-2β). In addition, an islet neuroendocrine ganglioside is also a target of autoantibodies.

It is now believed that the β-cell autoimmunity is primarily a T-cell mediated process based on the evidence of cellular infiltration (insulitis) in islets of NOD mice long before the development of overt diabetes and isolation of islet specific T-cell clones (Haskins and Wegmann, 1996). The majority of CD4+ T-cell clones isolated from NOD mouse islets are activated by insulin and a specific epitope of insulin B chain (Daniel et al. 1995). Potential mechanisms for indirect T-cell destruction of β-cells include the production of lymphokines, and free radicals (by cells such as macrophages) under the influence of activated T-lymphocytes and the presence of Fas ligands on T-lymphocytes (Rabinovitch, 1994). Such molecules may be then the final effectors of β-cell cytotoxicity. In addition, even though CD4+ T-lymphocyte clones are sufficient to destroy islets, additional effector mechanisms such as cytotoxic CD4+ T-lymphocytes are likely to be involved in the pathogenesis (Santamaria et al., 1995).

Given the complexity and chronicity of type IA diabetes mellitus, multiple interventions are able to prevent the disorder in animal models. One of the most whole B chain of insulin, or insulin prevented diabetes. Studies indicate that NOD mice transgenically producing proinsulin are protected from both diabetes and insulitis, consistent with this hypothesis (Daniel et al., 1995). Table 3 gives details of interventions in different animal models to prevent the diabetes.
### Table 3: Examples of interventions preventing IDDM in animal models

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Specificity</th>
<th>Animal</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune System</td>
<td>None</td>
<td>NOD, BB</td>
<td>Non-specific suppression of cell-mediated immunity (e.g., with cyclosporine or rapamycin) prevents type 1 DM in these animals, but the degree of immunosuppression would be unacceptable for long term human</td>
</tr>
<tr>
<td>Monoclonal anti CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CD4 cells</td>
<td>NOD</td>
<td>CD4 depletion prevents type 1 DM at least transiently; non-depleting CD4 antibody may have a longer action, as has been found in transplantation</td>
</tr>
<tr>
<td>Anti-CD&lt;sub&gt;3&lt;/sub&gt;</td>
<td>T Cells</td>
<td>NOD</td>
<td>Treatment at diabetes onset (only at onset), long term remission</td>
</tr>
<tr>
<td>IL-2 toxin</td>
<td>Activated T Cells</td>
<td>NOD</td>
<td>Short term efficacy</td>
</tr>
<tr>
<td>CTLA4-Ig</td>
<td>Accessory T cell molecule</td>
<td>NOD</td>
<td>Prevention of diabetes by blocking accession of molecules</td>
</tr>
<tr>
<td>Transplantation</td>
<td>Unknown</td>
<td>NOD, BB</td>
<td>Mice that received grafts of marrow, dendritic cells, fetal liver and thymus are protected</td>
</tr>
<tr>
<td>Immune stimulation</td>
<td>Unknown</td>
<td>NOD</td>
<td>Intervention ranging from injection of monoclonal antibodies, Freund’s adjuvant, BCG vaccine, or event attenuated mycobacterial organisms might have this effect</td>
</tr>
<tr>
<td>Oral/nasal tolerance</td>
<td>Insulin/GAD</td>
<td>NOD</td>
<td>Mice fed insulin or receiving intranasal insulin or GAD have delayed or reduced onset of diabetes; no effect on BB rat</td>
</tr>
<tr>
<td>Diet and nutritional therapies</td>
<td>Unknown</td>
<td>NOD, BB</td>
<td>These diets can be divided into those with a hypoththesized mechanism such as radical scaventing and those that are nutritionally special (e.g., eliminating proteins)</td>
</tr>
<tr>
<td>Cytokines/nicotinamide</td>
<td>Effector pathways</td>
<td>NOD</td>
<td>These agents alter levels or activities of interleukins; conflicting data, depending on timing, transgenic islet expression or peripheral administration</td>
</tr>
<tr>
<td>Immune modulation</td>
<td>Insulin/GAD</td>
<td>NOD</td>
<td>Insulin peptide vaccination prevents type 1 DM; intrathymic injection of GAD is associated with reduced T-cell response to GAD</td>
</tr>
<tr>
<td>Insulin/metabolically active</td>
<td>Islet targeting</td>
<td>NOD, BB</td>
<td>Prevents diabetes, probably by β-cell rest and immune modulation</td>
</tr>
</tbody>
</table>

NOD – Non-Obese Diabetic mouse, BB – Biobreeding rat; IL – Interleukin

**The development and course of autoimmune Type 1 Diabetes mellitus**

The development and course of autoimmune Type 1 Diabetes mellitus has been divided into a series of stages (Eisenbarth, 1986).

Stage 1, genetic predisposition  
Stage 2, triggering of autoimmunity  
Stage 3, development of series of autoantibodies  
Stage 4, loss of β-cells  
Stage 5, overt DM  
Stage 6, total or near total β-cell destruction with complete insulin dependence.
The pathogenic events unique to each stage are potential target for intervention. For example, if triggering environmental factors were identified, their removal might provide a means of disease prevention.

**Stage 1: Genetic Predisposition**

Type 1A diabetes mellitus develops in the presence of genetic susceptibility even though more than 85% of patients with type 1A DM do not have a close relative with the disorder. The risk for type 1A DM in first degree relatives of patients is approximately 6%. For monozygotic co-twin of a patient, the lifetime risk is greater than 70% and for dizygotic twins or siblings of patients it is less than 10%.

The human leukocyte antigen (HLA) region, also known as the major histocompatibility complex (MHC), is a cluster of over 150 genes containing about 3.5 million bases of DNA on the short arm of chromosome 6 (6p 2 1.3). The highest risk alleles DR3 or DR4 are present in about 95% Caucasian type 1 DM patients compared to 50% of normal controls. On the other hand, the DR2 allele is rarely seen in type 1 DM patients, suggesting that this allele is associated with protection against DM (Pugliese et al., 1995).

**Genetic markers as predictive markers**

Despite the familial aggregation of type 1 DM, there is no identifiable pattern of inheritance and most cases occur in the absence of any family history. The genetic markers of type 1 DM, although enriched in those with disease or destined to develop it, are nonetheless present in sufficiently high numbers in general population. The DR3-DQ2/DR4-DQ8 heterozygous genotype confers a particularly high risk (Bingley et al., 1997). Table 4 gives idea about the risk for diabetes in relatives of patients. Genetic markers identify a population at potential risk. However, protective genes, which are dominant even in the presence of susceptibility genes or the absence of genetic risk may be better predictors.

**Table 4: Risk for diabetes in relatives of patients**

<table>
<thead>
<tr>
<th>Type of Relative</th>
<th>Point estimate</th>
<th>Long term follow-up</th>
<th>HLA identical</th>
<th>HLA DR3/DR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MZ Twins</td>
<td>10-55%</td>
<td>~ 70%</td>
<td>-</td>
<td>~70%</td>
</tr>
<tr>
<td>DZ Twins</td>
<td>5-10%</td>
<td>10-15%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Siblings</td>
<td>5-10%</td>
<td>10-15%</td>
<td>15-20%</td>
<td>20-25%</td>
</tr>
</tbody>
</table>

**Stage 2: Triggering of autoimmunity**

In the second stage, an environmental trigger probably initiates autoimmunity. This results in anti β-cell immune responses leading to islet infiltrates (insulitis), with the consequence of β-cell injury, impairment of β-cell function and some loss of β-cell mass. As β-cells are injured, a presumably secondary humoral response develops, with the appearance of β-cell autoantibodies that characterize this stage.

The theory that DM incidence is affected by infant diet is stimulated by Scandinavian studies showing correlation between the decline in breast-feeding and increase in DM incidence. Numerous subsequent case-control studies provide conflicting results about the association, but a meta-analysis of these studies indicated a significant but modest effect of exposure to breast milk substitutes or cow’s milk-based substitutes (Norris et al., 1996). Type 1 DM, as well as thyroiditis is clearly linked with congenital rubella. Congenital rubella may
alter the T-cell development, thus creating susceptibility to a series of autoimmune disorders. An alternative hypothesis is that the rubella virus mimics an islet autoantigen leading to anti-islet autoimmunity. Sequence homology between the PC2-C protein of Coxsackie B viruses and the GAD65 autoantigen has led to the theory of induction of autoimmunity by molecular mimicry (Graves and Rewers, 1997). Given genetic susceptibility a number of environmental factors may influence the development of DM in the NOD mouse.

Stage 3: Expression of autoantibodies

Over the past decade, investigators have defined a large family of islet autoantigens. Table 2 gives a list of autoantigens associated with immune related type 1A diabetes mellitus. GAD65 is a cytoplasmic enzyme expressed in all islet cells of humans and a series of neuroendocrine tissues. Autoantibodies to GAD65 led to the identification of GAD65 as elusive islet 64-kd autoantigen (Kaprio et al., 1993). The autoantigen ICA5121 was originally discovered by Rabin and co-workers following the screening of an islet expression library with sera from type 1 DM patients. The same molecule has been termed as IA-2 (Rabin et al., 1992). Prior to characterization of ICA512/IA-2, Christie had identified autoantibodies reacting with 40-kd and 37-kd tryptic fragments of labeled islets. It is now understood that the 40-kd protein is ICA512/IA-2 and the 37-kd molecule is a tryptic fragment of a molecule termed as phogrin (Christie, 1996). Antibodies to carboxypeptidase are too infrequent to standard panel of autoantibodies. Currently the best predictor of future type 1A DM is the expression of multiple, biochemically determined autoantibodies. Among 50 first-degree relatives of patients with type 1A DM followed to the onset of overt DM, 49/50 expressed one or more autoantibodies.

Stage 4: Loss of β-cells

By this stage, there is sufficient impairment of β-cell function and/or loss of β-cell mass resulting in a loss of first-phase insulin response (FPIR) during intravenous glucose tolerance test (IVGTT). More than 50% of these individuals will progress to type 1 DM within 5 years and more than 90% will do so in 10 years (Bingley et al., 1993). FPIR usually calculated as the sum of the plasma insulin levels on samples drawn at and 3 minutes after a standard intravenous glucose injection. Studies indicate that islet cells from newly-onset patients expressed increased class I molecules and that the most common infiltrating T cell is CD8+. CD8+ cells react with class I HLA molecules, in contrast to CD4+ cells which recognize antigen presented by class I HLA molecules (Itoh et al., 1993).

Given the presence of anti-islet autoantibodies, the FPIR measured with IVGTT is the best predictor of both the risk for overt DM and the time to onset of overt DM. A standardized procedure for performing the IVGTT has been proposed by the Islet Cell Antibody Registry of Users (ICARUS) working group. This common registry has facilitated consensus concerning the role of IVGTT in DM prediction. A dualparameter model has been described recently for the prediction of type 1A DM that takes into account both first-phase insulin release and levels of anti-insulin autoantibodies (Eisenberth et al., 1998).

Stage 5: Overt Diabetes

This stage is marked by the clinical onset of type 1 DM. At the beginning of this stage, it is estimated that over 80% of β-cell function and/or mass has been lost, but the residual β-cell function (evidenced by c-peptide production) remains an important
contributor to metabolic homeostasis. β-cell destruction continues after the diagnosis of type 1A DM, although a metabolic remission, termed honeymoon phase may occur after the institution of insulin therapy. As β-cell function is lost and hyperglycaemia prevails, antibodies tend to decrease in titre and/or disappear, although majority of retain GAD65 or ICA512/IA-2 autoantibodies for more than a decade. During early phases, very low doses of insulin may suffice to maintain normal blood glucose and approximately 27% of patients become temporarily independent of insulin. As time goes by, complete loss of β-cell function and mass occurs and the DM becomes more difficult to control. A number of trials using less toxic immunosuppressants are continuing in newly-onset patients. There is a consensus that maintenance of β-cell mass is worthwhile to attain metabolic control and may reduce risk of complications. Nicotinamide which may act as free radical scavenger and may protect the β-cell from autoimmune attack, preserved β-cell function in one trial (Lampeter et al., 1998).

Role of cytokines

Cytokines are peptide molecules synthesized and secreted by activated lymphocytes (lymphokines), macrophages/monocytes, and cells outside the immune system such as endothelial cells, bone marrow stromal cells and fibroblasts. Cytokines are mainly used by immune system cells to communicate with each other and to control local and systemic events of immune and inflammatory responses. More than 30 immunologically active cytokines exist and are grouped as interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs) and colony stimulation factors (CSFs). Both the production of cytokines by cells and the action of cytokines on cells complex: a single cell can produce several different cytokines and a given cytokine can act on one or more cell types. Cytokine actions are usually local:

- a) between two cells that are conjugated to one another
- b) on neighboring cells (paracrine)
- c) on cell that secretes cytokine (autocrine)

In some case macrophage derived inflammatory cytokines; such IL-1, IL6 and TNF-α exert actions on distant organs (endocrine).

Antigen activated T-cells are termed as T helper cells (Th) because they help to mediate both cellular and humoral (antibody) immune responses. Th cells are divided in two populations with contrasting and cross-regulating cytokine profiles. The mouse Th1 cells produce IL-2, IFN-γ and TNF-β (also termed as lymphotoxin), whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Cytokine production by human Th1 and Th2 cells follow similar pattern.

The functional significance of Th1 and Th2 cell subsets is that their distinct patterns of cytokine secretion lead to strikingly different T-cell actions. Thl cells and their cytokine products 1L-2, IFN-γ and TNF-β are the mediators in cell mediated immunity. IFN-γ and TNF-β activate vascular endothelial cells to recruit circulating leukocytes into the tissues at the local site of antigen challenge and they activate macrophages to eliminate the antigen-bearing cell. In addition, IFN-γ and TNF-β activate a) cytotoxic T-cells to destroy target cells expressing appropriate MHC-associated antigen and b) natural killer (NK) cells to destroy target cells in MHC-independent fashion. In contrast, Th2 cells are much more effective stimulators of immune response that is immunoglobulin production, especially immunoglobulin E by β cells. Furthermore, responses of Th1 and Th2 cells are mutually
inhibitory. Thus, Th1 cytokine IFN-γ inhibits the production of the Th2 cytokine IL-4 and IL-10; these in turn, inhibit Th1 cytokine production.

**Approaches used to study role of cytokines in type 1 diabetes**

Studies over the last 15 years have examined the possible role of the involvement of cytokines in the pathogenesis of type 1 diabetes through a variety of approaches:

a) correlation studies of cytokines expressed in islets in relation to DM development
b) cytokine augmentation studies
   i) adding cytokines to islets in vitro
   ii) administering cytokines and cytokine producing cells
c) cytokine deficiency studies
   i) disrupting genes encoding cytokines or their receptors
   ii) neutralizing cytokines by anticytokine antibodies or soluble cytokine receptors
   iii) blocking cytokine receptors by receptor antagonists or antibodies
   iv) deleting cytokine receptor-positive cells

**Effect of cytokine expression**

Effects of cytokines expressed in the insulitis lesion are given in table 5 (Rabinovitch 1994). A given cytokine might promote autoimmunity and β-cell destruction, or alternatively may regulate (i.e., suppress) the autoimmune and/or inflammatory processes that would otherwise result in β-cell destruction (Kolb, 1997).

**Effects of cytokine addition**

It is well documented that certain cytokines are cytotoxic to pancreatic islets in vitro. IL-1, TNF-α, TNF-β and IFN-γ are cytostatic to β-cells, in that they inhibit insulin synthesis and secretion. They destroy β-cells, in that they inhibit insulin synthesis and secretion. They destroy β-cells in both rodent and human islets. Because the cytodestructive effects of cytokines on islets cells in vitro are not specific to β-cells, α-cells in the islets are also damaged, cytokines may not qualify as the mediators of β-cell destruction in type 1 DM. Table 6 gives details of the effects of cytokine additions to islets in vitro, to β-cell destruction in type 1 DM. Table 6 gives details of the effects of cytokine additions to islets in vitro, to β-cells transgenically and to NOD mice and BB rats by systemic administration (Rabinovitch, 1996).
Table 5: Correlation of cytokines expressed in islets with β-cell destructive of benign insulitis

<table>
<thead>
<tr>
<th></th>
<th>Proinflammatory Cytokines</th>
<th>Type-1 Cytokines</th>
<th>Type-2 Cytokines</th>
<th>Type-3 Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1</td>
<td>TNF-α</td>
<td>IFN-α</td>
<td>IL-12</td>
</tr>
<tr>
<td>NOD mice</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>BB Rats</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Humans</td>
<td>0</td>
<td>nd</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, Tumor necrosis factor
+: cytokine presence correlates with β-cell destructive insulitis;
-: cytokine presence correlates with benign insulitis;
O: cytokine presence does not correlate with either destructive or benign insulitis;
Nd: not detected;
?: not reported

Transgenic expression of IFN-α, IFN-γ and IL-2 by β-cells in non-DM prone mice induced β-cell destruction and DM, whereas expression of TNF-α, IL-4, IL-6 induced insulitis that did not progress to β-cell destruction and IL-10 and TGF-β induced only peri-islet inflammatory reDM-prone NOD mice protected against DM development.

Table 6: Effects of cytokine additions to islets in vitro and to NOD mice and BB rats by systemic administration

<table>
<thead>
<tr>
<th>Cytokine Additions</th>
<th>Proinflammatory cytokines</th>
<th>Type 1 cytokines</th>
<th>Type 2 cytokines</th>
<th>Type 3 cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1</td>
<td>TNF-α</td>
<td>IFN-α</td>
<td>IL-12</td>
</tr>
<tr>
<td>Toxicity to β-cells in vitro (rodents and humans)</td>
<td>Toxic</td>
<td>Toxic</td>
<td>0</td>
<td>?</td>
</tr>
<tr>
<td>Systemic administration to NOD mice</td>
<td>-</td>
<td>+(≤3wks)</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Systemic administration to BB Rats</td>
<td>±</td>
<td>-</td>
<td>±</td>
<td>?</td>
</tr>
</tbody>
</table>

IFN: interferon; IL: interleukin; TGF: transforming growth factor; TNF: tumor necrosis factor
+: cytokine presence correlates with β-cell destructive insulitis;
-: cytokine presence correlates with benign insulitis;
O: cytokine presence does not correlate with either destructive or benign insulitis;
Nd: not detected;
?: not reported

Systemic administration of a wide variety of cytokines has been shown to prevent DM development in NOD mice and/or BB rats. Deficiencies in endogenous production of IL-1, IL-2, IL-4, TNF-α and TNF-B have been reported in DM-prone NOD mice and/or BB rats. Systemic administration of cytokines however, produces gradient for the cytokine that is higher outside than inside the islet and this may result in immunologic effect different from those induced by the same cytokine secreted in the islet. The effect of cytokines autoimmune DM development largely depends on the dose, the frequency and the route of cytokine administration, as well as the time of administration in relation to disease development.
Effects of cytokine deletion

Studies in which the cytokines are deleted from expression in autoimmune DM-prone animals have the potential of revealing whether the cytokine plays an essential (necessary) role in type 1 DM development. Cytokine deficiencies have been created in DM-prone animals by disrupting genes encoding cytokines or their receptors (gene knockout), neutralizing cytokines by anticytokine antibodies or soluble cytokine receptors, blocking cytokine receptor by receptor antagonists or antibodies and deleting cytokine receptor positive cells. Deletions of wide variety of cytokines (IL-1, TNF-α, IL-12, IFN-γ, IL-2 and IL-6), by one or more of the approaches described above have been reported to delay or decrease DM incidence or both, in NOD mice and deletion of IL-1. IFN-γ has decreased DM incidence in BB rats (Rabinovitch, 1996). Table 7 gives an account of effect of deletions of cytokines.

These findings reveal that multiple cytokines may likely participate in the autoimmune response that leads to β-cell destruction and the deletion of single pathogenic cytokine does not appear to be sufficient to prevent DM development completely. Therefore, therapy of autoimmune DM might require neutralizing or locking more than one cytokine. Alternatively, a pathogenic mechanism common to the diabetogenic cytokines may be identified.

Table 7: Effects of cytokine deletion in NOD mice and BB rats

<table>
<thead>
<tr>
<th>Cytokine deletion</th>
<th>Proinflammatory cytokines</th>
<th>Type 1 cytokines</th>
<th>Type 2 cytokines</th>
<th>Type 3 cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1</td>
<td>TNF-α</td>
<td>IFN-γ</td>
<td>IL-12</td>
</tr>
<tr>
<td>By breakout of gene for cytokine/or its receptor in NOD mice</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>0</td>
</tr>
<tr>
<td>By neutralization of cytokine, blockage of receptor, or deletion of receptor-positive cells in NOD mice</td>
<td>-</td>
<td>(&lt;3 wks)</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+&gt;4 wks)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IFN: interferon; IL: interleukin; TGF: transforming growth factor; TNF: tumor necrosis factor
+: cytokine presence correlates with β-cell destructive insulitis;
-: cytokine presence correlates with benign insulitis;
O: cytokine presence does not correlate with either destructive or benign insulitis;
Nd: not detected;
?: not reported

Role of viruses in the pathogenesis of Type 1 Diabetes Mellitus

Although genetic predisposition appears to be necessary for the development of type 1 DM, non genetic environmental factors play a critical role in the expression of the disease. Viruses as one environmental factor may directly infect and destroy pancreatic β-cells or trigger β-cells specific autoimmunity.
The etiology of Type 1 DM is believed to have a major genetic component. However, cumulative evidence suggests that environmental factors play an important role in the development of Type 1 DM by influencing the penetrance of diabetes susceptibility genes. The environmental factors thought to play a role this disease include viral infections, certain dietary components and toxins (Yoon, 1997). There appears to be a seasonal variation in the onset of acute type 1 DM, with a peak in autumn. Diseases with seasonal incidents are often caused by viruses. In some cases it is believed that viruses directly destroy pancreatic β-cells; in other cases, viruses are thought to trigger or somehow contribute to β-cells specific autoimmunity leading to development of type 1 DM (Yoon, 1997). In addition, there is evidence that viruses can also protect against the development of diabetes in the spontaneously diabetic BB rats and NOD mouse (Oldstone, 1988).

Virus-induced Diabetes in Animals

Several viruses have been demonstrated to cause diabetes in animals, including encephalomyocarditis (EMC) virus, Coxsackievirus B4 (Toniolo et al., 1982), Kitham rat virus (KRV) (Guberski et al., 1991), rubella virus (Rayfield et al., 1986), retrovirus (Suena and Yoon, 1988), bovine viral diarrhoea-mucosal virus (Tajima et al., 1992) are suspected of causing DM. Studies using transgenic mice that express novel surface antigens, including viral proteins, have been undertaken to target the antigen involved in the pathogenesis of autoimmune type 1 DM.

Virus-induced Diabetes mellitus in humans

Several viruses including mumps virus and coxsackie viruses B3 and B4, were found to infect human β-cells. However, there is as yet no way to prove that these viruses actually infect and destroy human pancreatic β-cells in vivo and cause DM. In vitro susceptibility of β-cells to certain viruses may not truly reflect in vivo susceptibility because it is known that some viruses grow in cultured cells derived from animals normally resistant to viral infection. Several studies involving the isolation of viruses from pancreas of patients with acute-onset type 1 DM, followed by development of DM in susceptible mice infected with the viral isolates, have provided support for the hypothesis that viruses play a role in pathogenesis of human type 1 DM.

Coxsackie virus B subtypes

Direct evidence supporting a role for these viruses in type 1 DM has come from the isolation of coxsackievirus B subtypes from, or the presence of coxsackievirus antigens in pancreas of patients with recent onset type 1 DM. Lymphocytic infiltration, β-cells necrosis, and high levels of serum anti-coxsackievirus B4 antibody were found in the islets of a 5 year old girl in whom DM developed 2 weeks after open heart surgery. Similar findings were reported by several researchers (Champsaur et al., 1982). Those show that coxsackievirus B subtypes are able to infect human and murine islets and suggests that these viruses may contribute to the development of type 1 DM.

Three mechanisms have been proposed so far to explain the coxsackievirus B-induced type 1 DM. The first one involves direct infection of pancreatic β-cells in vivo, viral replication resulting in cell lysis and subsequent development of hypoinsulinemia and hyperglycaemia (Foulis et al., 1988). Second mechanism is molecular mimicry between coxsackievirus B4 antigen and a β-cells antigen. It was fixed that homology exists between
the P2-C protein of coxsackievirus B4 and GAD65 the best defined and extensively studied type 1 DM autoantigens. Atkinson et al. (1994) identified T-cell reactive determinants of GAD and found that major determinant of GAD in people at risk for development of type 1 DM had significant sequence homology with coxsackievirus B4 protein P2-C. Third mechanism suggested that a chronic β-cells, infection may result in β-cells specific autoimmunity leading to type 1 DM.

Cytomegalovirus

Cytomegalovirus (CMV) has also been implicated in type 1 DM, as evidenced by a case report of 13 month old baby with congenital CMV infection in whom type 1 DM developed (Ward et al., 1979; Pak et al. 1988) showed 20% patients with type 1 DM appear to have CMV genomic material in their pancreatic islets.

Mumps Virus

Mumps virus was one of the first viruses implicated in the development of type 1 DM and there continue to be cases in which mumps infection appears to precede the onset of type 1 DM. Hyoty et al. (1993) investigated whether vaccination against mumps has had any impact on anti-mumps antibody activity in children with type 1 DM or on the incidence of type 1 DM and concluded that elimination of natural mumps virus infection by vaccination may have been responsible for the decreased risk of type 1 DM.

Epstein-Barr Virus

A temporal link between Epstein-Barr virus (EBV) infection and the onset of type 1 has been reported in rare cases, including one in which the patient also had concurrent adenovirus and coxsackievirus B infections. In children with new-onset type 1 DM, EBV capsid antigen IgG antibody levels were significantly lower than in age-matched non-diabetic control subjects, suggesting that the diabetic children had abnormalities in their EBV-specific immune responses (Yoon and Hee-Sook, 2000).

Retroviruses

It has been shown that human insulin antibody-positive sera contain antibodies that both insulin and retroviral antigen p73. Two thirds of sera from newly diagnosed patients that bound insulin in ELISA also bound retroviral protein p73. Sera from 75% of insulin autoantibody positive, non diabetic, first degree relatives also bound p73. In contrast, only 2.7% of sera from non diabetic healthy control subjects bound p73 (Yoon and Hee-Sook, 2000). These suggests that endogenous retroviruses may be involved in the pathogenesis of autoimmune type 1 DM.

3.2.1.2 Animal Models of Type 1 Diabetes Mellitus

Useful analogies exist between human type 1 DM and similar disorders in rodents. These analogies engendered creative experimentation and powerful insights into the processes that generate and regulate autoreactivity. Nevertheless, autoimmune DM syndromes are not identical in different species. From a clinical perspective, mice and rats sometimes seem to be giving different lessons about human type 1 DM. Particularly with
respect to the generation of human therapeutics, animal data need to be extrapolated with considerable caution. To date, therapeutics effective in rodents for the prevention of disease have proven to be either ineffective or unacceptably toxic in humans (Atkinson and Leiter, 1999). Table 8 gives comparison of various parameters of different animal models of type 1 DM.

**Animals with spontaneous Onset of Insulitis and Hyperglycaemia**

Two animal models in this category have consistently provided data relevant to human type 1 DM: BioBreeding (BB) rats and the non obese diabetic (NOD) mouse. The clinical presentation and underlying microscopic islet disease observed in both rodents are similar to what is observed in humans. Spontaneous insulitis is followed by selective β-cell destruction with ensuing insulin deficiency and hyperglycaemia, all of which can be prevented by immunosuppression and immunomodulation.

**Diabetes mellitus prone and diabetes mellitus resistant BB rats**

Routine surveillance at the BioBreeding Laboratories in Canada led to the detection of spontaneous hyperglycaemia and ketoacidosis in a colony of outbred Wistar rats in the 1970s (Crisa et al., 1992). The first colony (BBDP/Wor) was established at the University of Massachusetts in Worcester. Results of studies that used BB rats of different origins may not be comparable. Non-diabetic BB control rats are also available. In Worcester colony, DM-resistant BB (BBDR/Wor) rats comprise a distinct inbred strain. BBDR/Wor rats never become spontaneously diabetic if maintained in viral antibody free vivaria, but they retain susceptibility to the induction of both DM and thyroiditis. The expression of DM in BB rats is a function of the relative balance between RT6- autoreactive (A) cells and RT6+ regulatory (R) cells (Rossini et al., 1993).

**NOD mice**

Makino and colleagues discovered this mouse of spontaneous autoimmune DM in the late 70s. These nonobese diabetic (NOD) animals acquire pancreatic insulitis by 4 or 5 weeks of age. By 7 months of age, 80-90% of female mice and 20-50% of male ice become diabetic. Ketoacidosis in affected animals is mild. Diabetic mice can survive up to a month without exogenous insulin. NOD mice also acquire sialadenitis and thyroiditis. Presence of insulitis and the response to immunosuppression suggest that NOD mouse DM is an autoimmune disorder. NOD mice appear to have a generalized defect in tolerance mechanisms. Splenocytes from adult NOD mice can adoptively transfer DM to MHC-compatible, immunodeficient recipients; efficient transfer is observed using 'both CD4+ and CD8+ lymphocytes (Christianson et al. 1993). Islet specific CD4+ and CD8+ T-cell clones have been obtained from affected NOD mice; these can induce DM in appropriate recipients (Mordes et al., 2000).

**Table 8: Comparative features of type 1 DM in different animal models and human**
**Features**  

<table>
<thead>
<tr>
<th>Human</th>
<th>Spontaneously diabetic animals</th>
<th>Animals with induced diabetes mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD Mouse</td>
<td>DP-BB Rat</td>
<td>DR-BB Rat</td>
</tr>
<tr>
<td><strong>Onset</strong></td>
<td>Spontaneous</td>
<td>Spontaneous</td>
</tr>
<tr>
<td><strong>Latency</strong></td>
<td>Severe</td>
<td>Up to 6 months</td>
</tr>
<tr>
<td><strong>Ketosis</strong></td>
<td>Severe</td>
<td>Mild</td>
</tr>
<tr>
<td><strong>Insulin deficiency</strong></td>
<td>Absolute</td>
<td>Mild to Severe</td>
</tr>
<tr>
<td><strong>Associated autoimmune diseases</strong></td>
<td>Thyroiditis, adrenalitis</td>
<td>Salodentis, thyroiditis</td>
</tr>
<tr>
<td><strong>Autoantibodies</strong></td>
<td>Insulin, GAD, ICA, islet cell surface antibody, carboxypeptidase H, IA-2</td>
<td>Insulin, GAD, ICA</td>
</tr>
<tr>
<td><strong>MHC genes</strong></td>
<td>HLA-DQ</td>
<td>Unique I-A</td>
</tr>
<tr>
<td><strong>Gender effect</strong></td>
<td>Male = Female</td>
<td>Female &gt; Male</td>
</tr>
<tr>
<td><strong>Response to general immunosuppression</strong></td>
<td>Cyclosporine prolongs endogenous insulin production</td>
<td>Cyclosporine prevent diabetes</td>
</tr>
</tbody>
</table>

β2-Microglobulin knockout NOD mice, which are deficient in MHC class I-restricted CD8+ cells, failed to become spontaneously diabetic. Further studies based on these observations suggest that CD8+ cells are involved in the initiation of autoimmune diathesis and CD4+ cells may amplify that response (Serreze et al., 1997). The progressive involvement of different classes of autoreactive cell populations may in part explain the progress of NOD disease through a series of checkpoints leading to insulitis to DM. It is likely that progression through these checkpoints involves co-stimulatory pathways. Co-stimulatory molecules in part determine whether an autoantigen-activated diabetogenic T cell differentiates into a β-cell cytotoxic effector cell or becomes nonresponsive. Co-stimulatory blockade prevents DM but not insulin in NOD mice (Balasa et al., 1997).

Additional data indicate that β-lymphocytes, functioning as antigen-presenting cells are critical to the development of DM in NOD mice. They may act by trapping islet antigen present in low concentration for presentation to autoreactive T cells and by providing important co-stimulatory signals. Anti-GAD antibodies are detectable in NOD mouse but levels of both anti-GAD and anti-insulin antibodies appear to be low (Velloso et al., 1994).

The preponderance of DM in female compared with male NOD mice have prompted speculation that androgens exert a protective effect and this has been shown experimentally. Male castration does not increase the frequency of NOD DM, but sex-specific differences in anti-oxidant enzyme activities have also been described and could contribute to differential susceptibility. In human type 1 DM both sexes are affected approximately equally (Karvonen et al., 1993). In the BB rat, DM occurs with equal frequency in both sexes and neither gonadectomy nor hypophysectomy changes the frequency in DM (Crisa et al., 1992).

### Toxin-induced diabetes mellitus and insulitis

#### Single high-dose streptozotocin model

Streptozotocin (STZ), a commonly used substance in animals for the study of diabetes, originally was used as antibiotic. STZ was isolated from *Streptomyces achromogens* in the early 1960's and it was found to be effective broad-spectrum antibiotic drug. Rakietan and colleagues (1963) reported about the diabetogenic property of STZ. It
also possesses anti-tumor and oncogenic properties. Injection of a high dose STZ induces β-cell necrosis within 4 h of administration and hyperglycaemia is achieved rapidly. Alloxan and streptozotocin (STZ), at high doses are selective β-cell cytotoxins. STZ is also used in the chemotherapy for insulinomas. It has a short biological half-life of 5-15 min in-vivo. STZ is a D-glucopyranose derivative of N-methyl-N-nitroso urea. Both are potent alkylating agents (Bennett and Pegg, 1981), highly toxic and carcinogenic (Schein et al., 1974) but only STZ is diabetogenic because of the selective destruction of insulin-producing β-cells resulting from necrosis. It has been suggested that β-cell toxicity of STZ is related to the glucose moiety in its chemical structure which enables STZ to the β-cell via the low affinity glucose transporters. This hypothesis is supported by the observation that the RINm5F rat insulinoma cell line, which does not express GLUT 2 resists STZ toxicity and becomes sensitive to the toxic action of STZ only after expression of GLUT 2 glucose transporters in this cell line (Schnedl et al., 1994).

Inside the cell, STZ is decomposed. During this procedure reactive methylcarbonium ions are produced, which may alkylate the DNA and cause cross-links between DNA strands. This initiates DNA repair mechanisms in the cell. The damaged sections of the DNA are recognized and excised by DNA repair nucleases, leading to DNA strand interruptions. It has been suggested that a nuclear enzyme called poly ADP-ribose polymerase (PARP) participates in this process (de Murcia, 1994). PARP and DNA polymerases compete for binding to the strand breaks in the DNA, but PARP binds stronger which affects the access for repair enzymes to the damaged site. It is thought that the binding PARP prevents replication and transcription of damaged DNA (Satoh and Lindahl, 1992) and it may also constitute an emergency signal. If PARP binds, it leads to poly (ADP-ribose) synthesis and long chains of NAD become attached to PARP and other acceptor proteins and thereby these proteins are modified (de Murcia, 1994). This kind of modulation by ribosylation is a way of changing binding properties of proteins (Simbulan-Rosenthal, 1996). The auto-modified PARP has reduced affinity for the DNA and therefore it dissociates, allowing the DNA repair enzymes to reach the damaged area (Satoh and Lindahl, 1992). DNA polymerase then synthesizes a new strand, and the DNA parts are finally joined together by DNA ligase. The DNA damage caused by STZ and the following activation of DNA repair mechanisms sooner or later depletes the cell content of NAD (Kullin et al., 2000) in β-cells. This causes a deficiency in cofactors for oxidative phosphorylation with subsequent lack of ATP, which in turn causes diminished protein synthesis, insulin and reduced activity of ion pumps, which can also lead to cell death. In this context, it has been shown that the action of PARP can be inhibited by nicotinamide and theophylline, thereby saving the cellular NAD stores but at the same time deteriorating the DNA repair. Furthermore, it has recently been reported that mice lacking the PARP gene are resistant to pancreatic β-cell destruction and diabetes development induced by STZ (Burkart et al., 1999).

**Low-dose streptozotocin model**

When mice receive multiple small subdiabetogenic doses of STZ, pancreatic insulitis, selective β-cell destruction and DM ensue after a delay of several days (Like and Rossini, 1976). The process depends on the glucose transporter GLUT 2 on β-cells, probably involves the poly (ADP-ribose) polymerase gene (Burkart et al., 1999) and involves the CD28-B7 costimulatory pathway for T cell activation (Herold et al., 1997). Insulitis consists of T cells and macrophages (Kolb and Kroncke, 1993). Various MHC associations have been
identified, but no one haplotype uniformly confers resistance or susceptibility to all strains (Kolb and Kroncke, 1993).

Islet autoantibodies appear after STZ treatment, but there is no evidence for an etiologic role for humoral immunity. Treatment with low-dose STZ is also associated with induction of pancreatic retroviruses but there is no evidence that they play an active role in pathogenesis. Athymic mice are resistant to low-dose STZ DM (Paik et al., 1980), but this can be reversed by T-cell reconstitution from normal donors. Many immunosuppressive interventions ameliorate low-dose STZ DM (Kolb and Kroncke, 1993).

The low-dose STZ model has two distinct components. One is a direct β-cell cytotoxic effect, which can be overcome by antioxidant therapy (Hotta et al., 1998). The other is generation of immunologic recognition of residual, altered β-cells, regardless of whether those β-cells have been lethally injured by STZ. Islet transplantation data support these concepts (Weide and Lacy, 1991). Insulitis develops in syngeneic islet transplants in low dose STZ diabetic mice if they are transplanted 10 to 14 days before injection of the drug. In contrast, there is no graft insulitis if the transplants are done 3 days after the injections of STZ, indicating that some exposure to the toxin is needed to facilitate immune recognition of islet grafts. Low-dose STZ appears to induce autoimmunity in susceptible hosts by altering β-cells and inducing autoantigenicity.

**Transgenic mouse models of type 1 diabetes mellitus**

**Transgenic strategy**

Transgenic expression involves introducing functional gene into the germline, in most cases to obtain expression of specific gene in a target tissue of interest. Microinjection of cloned DNA directly into the male pronuclei of recently fertilized one-cell stage oocytes is the most common method used for generating transgenic mice. The DNA transgene may contain the coding sequence as well the promoter-enhancer sequence that normally directs tissue-specific expression of the gene. Alternatively, the coding sequence can be ligated to other gene's promoter-enhancer sequence specific for the tissue of interest to which the coding sequence is to be targeted. After microinjection of several copies of DNA, eggs are implanted into the oviducts of gonadotropin-primed pseudopregnant foster mothers. This germline injection results in the random chromosomal integration of the DNA into all cells of the offspring, but gene expression to RNA and protein should normally occur only in the tissue for which the promoter-enhancer sequence is active (Jaeneisch, 1988).

Post-natally the presence of the transgene in the offspring is detected by analysis of DNA extracted from a segment of the mouse tail or from blood cells, by the Southern blot test the polymerase chain reaction (PCR) technique. Transgene-positive mice can then be mated with non-transgenic mice to establish several transgenic linkages.

The history of transgenes and β-cell began with the isolation of the rat insulin II promoter (RIP) sequence by Hanahan (1985). Table 9 gives an account of different transgenic models in the NOD mouse.
Table 9: Different transgenic models in NOD mouse

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Insulitis</th>
<th>Diabetes mellitus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Increase</td>
<td>Increase</td>
<td>Allison et al., 1994</td>
</tr>
<tr>
<td>IL-6</td>
<td>Increase</td>
<td>Decrease</td>
<td>DiCosmo et al., 1994</td>
</tr>
<tr>
<td>IL-10</td>
<td>Increase</td>
<td>Increase</td>
<td>Wogensen et al., 1994</td>
</tr>
<tr>
<td>TNF-α (1)</td>
<td>Increase</td>
<td>Decrease</td>
<td>Grewal et al., 1996</td>
</tr>
<tr>
<td>TNF-α (2)</td>
<td>Increase</td>
<td>Increase</td>
<td>Green et al., 1998</td>
</tr>
<tr>
<td><strong>T-cell receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDC2.5 (CD4+)</td>
<td>Increase</td>
<td>Increase</td>
<td>Katz et al., 1993</td>
</tr>
<tr>
<td>8.3 (CD8+)</td>
<td>Increase</td>
<td>Increase</td>
<td>Verdaguer et al., 1997</td>
</tr>
<tr>
<td><strong>Antigens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proinsulin</td>
<td>Decrease</td>
<td>Decrease</td>
<td>French et al., 1997</td>
</tr>
<tr>
<td>Hsp60</td>
<td>Decrease</td>
<td>Decrease</td>
<td>Birk et al., 1996</td>
</tr>
<tr>
<td>GAD</td>
<td>No change</td>
<td>No change</td>
<td>Geng et al., 1998</td>
</tr>
</tbody>
</table>

Knockout Strategy

There are limitations of transgenic technology which include the potential for non-specificity and unpredictable effects owing to random integration of foreign DNA, the inability of promoter sequences to mimic complex natural regulatory sequences that may be much longer or dispersed and in some cases pathologic over expression of the transgene that may not reflect the usual functions of the gene product in health or disease. These lead to the development of another technique known as gene knock out which involves gene targeting, gene knock out or gene replacement. Here, a gene of interest is replaced by the sequences engineered in the laboratory to potentially alter it in a defined. When the engineered DNA contains a sequence homologous to normal cellular DNA, it manages to find its complementary endogenous sequence, resulting in homologous recombination.

Engineered DNA molecules (called DNA targeting vectors) designed to carry such red genes to cells contains sequences encoding selectable markers that allow cells containing foreign DNA to be distinguished from other cells.

The targeting vector is inserted into embryonic stem (ES) cell lines by electroporation. ES cell lines are maintained in an undifferentiated state by culturing with leukemia inhibitory factor. After electroporation ES cells are grown in a medium containing G418 (a marker gene encoding resistance to neomycin) and the resistant clones are tested for the Presence of electroporated DNA by PCR. ES cells with homologous integration of the foreign DNA are reintroduced into blastocytes. The reconstituted blastocytes are placed in the uterus of a pseudo-pregnant foster mother. The chimeric progeny are then mated with normal mice, resulting in heterozygote knockout mice which are then mated to produce homozygote knockout mice. Cytokines and their receptors, components of T-cell and B-cell receptors for antigen MHC molecules, co-stimulatory molecules, transcription factors, and effector molecules are the immunologically relevant genes used for targeting. Table 10 gives details of effects of different gene targeting on NOD mice.
Table 10: Different genes knock-out models in NOD mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Insulitis</th>
<th>Diabetes mellitus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>→</td>
<td>→</td>
<td>Wang et al., 1997</td>
</tr>
<tr>
<td>IFN-γ γ</td>
<td>→</td>
<td>→</td>
<td>Hultgren et al., 1996</td>
</tr>
<tr>
<td>IFN-γR γ</td>
<td>↓</td>
<td>↓</td>
<td>Wang et al., 1998</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>→</td>
<td>↓</td>
<td>Kagi et al., 1999</td>
</tr>
</tbody>
</table>

↓: Decrease; →: No change

The uses of transgenic technology in the analysis of autoimmune DM in mice are many and varied, but it is important to recognize the limitations of these complex systems as models of human disease. Transgenes can alter the intracellular metabolism, affecting insulin secretion and they can induce expression of molecules that do not naturally occur in β-cells. Tissue-specific promoters can drive the expressions of transgenes in expected sites with unforeseen consequences. In the transgenic mouse both copy number and site of insertion can affect phenotype. Although the promise of these early breakthroughs may not be fulfilled, ongoing research will continue to generate exciting new concepts and reveal new mechanisms, which will ultimately lead to the better understanding.

3.2.2 Type 2 Diabetes Mellitus

Type 2 DM is the most common form of diabetes. It is characterized by disorders of insulin action and insulin secretion, either of which may be the predominant feature and usually present, by the time the disease becomes clinically manifested. Much of the information on risk factors and knowledge of pathogenesis have been derived from studies of high frequency populations such as Pima Indians and Mexican Americans but similar patterns have been confirmed in other racial groups and other continents (Zimmet, 1992).

3.2.2.1 Risk Factors

Type 2 DM result from the interaction of genetic and environmental factors. The disease long has been recognized as showing familial aggregation. Most convincing evidence came from the study of twins. Concordance rates for type 2 DM in older identical twins range from 50% to 90% much higher than among similarly aged non-identical twins, siblings or first degree relatives (Poulsen et al., 1999). Although the evidence that susceptibility to type 2 DM is genetically determined, the extensive studies have not provided a clear picture of genetic determinants.

Environmental Factors

The development of type 2 DM is influenced by exposure to different environments. The prevalence of type 2 DM in those aged 15 years and older in India has been estimated to be approximately 2% in urban populations and 1.5% in rural ones. Studies of similar age groups in Indian populations show much higher rates of incidence. In Singapore 9% of Asian Indians are reported to have type 2 DM, compared with 5% in South Africa and 13% in Fiji. Similar incidents have been reported for migrants from other races and continents (Taylor et al., 1983). This increase is partially attributable to a rise in the average age of the population and to the widespread use of the automated methods for determining glucose levels, a practice that has increased the recognition of previously undiagnosed type 2 DM. Moreover,
some part of the increase can probably be attributed to decreased mortality rate from DM and cardiovascular disease, which resulted in increased survival among diabetic patients. Nevertheless, most of the increase appears to be due to a true increase in the incidence of the disease.

**Obesity**

Obesity is a major determinant of the incidence of type 2 DM, but in most populations, a small proportion of obese people contract the disease. Among obese subjects with diabetic parent, the incidence of type 2 DM is many times higher than among equally obese people whose parents do not have diabetes. Among those who are not obese, but even if one or both the parents are diabetic, the incidence of type 2 DM is much lower. Not only the presence but the distribution of obesity influences the risk for development of type 2 DM. Upper body or central obesity is associated with an increased risk for development of type 2 DM, as has been shown in many ethnic and racial groups (Haffner et al., 1990). Hyperinsulinemia or insulin resistance appears to be a central feature of this cluster of abnormalities related to truncal or abdominal obesity (DeFronzo and Ferrannini, 1991).

**Physical Activity**

Cross-sectional studies in several ethnic groups show that prevalence of DM among physically inactive people is typically two to three times higher than among physically active people in the same population. Studies in Pima Indians indicated a similar pattern (Kriska et al., 1993). Moreover, in a randomized trial in subjects with impaired glucose tolerance (IGT) in China, the incidence of type 2 DM was reduced by exercise intervention (Pan et al., 1997). Increased obesity and reduced physical activity both favour the development of insulin resistance, which appears to be a critical component in the pathogenesis of type 2 DM.

**Diet**

Another factor that may contribute to the development of insulin resistance is diet with a high percentage of calories from fat, decreased fiber content and decreased unrefined carbohydrate content. Populations with high prevalence of DM characteristically consume a diet that contains more fat, particularly saturated fat, than they ate when they followed a more traditional way of life (Bennet, 2000). There is some evidence that the type of carbohydrate, the fiber content, alcohol consumption and the type of fats consumed may influence the risk for development of type 2 DM (Salmeron et al., 1997).

**Birth Weight**

Low birth weight is associated with an increased risk for development of type 2 DM in later life (Lithell et al., 1996). This relationship was first postulated to be the result of under-nutrition in utero, giving rise to limited development of pancreatic β-cells, whose number is fixed at or about the time of birth (Hales and Barker, 1992). Low birth weight is also associated with an increased risk of hypertension, hypertriglyceridemia and coronary heart disease, suggesting that the relationship of low birth weight with type 2 DM and IGT may be mediated through a mechanism of increased insulin resistance. Several studies indicate that low-birth weight infants acquire insulin resistance. Furthermore, birth weight may be determined by genetic factors that regulate glucose homeostasis (Hattersley et al.,...
Another clear environmentally determined early life event is the choice of infant feeding. Breast feeding exclusively for several months is associated with a lower prevalence of DM in young adult Pima Indians (Pettitt et al., 1997).

**Diabetic Pregnancy**

Offspring whose mothers had type 2 DM during pregnancy have a high risk for development of type 2 DM (Pettitt et al., 1988). Although such offspring have increased genetic susceptibility to the disease, those born before the development of DM have a much lower risk than offspring born after the mother acquires the disease. The exposure to the diabetic intrauterine environment predisposes the child to the development of the disease. The effect is beyond any that can be explained by genetic factors alone. Infants of diabetic mothers typically have a higher than normal birth weight, but the specific reason why they are prone to development of DM is uncertain. In populations with a high prevalence of type 2 DM in the child bearing age range, such as the Pima Indians, one third of children in whom type 2 DM developed before 20 years of age were the product of diabetic pregnancy (Debelea et al., 1998).

### 3.2.2.2 Pathophysiology of Type 2 Diabetes Mellitus

The type 2 DM is preceded by hyperinsulinemia and IGT. Hyperinsulinemia in fasting state represents an index of insulin resistance. Insulin resistance is usually present for many years before evidence of glucose intolerance appears and its presence can be regarded as a stage in the development of type 2 DM (Haffner et al., 1990). After IGT develops, decompensation to DM associated with reduced early insulin secretion. Nevertheless, fasting insulin levels which remain high in recent-onset DM diminish later in course of disease.

Insulin action and secretion are two key players in the development of frank diabetes in obese subjects. The two variables regulate glucose uptake and metabolism in skeletal muscles together with hepatic glucose production (HPG). For the development of diabetes, defects in both variables are necessary. Hyperglycaemia first develops when insulin resistance cannot be compensated for by appropriate elevation of insulin secretion. It is important to note that, as long as β-cell function is normal, even severe insulin resistance may not lead to frank diabetes.

**Insulin resistance**

Defects in insulin action result in tissue insulin resistance. Insulin resistance is a metabolic state in which physiologic concentrations of insulin produce less than normal biologic response. This could be due to:

- a) abnormal insulin molecules
- b) incomplete conversion of proinsulin to insulin
- c) elevated levels of growth hormone, cortisol, glucagons or catecholamines
- d) the presence of insulin or insulin receptor antibodies
- e) decreased capillary density or the failure of insulin to facilitate its own delivery by increasing blood flow
- f) impaired transcapillary passage from the intravascular compartment to interstitial compartment
g) insulin resistance at cellular level

The major cause of insulin resistance in the majority of patients with type 2 DM is at the cellular level. The major metabolic consequence of insulin resistance is hyperglycaemia caused both by the failure of insulin to inhibit hepatic glucose production and by the glucose utilization by peripheral tissues.

**Neuroendocrine and Humoral Factors in Insulin Resistance**

**Glucocorticoids**

Glucocorticoids are produced in the adrenal cortex and the production is governed by the thalamic-pituitary-adrenal axis via adrenocorticotropic hormone (ACTH) that is released by pituitary. Glucocorticoids play a key role in regulating salt and water homoestasis, blood pressure, immune function and metabolism. The main glucocorticoid in man is cortisol. The clinical syndrome of glucocorticoid excess, Cushing's syndrome, is associated with insulin resistance, glucose intolerance, central obesity and hypertension. Pharmacological treatment with high doses of glucocorticoids also leads to an impairment of insulin sensitivity. In clinical obesity, there are alterations in cortisol metabolism, and local activation of cortisol in the adipose tissue may be an important link between glucocorticoids and development of the so-called metabolic syndrome (Rask *et al.*, 2001). The metabolic effects of cortisol are partly explained by its effects to oppose the actions of insulin, i.e. to induce a state of insulin resistance. The effects of glucocorticoids in vivo appear to include both an impairment of insulin-dependent glucose uptake in peripheral tissues and a stimulation of gluconeogenesis in the liver (Rooney *et al.*, 1993). In addition to their effects on insulin sensitivity, glucocorticoids may also inhibit insulin secretion from pancreatic β-cells (Delaunay *et al.*, 1997). For a long time it has been known that glucocorticoids inhibit insulin-stimulated glucose metabolism in adipocytes (Fain *et al.*, 1963) and muscle (Riddick *et al.*, 1962).

This appears to be mediated primarily by an impairment of glucose transport, and dexamethasone-induced insulin resistance in 3T3-L1 adipocytes probably involves the GLUT4 translocation machinery (Sakoda *et al.*, 2000). Previously, it has been reported that insulin-stimulated recruitment of GLUT4 to the cell surface is inhibited in rat skeletal muscle following dexamethasone treatment. Glucocorticoids are reported to activate adipose tissue lipolysis, and this is probably also an important factor in promoting insulin resistance, since insulin sensitivity was normalized when lipolysis or lipid oxidation (Guillaume-Gentil *et al.*, 1993) was inhibited. There are several synthetic cortisol analogues available for research purposes and in clinical practice. The glucocorticoid activity of dexamethasone is approximately 25 times stronger compared to that of cortisol.

**Catecholamines**

Catecholamines mainly adrenaline and noradrenalin, are secreted by the adrenal medulla and sympathetic nerve endings, respectively, and this is stimulated by physical and mental stress. An acute injection of catecholamines decreases the sensitivity to insulin’s effect on glucose utilization, and leads to elevation of blood glucose. This is not only mediated by an enhanced rate of glycogen breakdown in liver and an increased rate of fatty acid mobilization, but also by inhibition of insulin secretion and stimulation of glucagon release. Some physiological situations with a long-term increase in catecholamine-levels,
however, are in fact associated with an increase in the sensitivity of glucose metabolism to insulin (e.g. physical exercise) (Cherrington et al., 1984).

In fact, catecholamines increase lipolysis by stimulating plasma membrane adenylyl cyclase activity through β-adrenergic receptors, which leads to increased intracellular levels of cyclic adenosine monophosphate (cAMP) and then activation of cAMP-dependent protein kinase A (PKA). Hormone-sensitive lipase (HSL), the rate-limiting enzyme in lipolysis, is in turn phosphorylated and activated by PKA.

**Growth hormone**

Growth hormone (GH) is secreted from the anterior pituitary. GH impairs insulin binding and glucose uptake in some tissues, increases hepatic glucose output, and mobilizes FFA from adipose tissue. Moreover, GH treatment in adults with growth hormone deficiency was associated with a measurable increase in insulin and glucose levels, indicating mild insulin resistance. However, GH may also reduce adiposity, since GH deficient individuals have an increased fat mass. This might also be of importance in humans with visceral obesity, since multiple endocrine perturbations are found, including low GH and elevated cortisol and androgens in women, as well as low testosterone secretion in men.

However, the role of GH in insulin resistance is not yet fully understood. The autonomic nervous system could potentially contribute to insulin resistance in type 2 diabetes, and this would mainly be mediated via the release of catecholamines. Studies in animal models of type 2 diabetes as well as patients with type 2 diabetes have revealed an altered sympathetic activity and, moreover, their carbohydrate metabolism seems abnormally sensitive to sympathetic stimulation (Bruce et al., 1992). In healthy humans with or without a family history of type 2 diabetes, recent data suggest that insulin resistance is associated with an altered balance in the autonomic nervous system with a relative ease in sympathetic Vs parasympathetic activity following standardized stress or owing hyperinsulinemia (Laitinen et al., 1999). Thus, dysregulation of the autonomic nervous system might be a potential mechanism for early insulin resistance in the development of type 2 diabetes.

**Hyperglycemia and hyperinsulinemia**

In diabetes, glucose levels are chronically elevated, and insulin levels are naturally often abnormal, e.g. high in early type 2 diabetes but low in later type 2 diabetes and in type 1 diabetes. Experimental hyperinsulinemia has been shown to cause insulin resistance both in vitro (Garvey et al., 1986) and in vivo (Bonadonna et al., 1993). In isolated rat adipocytes, long-term exposure (24 h) to high glucose in the presence of insulin downregulates subsequent basal and acutely insulin-stimulated glucose transport. Hyperglycemia alone exerts detrimental effects on insulin secretion and insulin action, a phenomenon commonly referred to as glucose toxicity (Rossetti et al., 1990), and in muscle from patients with type 2 diabetes, insulin-stimulated glucose transport is impaired under hyperglycemic conditions (Zierath et al., 1994). Moreover, glucose transport capacity of isolated muscle strips can be restored in vitro following incubation at a physiological glucose level, supporting that glucose levels per se have regulatory effects on the glucose transport machinery and that these effects are reversible (Zierath et al., 1994). Accordingly, reversal of hyperglycemia in rats by phlorizin treatment improves insulin sensitivity (Kahn et al., 1991). Several studies in rats have suggested that increased hexosamine biosynthesis leads to skeletal muscle insulin resistance in vivo and in vitro and that this may be a mechanism involved in glucotoxicity (Hawkins et al., 1996). Moreover, glucose-induced activation of different PKC isoforms has
been shown to interfere with insulin receptor signaling and produce insulin resistance (Kawano et al., 1999). However, the mechanisms by which hyperglycemia causes insulin resistance still remain incompletely understood.

**Free Fatty acids**

Elevated free fatty acids (FFAs) might promote accumulation of fat depots in muscle, liver and/or β-cells, and the accumulated triglycerides might provide an environment that could interfere with metabolic signaling and thus action in these different tissues (Nyholf et al., 1999). A link between insulin resistance and triglyceride content in muscle biopsies has been established (Pan et al., 1997a). Moreover, it was shown that elevations in free fatty acid concentrations can lead to an attenuated effect of insulin to stimulate Insulin receptor substrate 1 (IRS-1)- associated PI-3 kinase activity in muscle (Dresner et al., 1999). The reduced PI-3 kinase activity may be due to a direct effect of intracellular free fatty acids or some fatty acid metabolite, or it may be secondary to alterations in upstream signaling events. Recent data have suggested that fatty acid metabolites activate a kinase that phosphorylates serine/threonine sites on IRSs, which in turn may reduce the ability of the IRSs to activate PI-3 kinase and glucose transport (Griffin et al., 1999).

It is well known that FFAs are important substrates for skeletal muscle energy production. In the fasting state, skeletal muscle has a high fractional extraction of plasma FFAs, and lipid oxidation accounts for the majority of energy production. The capacity of skeletal muscle to utilize lipid-or carbohydrate fuels, as well as the potential for substrate competition between fatty acids and glucose, is of interest in insulin resistance.

A potential implication of the glucose-fatty acid cycle, originally postulated by Randle and colleagues (Randle et al., 1963), is that increased lipid availability could interfere with muscle glucose metabolism and contributes to insulin resistance for example in obesity and type 2 diabetes. Several studies support the concept that elevated free fatty acids produce an impairment of insulin-stimulated glucose metabolism (Roden et al., 1996).

Another concept is that of metabolic inflexibility in insulin resistance. In the fasting condition, skeletal muscle predominantly utilizes lipid oxidation for energy production. Upon insulin stimulation in the fed condition, healthy skeletal muscle rapidly switches to increased uptake, oxidation and storage of glucose and, moreover, lipid oxidation is suppressed. Obese individuals and those with type 2 diabetes manifest higher lipid oxidation during insulin-stimulated conditions as compared to control subjects (Felber et al., 1987), despite lower rates of lipid oxidation during fasting conditions. This suggests that a key feature in insulin resistance of skeletal muscle is an impaired ability to switch between fuels.

**Renin-angiotensin system (RAS)**

RAS must also be considered in the development of insulin resistance. It has been shown that treatment with angiotensin converting enzyme inhibitors (ACE-I) and angiotensin receptor blockers (ARB) may improve insulin sensitivity (Iimura et al., 1995) and prevent the development of type 2 diabetes (Lindholm et al., 2002). Angiotensin II (AT II) exerts many effects that can be related to insulin resistance, e.g. increases hepatic glucose production, inflammation and the activity of the sympathetic nervous system (McFarlane et al., 2003).
Mechanisms of Insulin Resistance

Insulin receptor

Following secretion from the β-cells of the pancreas, insulin binds to its specific cell-surface receptor. The highest numbers of receptors are present in insulin-sensitive tissues such as liver and adipose and significantly fewer receptors are present in non-classic target tissues such as circulating erythrocytes and in brain (Rosen, 1987). In its native conformation, the insulin receptor is a transmembrane glycoprotein composed of two α-subunits (135 kd) and two β-subunits (95 kd) covalently linked through disulfide bonds to form an α₂β₂ heterotetramer. The α-subunit is entirely extracellular and contains the site for insulin binding, whereas the β-subunit has small extracellular portion, a transmembrane domain and an intracellular insulin-regulated tyrosine protein kinase activity. Both subunits are derived from a single gene via a large proreceptor polypeptide (Hedo et al., 1983).

α-subunit is responsible for ligand binding (Ullrich et al., 1985). It may also be viewed as the regulatory subunit for this classic allosteric enzyme. Removal of a portion of the α-subunit by digestion with trypsin or in vitro mutagenesis releases the inhibitory effect and activates the kinase (Shoelson et al., 1988). Thus, in the absence of insulin, the α-subunit maintains a structural constraint on the β subunit which inhibits an otherwise constitutively active kinase. Under basal conditions, the α-subunit serves as an allosteric inhibitor of the β subunit. It is thought that this oligomerization step provides environment for the phosphorylation of neighboring ligand-bound receptors which leads to kinase activation. In all cases of ligand-induced receptor oligomerization, the result is increased kinase activity. There is evidence that insulin receptors undergo dimerization or oligomerization during signaling (Heffetz and Zick, 1986).

Insulin binding to the α-subunit rapidly induces a complex cascade of Tyr (tyrosine) phosphorylation in β-subunit in both intact cells and in purified receptor preparations (White et al., 1988).

A reduced auto-activation status of the insulin receptor from skeletal muscle of type 2 Diabetic patients has been described. Some of these studies have shown that obesity was the major factor for the development of reduced insulin receptor activity and the defective insulin receptor kinase activity is secondarily acquired due to obesity and metabolic changes such as hyperinsulinemia and hyperglycemia (Caro et al., 1987).

Insulin receptor substrates

Recent advances after identification of the IRS-1, has shifted the focus towards the association model of protein-protein interaction for signal transduction (Cheetham and Kahn, 1995). IRS-1 is phosphorylated by the insulin receptor activated by binding of insulin. IRS-1 is shown to contain at least eight Tyr residues which act as potential sites for phosphorylation. IRS-1 is also found to be the substrate for insulin like growth factor-1 (IGF-1) receptor.

There are presently nine insulin receptor substrate proteins (IRS-1, IRS-2, IRS-3, IRS-4, Dok, Gab-1, Cbl, APS and isoforms of She) (Ross et al., 2004). IRS proteins can recruit other proteins (PI3K, Nck, Grb2 and Crk II), thereby forming a multifunctional signaling centre from which insulin action emanates. Despite significant homology among the IRS proteins, experiments with both transgenic animals and cultured cells imply their functions are complementary in nature rather than redundant. Ablation of IRS-1 in mice
manifests as insulin resistance in peripheral tissues; impaired glucose tolerance and pre-and postnatal growth retardation (Tamemoto et al., 1994). Similarly IRS-2 knockout mice are also characterized as having peripheral insulin resistance. In contrast to IRS-1 (−/−) mice, IRS-2 (−/−) mice also demonstrate hepatic insulin resistance and diminished β-cell mass culminating in frank type 2 diabetes (Withers et al., 1998), whereas, knockouts of IRS-3 and IRS-4 display apparently normal growth and glucose homeostasis (Fantin et al., 2000).

Emerging data indicate that serine phosphorylation of IRS-1 attenuates IRS-1 signaling activity via prevention of tyrosine phosphorylation (Ross et al., 2004). The precise identification of all the candidate regulatory phospho-serine sites within IRS-1 and the kinases that phosphorylate them is under intense investigation. Recently Ser\(^{307}\) has been implicated as a potentially critical residue as a candidate regulatory phosphorylation site, whereby phosphorylation of this site prevents insulin elicited tyrosine phosphorylation of IRS-1.

A role for the dysregulated c-Jun NH2-terminal kinase isotype 1 (JNK-1) in mediating obesity and insulin resistance has come into light with the characterization of JNK-1 knockout mice. The absence of JNK-1 resulted in decreased adiposity coupled with enhanced insulin signalling. Insulin induced a greater extent of tyrosine phosphorylation of the insulin receptor and IRS-1 in JNK-1 (−/−) mice when compared with control. Furthermore, obese mice showed an increase in IRS-1 Ser\(^{307}\) phosphorylation relative to lean control, whereas, there was no evidence of Ser\(^{307}\) phosphorylation relative to either lean or obese JNK-1 (−/−) mice (Hirosumi et al., 2002).

**Phosphatidylinositol 3-Kinase**

Phosphatidylinositol 3-kinase (PI3K) comprises of two protein subunits designated as p85 and p110 with molecular weights of 85 and 110 kDa respectively. Upon insulin treatment, the p85 subunit associates via its SH2 domain with tyrosine-phosphorylated IRS proteins. This interaction activates the catalytic p110 subunit to phosphorylate the 3- position of phosphatidylinositol 4-phosphate and phosphatidylinositol 4, 5-bisphosphate to form phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate respectively.

Several studies have defined a central role for the activation of PI3K in mediating GLUT 4 vesicle translocation and thereby, increasing glucose transport in response to insulin. Treatment of rat and 3T3-L1 adipocytes with inhibitors of PI3K such as the fungal metabolite wortmannin (Okada et al., 1994) or expression of dominant interfering mutants of PI3K (Katagiri et al., 1996) completely blocks insulin stimulated GLUT 4 translocation and glucose transport. Microinjection of a neutralizing antibody to the p110 subunit of PI3K into 3T3-L1 adipocytes completely abolished GLUT 4 translocation (Hausdorff et al., 1999).

 Whereas inhibition of PI3K inhibits insulin stimulated glucose transport (Fig. 1), over-expression of wild type PI3K in 3T3-L1 promoted glucose transport activity and GLUT 4 vesicle translocation comparable to the maximal effect induced by insulin (Martin et al., 1996). Interestingly growth factors such as platelet-derived growth factor (PDGF) as well as certain cytokines activate PI3K to a similar extent as insulin, but they do not stimulate GLUT 4 translocation or other metabolic effects of insulin (Wiese et al., 1995). Current thinking suggests the exquisite sensitivity to insulin is a matter of intracellular location, where insulin-stimulated PI3K activity is localized to an intracellular low density microsomal compartment at or near the GLUT 4 storage vesicle versus PDGF-stimulated PI3K activity localized in plasma membrane (Yang et al., 1996). But a study reported that a constitutively active PI3K specifically targeted to GLUT 4 vesicles was without effect on GLUT 4 translocation.
(Frevert et al., 1998). A gap in understanding still exists in the link between the coupling of insulin-stimulated PI3K activation and GLUT 4 vesicle translocation.

**Downstream signaling of PI3K**

**Isoforms of Protein Kinase C**

The atypical isoforms of protein kinase C such as PKCα and PKCζ are so called because they are not activated by either diacylglycerol or phorbol ester and are activated in vitro in the presence of PI3K products (Toker et al., 1994) or PDGF or EGF stimulation (Akimoto et al., 1996). Either or both PKCα and PKCζ have been implicated in insulin stimulated glucose transport in 3T3-L1 adipocytes (Bandyopadhyay et al., 1997), rat skeletal muscle (Etgen et al., 1999) and rat (Bandyopadhyay et al., 1997) and human (Bandyopadhyay et al., 2002) adipocytes. Interestingly following activation by insulin, PKCα and ζ may be targeted to GLUT 4 containing vesicles (Standaert et al., 1999). A third isoform PKCδ, a novel but not atypical isoform, was shown to participate in the insulin-elicted glucose transport in rat skeletal muscle (Braiman et al., 1999). However, a recent study does not support a role of the atypical PKC isoforms in mediating glucose transport. Robust over expression of PKCα, PKCζ and PKCδ wild type isoforms in 3T3-L1 adipocytes did not alter basal or insulin-stimulated glucose transport (Tsuru et al., 2002). The role of atypical PKCs in mediating insulin-stimulated glucose transport requires further clarification.

**Protein Kinase B (AKT)**

AKT also known as protein kinase B (PKB) is activated by insulin in a variety of cell types through phosphorylation of its serine and threonine residues (Kohn et al., 1995). PI3K inhibitors block this activation (Kohn et al., 1996). Ablation of Akt2 (an isoform of Akt), in mice resulted in animals that were insulin resistant as characterized by elevated blood glucose and insulin concentrations, defective suppression of hepatic gluconeogenesis and modestly decreased insulin stimulated glucose uptake in skeletal muscle (Cho et al., 2001).

**c-Cb1/CAP Signaling Pathway**

There is a general acceptance that PI3K activation is necessary for GLUT 4 translocation and glucose transport, but multiple lines of evidence suggest that stimulation of PI3K is not solely sufficient to mediate the process. One or more additional pathways may be required. Furthermore, introduction into 3T3-L1 adipocytes of membrane-permanent forms of PIP3, a product of the PI3K reaction, had no effect in increasing basal glucose transport, whereas pretreatment of adipocytes with the PI3K inhibitor wortmannin followed by addition of the PIP3 analogue in combination with insulin did elicit maximal glucose transport (Jiang et al., 1998).These data are consistent with the presence of a second PI3K-independent pathway for stimulating glucose transport.

Recently, potential upstream mediators of this pathway have been described. Insulin stimulates the tyrosin phosphorylation of the Cbl proto-oncogene (Ribon and Saltiel, 1997) that associates with the c-Cbl associating protein (CAP) (Ribon et al., 1998) and flotillin in caveolae and/or lipid rafts, known plasma membrane microdomains. Caveolae are present in many cell types, are thought to be involved in the coordination of signaling events and, like CAP and flotillin, proliferate dramatically during adipogenesis. Upon phosphorylation, the c-Cbl-CAP complex is recruited to lipid rafts, where it forms a ternary complex with flotillin.
This process is blocked in adipocytes via expression of a dominant-interfering mutant of CAP (Baumann et al., 2000).

Lipid raft localization of TC10 appears to be involved in its activation by insulin (Watson et al., 2001), and this somehow initiates a second signaling pathway leading to GLUT 4 translocation. Recently, a TC10 interacting protein, CIP42 (Cdc42-interacting protein 4/2), was identified in a yeast two-hybrid screen. Insulin causes the recruitment of CIP4/2 to the plasma membrane, where it interacts with TC10, and over-expression of TC10 in adipocytes prevents this translocation event. Finally, expression of mutant forms of CIP4/2 appeared to inhibit GLUT 4 translocation (Chang et al., 2002). Presently, the signaling events between TC10-CIP4/2 and the GLUT 4 storage vesicle are being delineated, and whether a convergence exists with the PI3K-dependent pathway remains to be determined.

Protein tyrosine Phosphatases

Specific protein tyrosine phosphatases (PTP) regulate insulin signaling in normal physiology and these enzymes are involved in the pathogenesis of tissue insulin resistance in human obesity and type 2 DM. The observation that the relative amount of tris-phosphorylated receptors present after insulin stimulation in intact cells is markedly less than that seen after activation of partially purified receptors in vitro suggests that the pool size of fully activated insulin receptors is tightly regulated in cells (White et al., 1989). Because cellular PTPases can determine the balance of receptor tyrosine phosphorylation, the tris-phosphorylated form of the insulin receptor may be a preferential substrate for PTPases that can deactivate the kinase and determine the steady-state level of receptor activation in vivo.

Additional support for the concept that PTPases are integrally involved in the reversal of insulin receptor activation and dephosphorylation of receptor substrate proteins has been provided by studies using agents such as vanadate and related compounds that act as potent PTPase inhibitors, and enhance insulin signalling, perhaps by augmenting the phosphorylation of insulin receptor or insulin receptor substrates (Fantus et al., 1998). McGuire et al. (1991) showed that insulin infusion in vivo produced a rapid 25% suppression of soluble PTPase activity in muscle of insulin sensitive human subjects. The insulin responsiveness of tissue PTPases activity was severely impaired in insulin resistant subjects, suggesting a possibility of a pathogenic role for abnormal regulation of PTPase in muscle insulin resistance. Worm et al. (1996) showed a significant 1.5 fold increase in PTPase activity in the particulate fraction and an apparent 28% decrease in PTPase activity in soluble muscle fraction, in a 3 h hyperinsulinenmic clamp study. There are several PTPases shown to be active against autophosphorylated insulin receptor such as PTP1B, LAR (leukocyte antigen related), SHP-2 (Src homology containing phosphatase) and MKP-1 (microtubule-associated protein kinase phosphatase-1) (Goldstein et al., 2000). PTP1B and LAR have been implicated in negative regulation of insulin receptor and other PTPases may play a role in other aspects of insulin action.

Leukocyte antigen related (LAR) PTPase

This PTPase has been implicated in the regulation of insulin signaling because of its plasma membrane localization. Kulas et al. (1996) showed that LAR mass could be reduced by 63.5% in hepatoma cells transfected with LAR antisense mRNA. This reduction of LAR abundance resulted in a 3.5 fold amplification of insulin stimulated PI3K activity compared with cells transfected with the null expression vector as well as an augmentation of additional post receptor events, including IRS-1 tyrosine phosphorylation, IRS-1 complexing with the
p85 subunit of PI3K, IRS-1 associated PI3K activity and the activation of both MAP kinase kinase and MAP kinase.

Studies with LAR knockout mice provided data to support a role for this PTPase in the regulation of glucose metabolism and insulin action. The homozygous knockout mice have structurally abnormal mammary gland development and are significantly smaller with less adipose tissue than control mice, but otherwise appear to develop normally. The adult homozygous knockout mice had significantly lower fasting insulin levels, glucose and triglycerides, suggesting a heightened level of insulin sensitivity (Ren et al., 1998).

**Protein Tyrosine Phosphatase 1B**

PTP1B is a widely distributed intracellular enzyme. It is one of the candidate PTPases for the insulin-signalling pathway whose abundance is also regulated in cultured cells by changes in the ambient concentrations of insulin and glucose. Lammers et al. (1993) showed that over expression of PTP1B completely dephosphorylated insulin proreceptors and β subunits in a basal state. In rat KRC-7 hepatoma cells, PTP1B antibody loading significantly increased insulin stimulated DNA synthesis and PI3K activity, as well as insulin stimulated receptor kinase activity towards exogenous peptide substrate, insulin receptor autophosphorylation and IRS-1 tyrosine phosphorylation (Ahmad et al., 1995). Chen et al. (1997) showed that transfection of active PTP1B reduced the insulin stimulated translocation of GLUT 4.

PTP1B knockout mice provided strong evidence for an essential role for this PTPase. It had no obvious disease or tissue phenotype. It showed a 50% decrease in fasting insulin levels and 13% decrease in glucose levels suggesting a heightened level of insulin sensitivity (Elchebly et al., 1999). Incubation of rat fibroblasts in high glucose conditions not only impaired insulin receptor kinase activation and substrate phosphorylation, but was associated with a 2 fold increase PTP1B cytosolic abundance. The increase in cytosolic PTPase activity was blocked by incubation with anti-PTP1B antibodies. Treatment with thiazolidinedione insulin sensitizers however not only prevented the increase in PTP1B protein but ameliorated the phosphorylation defect induced by high glucose (Maegawa et al., 1995).

**Glucose transporters**

Since the cell membrane is impermeable to glucose, it is transported by specific carrier proteins or transporters that span the cell membrane and allow the binding and transfer of glucose across the hydrophobic lipid bilayer. Two general classes of glucose carriers have been described in mammalian cells: 1) the energy-dependent Na⁺/glucose cotransporter, which can concentrate glucose against a gradient, and 2) the facilitative glucose transporters, which work in the direction of the glucose gradient. The physiological function of the Na⁺/glucose cotransporter takes place in polarized epithelial cells where it transports glucose from the lumen of the intestine into the cell (Hediger et al., 1987). The subsequent facilitative transport of glucose out of the intestinal epithelium allows entry of glucose into the blood. The Na⁺/glucose cotransporter is also expressed in the kidney where it serves to retain glucose and prevent its spillage into urine. The entry of glucose into different tissues via facilitative glucose carriers must serve different physiological needs in these different tissues; these needs are met by a family of related transporter proteins, expressed in a tissue-specific manner, whose biochemical properties serve the appropriate physiological needs of the organism. These proteins are called GLUT for glucose transporters. Table 1 gives details about the different GLUTs found in mammalian tissues.
### Table 11: Different facilitative glucose transporters in mammals

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Tissue specificity</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT 1</td>
<td>Blood-brain barrier, red blood cells, all tissue culture cells-ubiquitous</td>
<td>Basal glucose uptake in most cells (excluding neuronal cells)</td>
<td>Mueckler et al., 1985</td>
</tr>
<tr>
<td>GLUT 2</td>
<td>Liver, kidney, pancreas, intestine</td>
<td>Bidirectional glucose flux in the liver; part of glucose-sensing machinery in the pancreas</td>
<td>Asano et al., 1989</td>
</tr>
<tr>
<td>GLUT 3</td>
<td>Many tissues, particularly in neurons in rats</td>
<td>Neuronal glucose uptake, High affinity glucose transporter</td>
<td>Asano et al., 1988</td>
</tr>
<tr>
<td>GLUT 4</td>
<td>Brown and white fat, skeletal and cardiac muscle</td>
<td>Insulin-responsive glucose uptake</td>
<td>Charron et al., 1989</td>
</tr>
<tr>
<td>GLUT 5</td>
<td>Small intestine, sperm</td>
<td>Fructose transport</td>
<td>Kayano et al., 1990</td>
</tr>
<tr>
<td>GLUT 6</td>
<td>Pseudogene</td>
<td>None</td>
<td>Kayano et al., 1990</td>
</tr>
<tr>
<td>GLUT 7</td>
<td>Liver, (other tissues?)</td>
<td>Glucose-6-phosphate transporter of the endoplasmic reticulum</td>
<td>Waddell et al., 1992</td>
</tr>
<tr>
<td>GLUT 8</td>
<td>High expression in Sperm</td>
<td>Likely to affects normal spermatogenesis and sperm function</td>
<td>Kim and Moley, 2007</td>
</tr>
<tr>
<td>GLUT 9a and 9b</td>
<td>High expression in Sperm</td>
<td>Likely to affects normal spermatogenesis and sperm function</td>
<td>Kim and Moley, 2007</td>
</tr>
</tbody>
</table>

### Table 12: Concentration of GLUT 4 in animal models with altered insulin levels and sensitivity (Shepherd et al., 2000)

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Fasting serum insulin</th>
<th>Fasting serum glucose</th>
<th>GLUT Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker obese (fa/fa) rats</td>
<td>+</td>
<td>0</td>
<td>0 ++</td>
</tr>
<tr>
<td>Young</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Old</td>
<td>++</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>Zucker diabetic fatty (ZDF/drt) rats</td>
<td>+</td>
<td>++</td>
<td>--</td>
</tr>
<tr>
<td>Rats with gold thioglucose-induced obesity</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Diabetic (KK/A^a) mice</td>
<td>++</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>Viable yellow (A^Y/a) mice</td>
<td>++</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>Brown-fat-ablated mice</td>
<td>++</td>
<td>+</td>
<td>0 ND</td>
</tr>
<tr>
<td>Obese diabetic (db/db) mice</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Rats with high-fat feeding</td>
<td>+</td>
<td>0</td>
<td>0 --</td>
</tr>
<tr>
<td>Dexamethasone-treated rats</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Rats and mice with STZ-induced diabetes</td>
<td>-</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>Spontaneously hypertensive rats</td>
<td>+</td>
<td>0</td>
<td>0 ND</td>
</tr>
<tr>
<td>Aged rats</td>
<td>+</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>Diabetic rats treated with Metformin</td>
<td>-</td>
<td>-</td>
<td>0 ND</td>
</tr>
<tr>
<td>Rats and mice given leptin</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Rats given thiazolidinediones</td>
<td>-</td>
<td>-</td>
<td>0 +</td>
</tr>
</tbody>
</table>

+: increased; ++: markedly increased; -: decreased; --: markedly decreased; 0: unchanged; ND: not determined

### Table 13: Changes in concentration of GLUT 4 in different conditions in human (Shepherd et al., 2000)
### Review of Literature

#### Table 1: GLUT 4 Expression in Different Conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Muscle</th>
<th>Adipose tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 diabetes</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Pancreatic transplantation in subjects with type 1 diabetes</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Insulin resistance in relatives of subjects with type diabetes</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Obesity</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>0</td>
<td>0 or -</td>
</tr>
<tr>
<td>Aging</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Uremia</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Polycystic ovary syndrome</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Pseudoacromegaly</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Exercise</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Sulfonylurea therapy</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Weight loss</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

+: increased; ++: markedly increased; -: decreased; --: markedly decreased; 0: unchanged; ND: not determined

GLUT 1 and GLUT 4 seem to be the most important glucose transporters with respect to whole-body glucose disposal. GLUT 1 is considered to account for basal glucose uptake (Gulve et al., 1994). It is expressed in most tissues and is relatively insensitive to insulin. GLUT4, on the other hand, is the major insulin-responsive glucose transporter, mainly expressed in muscle and adipose tissue. Table 12 and 13 give details of gene expression and concentrations of GLUT 4 in different animal models and humans with altered insulin levels and sensitivity.

GLUT 4 is the main insulin-responsive glucose transporter and is located primarily in muscle cells and adipocytes. Its Michaelis-Menten constant for glucose is 36 to 179 mg/dL (2 to 10 mM/L), which is within the range of physiologic blood glucose concentrations, so it can be saturated under ambient conditions. The importance of GLUT 4 in glucose homeostasis is best demonstrated by studies of mice in which one allele of the GLUT 4 gene has been disrupted. These mice have approximately a 50% reduction in GLUT 4 concentrations in skeletal muscle, heart, and adipocytes; they have severe insulin resistance (Rossetti et al., 1997) and in at least half the males, frank diabetes develops with age (Stenbit et al., 1997).

In normal muscle cells and adipocytes, GLUT 4 is recycled between the plasma membrane and intracellular storage pools. GLUT 4 differs from other glucose transporters in that about 90 percent of it is sequestered intracellularly in the absence of insulin or other stimuli such as exercise (Gould and Holman, 1993). In the presence of insulin or another stimulus, the equilibrium of this recycling process is altered to favour the translocation (regulated movement) of GLUT 4 from intracellular storage vesicles to the plasma membrane and, in the case of muscle, to the transverse tubules as well. The net effect is a rise in the maximal velocity of glucose transport into the cell (Gould and Holman 1993).

Insulin-stimulated intracellular movement of GLUT 4 is initiated by the binding of insulin to the extracellular portion of the transmembrane insulin receptor. Its binding activates trypsin kinase phosphorylation at the intracellular portion of the receptor. The chief substrates for this tyrosine kinase include insulin-receptor-substrate molecules (IRS-1, IRS-2, IRS-3, and IRS-4), Gab-1 (Grb2 [growth factor receptor-bound protein 2]-associated binder 1), and SHC (Src and collagen-homologous protein) (Holman and Kasuga 1997).

In both adipocytes and skeletal muscle, subsequent activation of phosphoinositide-3 kinase is necessary for the stimulation of glucose transport by insulin (Holman and Kasuga,
1997) and is sufficient to induce at least partial translocation of GLUT 4 to the plasma membrane (Tanti et al., 1996).

Intracellular translocation of GLUT 4 to the plasma membrane is stimulated by the expression of active forms of protein kinase B or atypical isoforms of protein kinase C in cultured cells (Standaert et al., 1997). This suggests that one or both of these kinases may be the in vivo mediator of the process in which insulin signals GLUT 4 translocation. The atypical isoforms of protein kinase C are good candidates: it has been found that blocking their action attenuates insulin-stimulated involvement of GLUT 4, (Standaert et al., 1997) whereas studies in which the activation of protein kinase B is blocked have had conflicting results with regard to GLUT 4 translocation (Wang et al., 1999). Furthermore, in muscle from diabetic subjects, stimulation of glucose transport is impaired at physiologic insulin concentrations, whereas the activation of protein kinase β is normal (Krook et al., 1998).

Several proteins have been identified in GLUT 4 containing vesicles, most of which are also present in other exocytic vesicles such as synaptic vesicles in neurons. One such protein, insulin-responsive aminopeptidase, is of particular interest because it is also localized in GLUT 4 containing vesicles in adipocytes and muscle cells, although its physiologic function is unknown (Rea and James, 1997). A model of the docking of GLUT 4 vesicles and their fusion with the plasma membrane has been developed on the basis mechanisms used by synaptic vesicles. This model proposes that proteins similar to those involved in synaptosome fusion form a specific complex that links the GLUT 4 vesicle with the plasma membrane (Rea and James, 1997). Proteins such as Rab-4, a small guanosine triphosphate-binding protein, may modify the retention or movement of GLUT 4 containing vesicle.

Theoretically, there are at least three ways in which insulin might modulate GLUT4 function. First, insulin could promote translocation to the cell surface of intracellular GLUT 4 (Suzuki and Kono 1980). Secondly, insulin could upregulate GLUT 4 expression by increased synthesis and/or decreased degradation (Yu et al., 2001). Finally, insulin could increase intrinsic transport activity of GLUT 4 proteins in the plasma membrane (Sweeney et al., 1999). It is established that, upon insulin stimulation, GLUT 4 containing vesicles are translocated from intracellular compartments to the plasma membrane (Cushman and Wardzala, 1980).
A general hypothesis called the SNARE (soluble NSF attachment protein receptors; NSF, N-ethylmaleimide-sensitive fusion protein) hypothesis postulates that the specificity of secretory vesicle targeting is generated by complexes that form between membrane proteins on the transport vesicle (v-SNARE’S) and membrane proteins located on the target membrane (t-SNARE’S). Several v- and t-SNARE’S have been identified in adipocytes and muscle. The SNARE’S are a family of membrane-associated proteins that selectively mediate membrane fusion events via protein-protein interactions (Pfeffer 1999).

**Possible causes of insulin resistance involving GLUT**

Although decreased production of GLUT 4 is not the cause of insulin resistance in obesity and diabetes, there may be a therapeutic advantage to increasing the concentrations of GLUT 4 in these conditions. Glucose tolerance and insulin sensitivity are increased by overproduction of GLUT 4 in muscle or adipose tissue, or both, of normal (Leturque *et al.*, 1996) or *db/db* obese, diabetic mice (Gibbs *et al.*, 1995). Furthermore, an increase in GLUT 4 reduces hyperglycemia and increases insulin sensitivity in mice with streptozotocin-induced diabetes (Leturque *et al.*, 1996). Different pathological conditions with altered GLUT levels, which may give rise to insulin resistance, are reviewed briefly here.

**Mutations in glucose transporters**

Mutations in GLUT-1 are associated with intractable seizures resulting from a reduction in glucose transport across the blood-brain barrier (Seidner *et al.*, 1998). Mutations
in GLUT 4 could theoretically cause insulin resistance. However, polymorphisms in the GLUT 4 gene are very rare in subjects with type 2 diabetes and have the same prevalence among non-diabetic subjects (Bjorbaek et al., 1995).

**Tissue-specific alterations in GLUT 4 production**

In various insulin-resistant states, expression of the GLUT 4 gene is regulated differently in muscle and adipose tissue as shown by studies in both animals (Table 12) and humans (Table 13) (Shepherd et al., 2000). GLUT 4 concentrations are reduced in adipocytes from obese subjects and those with impaired glucose tolerance or type 2 diabetes, but GLUT 4 concentrations are not reduced in skeletal muscle in obese subjects, subjects with type 1 or type 2 or gestational diabetes, or insulin-resistant relatives of subjects with type 2 diabetes (Shepherd et al., 2000). Since muscle is the primary site of insulin-stimulated disposal of glucose, the impairment of whole-body insulin sensitivity in these states cannot be explained by a decrease in the production of GLUT 4. In contrast, decreased GLUT 4 production in muscle with aging in normal subjects may play a part in age-related declines in insulin sensitivity (Shepherd et al., 2000).

**Defects in the intracellular translocation of GLUT 4**

The reduction in insulin-stimulated glucose uptake in skeletal muscle in obese subjects and those with diabetes is associated with impairment in insulin-stimulated movement of GLUT 4 from intracellular vesicles to the plasma membrane (Zierath et al., 1996). Since GLUT 4 concentrations are normal in skeletal muscle in these subjects, the most likely explanation for the insulin resistance is a defect in the insulin-signaling pathways that regulate the translocation of GLUT 4 or in the molecular machinery directly involved in the recruitment of GLUT 4 containing vesicles to the plasma membrane, their docking, and their function with the membrane (Rea and James, 1997). Glucose transport in insulin-resistant muscle is activated normally by inhibitors of both serine-threonine phosphatases (e.g., okadaic acid) and tyrosine phosphatases (e.g., vanadate) (Carey et al., 1995). Both classes of phosphatase inhibitors are thought to prolong the activation of distal components of the insulin-signaling cascade.

**Defects in signalling pathways**

Activation by insulin of phosphoinositide-3 kinase in muscle is reduced in severely obese subjects with insulin resistance and those with diabetes, (Bjomho'lm et al., 1997) and expression of the regulatory subunit of phosphoinositide-3 kinase is reduced in those who are morbidly obese. Impairment of insulin-stimulated glucose uptake may also result from the up-regulation of proteins that inhibit the signaling pathways. The expression and activity of several protein tyrosine phosphatases are increased in skeletal muscle and fat in obese subjects but not in those with type 2 diabetes (Ahmad et al., 1997).

Another candidate may be the 15-kd substrate of protein kinase C, described as “phosphoprotein enriched in diabetes,” which is over-expressed in insulin target tissues in both obese subjects and those with diabetes (Condonelli et al., 1997). Over-expression of this protein in cultured cells attenuates insulin-stimulated GLUT 4 translocation and thus attenuates insulin-stimulated glucose transport. Over-expression of Rad, a small guanosine triphosphate-binding protein, also inhibits GLUT 4 translocation in cultured cells (Moyers et al., 1996).
Adipose tissue and adipokines

Adipose tissue is currently considered as a hormonally active system in the control of metabolism and not only as a store of excess energy. The term 'adipocytokines' has been coined to refer to a series of adipocyte-derived biologically active molecules which may influence the function as well as the structural integrity of other tissues. Some examples of these substances are leptin, adiponectin, acylation-stimulating protein (ASP), tumor necrosis factor-alpha (TNF-α), plasminogen activator inhibitor-1 (PAI-1) and interleukin-6. It is also likely that some of these adipocytokines mediate the systemic effects of obesity on health. Leptin is considered to be a fundamental signal of satiety to the brain and has a variety of actions, ranging from interference with sympathetic activity to hematopoiesis and reproductive function (Mantzoros, 1999). ASP increases triglyceride synthesis by increasing adipocyte glucose uptake, activating diacylglycerol acytransferase, and inhibiting hormone-sensitive lipase (Murray et al., 1999).

Leptin

Leptin is the product of the ob gene and is secreted mainly from adipocytes (Tartaglia et al. 1995). It regulates energy balance by reducing food intake and increasing energy expenditure after binding to specific receptors in the hypothalamus. The production as well as the circulating levels of leptin is elevated in obese compared to lean subjects and leptin levels are decreased upon weight reduction (Ahren et al., 1997). Inherited defects in the leptin molecule or in the leptin receptor, respectively, have been detected in a few families with extreme obesity (Clement et al., 1998). Subjects with aberrant leptin have been successfully treated with leptin substitution, but in common obesity, leptin treatment has not been effective. Moreover, leptin resistance has been suggested as a mechanism behind obesity (El-Haschimi and Lehnert, 2003).

TNF-α and IL-6

Adipose tissue synthesize cytokines, for example TNF-α and interleukin-6 (IL-6) that have effects on metabolism in the adipose tissue and probably also in other organs. Cytokine release from adipose tissue appears to be stimulated by inflammatory stimuli and also by catecholamines and β-adrenergic stimulation. Insulin and cortisol have been suggested to regulate cytokine release, but data are conflicting (Fasshauer and Paschke 2003). It has been suggested that serum concentrations of TNF-α and IL-6 are elevated in obesity and that weight loss results in decreased levels (Dandona et al. 1998). High serum levels of TNF-α and IL-6 also seem to be associated with insulin resistance and type 2 diabetes (Pickup et al., 2000). Knock-out experiments have shown that insulin resistance is prevented in obese mice lacking TNF-α (Uysal et al., 1997).

Mice lacking IL-6, however, develop obesity which is partly reversed by IL-6 replacement (Wallenius et al., 2002). Furthermore, it has been shown that intracerebroventricular administration of IL-6 causes increased energy expenditure in rats (Wallenius et al., 2002a) and that IL-6 level in the cerebrospinal fluid is negatively correlated with fat mass in obese humans (Stenlof et al., 2003). In humans the effects of TNF-α appears to be mainly autocrine and paracrine whereas IL-6 is released systemically and acts for example on the hypothalamus and the liver (Coppack, 2001).
Both TNF-α and IL-6 inhibit lipoprotein lipase and TNF-α also stimulates hormone sensitive lipase leading to decreased lipid accumulation within the adipose tissue. In addition, it has been suggested that TNF-α interferes with intracellular insulin signaling and induces a down-regulation of glucose transport proteins (GLUT 4) (Coppack, 2001). TNF-α and IL-6 are also produced locally in the adrenal cortex where they modify adrenal steroid secretion and, in fact, they promote the secretion of cortisol. The production of TNF-α and IL-6 in the adrenals is regulated by the same factors that regulate other adrenal hormones, e.g. ACTH and angiotensin II. Cytokines are also believed to have influences on the HPA axis activity via direct actions on the pituitary and the hypothalamus (Judd et al., 2000).

Adiponectin

Adiponectin is a recently characterized, adipocyte-derived plasma protein (Scherer et al., 1995) with insulin sensitizing, anti-atherogenic and anti-inflammatory properties. Plasma levels of adiponectin are negatively associated with obesity and insulin resistance (Weyer et al., 2001) and low levels of adiponectin can predict the future risk of developing type 2 diabetes (Spranger et al., 2003). Adiponectin can interact directly with endothelial cells to improve vascular function. Administration of adiponectin to obese or diabetic mice reduces plasma non-esterified fatty acids (NEFA) levels and also glucose excursions and enhances insulin sensitivity (Berg et al., 2001). Adiponectin is secreted from both subcutaneous and visceral adipose tissue but, surprisingly, secretion appears to be generally higher from visceral adipose tissue (Motoshima et al., 2002). Secretion of adiponectin is positively regulated by insulin and IGF-1 and negatively regulated by glucocorticoids, β-adrenergic stimulation, TNF-α and IL-6. PPAR-γ agonists appear to increase plasma levels of adiponectin (Halleux et al., 2001).

Resistin

Resistin is a novel signaling molecule induced during adipogenesis. It was originally named for its resistance to insulin. Resistin circulates in the blood (Steppan et al., 2001) and it is a peptide hormone that belongs to a family of tissue-specific resistin-like molecules (Steppan et al., 2001a). Adipose tissue increases expression of multiple genes, including resistin, at the onset of high-fat-diet-induced obesity in rats (Li et al., 2002). Rosiglitazone treatment has been shown to decrease resistin mRNA (Haugen et al., 2001; Hartman et al., 2002) and serum levels (Steppan et al., 2001). Li and Lazar (2002) created a transcription factor that activated transcription of PPARγ-responsive genes in the absence of ligand by fusion of the potent viral transcriptional activator VP16 to PPARγ2 (VP16-PPARγ). Resistin gene expression was reduced in VP16-PPARγ adipocytes treated with thiazolidinediones. Insulin has been suggested as a major inhibitor of resistin production (Haugen et al., 2001), which may explain the low resistin mRNA levels in insulin resistance. Also tumor necrosis factor alpha, elevated in obesity, inhibits resistin gene expression (Fasshauer et al., 2001). In addition, the transgenic mice developing high-fat diet-induced obesity exhibited downregulated adipocyte resistin mRNA levels in isolated fat cells (Le Lay et al., 2001).

Beta 3-adrenergic agonists, shown to have anti-diabetic and anti-obesity properties, have been reported to produce an increase in resistin gene expression in diet-induced obesity in animals (Martinez et al., 2001). Human studies do not provide evidence that resistin is a key player in the development of insulin resistance. Resistin expression in human fat and muscle cells in relation to insulin resistance was studied by Nagaev & Smith (2001). The results suggested that resistin was not detectable at all in human muscle and fat cells.
Furthermore, no differences were found between normal, insulin-resistant or type 2 diabetic samples. Similar results were found by Savage et al. (2001). They did not detect resistin mRNA in adipocytes from a severely insulin-resistant subject.

Although the first report proposed resistin serum levels to be increased in the obese state, a number of later publications have demonstrated decreased resistin gene expression in obesity (Ukkola, 2002).

**Incretins**

After meal ingestion, nutrient entry into the stomach and transit through the proximal gastrointestinal (GI) tract stimulates activation of neural and hormonal signals that control gastric emptying and gut motility, nutrient absorption, and hormonal regulation of energy disposal and storage. The mucosal epithelium of the GI tract is one of the earliest integrators of information relevant to digestion and assimilation of nutrient loads. Highly specialized enteroendocrine cells dispersed along the length of the GI tract play an important role in controlling the rate of gastric emptying and small bowel motility, pancreatic enzyme secretion, and the growth and differentiated absorptive function of the small and large bowel epithelium.

Up to two-thirds of the insulin normally secreted in relation to meal intake is thought to be due to insulinotropic actions of the so-called incretin hormones. The incretin effect is defined as the increased stimulation of insulin secretion elicited by oral as compared with intravenous administration of glucose under similar plasma glucose levels. Indeed, patients with type 2 diabetes have been demonstrated to exhibit an almost total loss of incretin effect (Nauck et al., 1986). Two most important incretin hormones are glucose-dependent insulinotropic polypeptide (GIP), formerly known as gastric inhibitory polypeptide, and glucagon-like peptide (GLP)-1 (Fehmann et al., 1995). Both are potent insulinotropic hormones released by oral glucose as well as ingestion of mixed meals.

These peptides stimulate glucose-induced insulin secretion with the cyclic AMP accumulation in the h cells (Lu et al., 1993). Several reports conclude that GIP and GLP-1 may play a physiological role in maintaining glucose tolerance (Kieffer and Habener, 1999). Antagonists of GIP and GLP-1 inhibit glucose-induced insulin secretion in rats (Tseng et al., 1999).

**Glucagon-Like Peptide 1**

Glucagon-like peptide 1 (GLP-1) is released from the intestinal L cells during a meal and that, as an incretin factor, stimulates insulin secretion (Orskov, 1992). The peptide reduces circulating levels of glucose both in normal subjects and in subjects with type II diabetes (Nauck et al., 1998).

Mice with a null mutation in the GLP-1 receptor gene do not develop severe diabetes but exhibit defective glucose-stimulated insulin secretion and glucose intolerance (Flamez et al., 1998). The unexpectedly modest phenotypes of both GIPReceptor-/- and GLPReceptor-/- mice have prompted suggestions that one or more compensatory mechanisms have evolved to supplant the role normally subserved by individual incretin receptors in control of glucose homeostasis. Evidence supporting the upregulation of compensatory mechanisms derives from findings that GLP-1R-/- mice exhibit significantly enhanced β-cell sensitivity to the actions of GIP (Flamez et al., 1999), whereas GIPR-/- mice exhibit an enhanced insulin secretory response to GLP-1 (Pamir et al., 2003).
The administration of GLP-1 to type 2 diabetic subjects effectively lowers blood glucose levels when given either by intravenous, subcutaneous, or oral buccal routes (Gutniak et al., 1992; Nauck et al., 1993; Rachman et al., 1997; Byrne et al., 1998). GLP-I infusions are also effective in reducing blood glucose in insulin deprived states, including type 1 diabetics (Gutniak et al., 1992). These actions are perhaps attributable to increased glucose disposition in peripheral tissues, reduced gastric emptying, and reduced hepatic glucose output, possibly secondary to a reduction in glucagon concentrations. Most noteworthy is that improved glycemic control is achieved in diabetic subjects with the subcutaneous administration of GLP-1 for 1 or 3 weeks (Juntti-Berggren et al., 1996).

Enzyme DPP IV cleaves the histidine-alanine dipeptide from the amino terminus of GLP-I, thereby eliminating its biological activities (Kieffer et al., 1995). A prolongation of the effectiveness of GLP-1 can also be achieved by the coadministration of inhibitors of DPP IV (Pederson et al., 1998). GLP-1 receptor agonist exendin-4 is more resistant to degradation in vivo. Thus, exendin-4 has a longer duration of action than GLP-1, is far more potent, and effectively lowers plasma glucose concentrations in obese diabetic ob/ob and db/db mice, fatty Zucker rats, and diabetic rhesus monkeys (Young et al., 1999).

**Suppressors of Cytokine Signaling (SOCS) and Insulin resistance**

SOCS family is composed of SOCS-1 to 7. These are thought to participate in negative feedback loops in cytokine signaling by multiple mechanisms (Starr et al., 1997).

The mechanisms of cytokine-induced insulin resistance are not clearly defined. One possible mechanism is the serine phosphorylation of insulin receptor substrate I by cytokine-activated kinases and the subsequent direct inhibitory effect on the insulin-signaling cascade (Aguirre et al., 2002). An alternative mechanism is that cytokines induce the expression of cellular proteins, such as members of the SOCS family that inhibit insulin receptor signal transduction (Mooney et al., 2001). The SOCS proteins are induced by various cytokines, and participate in a classic feedback loop to modulate cytokine action. It has been demonstrated that SOCS proteins can also play a role in the negative regulation of the signaling of insulin and IGF-I receptors (Dey et al., 2000). Senn et al., (2003) reported that SOCS-3 is a potential mediator of IL-6 dependant insulin resistance in hepatocytes. SOCS-1 and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms (Ueki et al., 2004).

**3.2.2.3 Animal models of type 2 diabetes**

**Chemically Induced Diabetes**

**Neonatal Streptozotocin induced diabetes mellitus**

By intravenous or intraperitoneal administration of STZ (100 mg/kg) on the day of birth of Wistar rats, a-type 2 model of diabetes can be developed (Okamato, 1981). Injecting on 2nd day (Portha et al., 1979) or 5th day (Portha et al., 1979a) of the birth can also be used to develop type 2 diabetes in rats.

Rats treated with STZ on the day of birth exhibit insulin deficient acute diabetes mellitus 3-5 days after birth. They showed high plasma glucose and about 93% decrease in plasma and high glucagon content. The hyperglycaemia observed in the neonates following STZ is only transient. It is reported that neonatal rats are resistant to STZ (Bonnier-Weir et
It was found that only by 8 weeks of age and thereafter rats showed mild hyperglycaemia (Portha et al., 1979a). When Sprague-Dawley pups injected intraperitoneally on the 2nd day after birth with 90 mg/kg of STZ, showed basal hyperglycaemia and abnormal glucose tolerance by 6 weeks of age (Portha et al., 1979a). Both these models are found almost similar with respect to growth, basal plasma glucose, insulin levels, lack of insulin release in response to glucose in vivo, glucose intolerance and depletion of pancreatic insulin stores (Weir et al., 1981).

The 0 day neonatal STZ-diabetic rats are characterized by a low insulin release in vivo in response to glucose or amino acids (Okamato et al., 1981). In insulin secretion studies of the 16 week old 0 day neonatal STZ-diabetic rats, there was a complete loss of β-cell activity to glucose (Portha et al., 1979a). The impairment of glucose-induced insulin effect in this model is clearly related to a defect in oxidative glycolysis. This leads to decrease in the mitochondrial oxidative catabolism of glucose derived pyruvate. It coincides with lower ATP/ADP ratio in stimulated islets and their subsequent alteration of the events rightly coupled to the fuel function of the hexose in the islet cells (Weir et al., 1981). It has been found that neonatal STZ rats exhibited an increased amylin-insulin level. This has been identified as a major component of amyloid deposits in the pancreatic islets of patients with type 2 diabetes mellitus (Polonsky et al., 1988).

It has been reported that after 0 day STZ challenge in neonatal rats, signs of regeneration are apparent from day 4 onwards. But in 4 month old animals the regeneration process is incomplete (Portha et al., 1979a; Bonnier-Weir et al., 1981).

**Streptozotocin and Nicotinamide induced diabetes model**

Pellegrino et al., (1998) reported about the use of nicotinamide with streptozotocin to induce diabetes, which stimulates type 2 diabetes in certain aspects. Administration of with suitable dosages of nicotinamide to adult rats leads to the development of an interesting novel diabetic syndrome, characterized by moderate and stable hyperglycemia and reduced pancreatic insulin stores (approximately 40% of normal). This experimental syndrome appears closer to human NIDDM than other commonly used animal models, at least with regard to insulin responsiveness to glucose and sulfonylureas. Therefore, these diabetic animals could be useful in testing new pharmacological agents with potential insulinotropic action.

It is worthwhile to note that the protective effect of nicotinamide against STZ is thought to be dependent on the preservation of the intracellular NAD pool accomplished by this compound (Yamamoto et al., 1981). Indeed, on one side nicotinamide is precursor of NAD, and on the other side it is an inhibitor of poly (ADP-ribose) synthetase, an NAD-consuming enzyme activated by STZ-induced DNA injury (Yamamoto et al., 1981). The effectiveness of the protection depends on the effective dosage of STZ and nicotinamide. Rats administered STZ plus nicotinamide showed a well-preserved in vitro sensitivity to sulfonylureas, particularly those treated with 230 mg/kg nicotinamide, whose insulin response to 0.19 mmol/l tolbutamide in the presence of 5 mmol/l glucose was superimposable on that of controls. Such sensitivity to sulfonylureas, which is typical of NIDDM, was previously observed only for Goto-Kakizaki (GK) rats after static incubation of isolated islets with gliclazide (Giroix et al., 1993), whereas in neonatal-STZ rats it was reported in the absence of glucose only (Giroix et al., 1983).

A striking feature in rats administered STZ plus nicotinamide is the marked hypersensitivity of beta-cells to arginine; indeed, at glucose concentrations as low as 2.8 mmol/l, insulin release in response to 7 mmol/l arginine, a small amount in normal rats, has
drastically increased in diabetic animals, as has also been reported for other animal models of NIDDM (Giroix et al., 1983; Portha et al., 1991). IVGTT performed in animals administered STZ plus nicotinamide revealed marked glucose intolerance, as expected. With regard to post-load plasma insulin concentrations, an early small peak higher at 2 than at 5 min) occurred, with a tendency to return to basal values more slowly than control rats did from their sharp insulin peak. This behavior is most likely indicative of an altered but still operative ability of residual beta-cells to respond to glucose. These cells, however, maintain a much greater sensitivity to sulfonylurea stimulation, as can be argued from the effects of glucose plus tolbutamide intravenous loading, which strongly supports our in vitro data and implies that the defective insulin-secreting capabilities in these animals can be efficiently corrected by an appropriate stimulus. From this point of view, the analogy with human NIDDM is straightforward (Pellegrino et al., 1998).

It is also noteworthy that after tolbutamide stimulation, the rate of glucose disappearance from the blood in the diabetic rats paralleled that of controls and resulted in normalization of glycemia. Nevertheless, the magnitude of tolbutamine-stimulated insulin secretion was much larger in control than in diabetic animals and led to hypoglycemia. By comparison, in both neonatal-STZ (Blondel et al., 1989) and GK (Portha et al., 1991) rats, plasma insulin release in response to intravenous glucose was lacking.

Animal Models With Insulin Resistance

Fructose fed rats

The effects of fructose feeding are exerted on the liver. Golden Syrian hamsters fed diets containing 60% fructose or sucrose develops obesity and glucose intolerance (Kasim-Karakas et al., 1996). Fructose feeding also increases fasting plasma non-esterified fatty acids (NEFA), plasma and liver triglycerides (Kasim-karakas et al., 1996). Because it takes only two weeks to feed the animals with a high-fructose diet to induce insulin resistance, fructose feeding is a convenient way to produce insulin resistance in vivo. The insulin resistance induced by fructose feeding is reported due to the diminished ability of insulin to suppress hepatic glucose output but not a decreased insulin stimulated glucose uptake by the muscle, suggesting the phenotype is characterized primarily by hepatic insulin resistance. Thus, this model has limited value for testing drug candidates that act primarily on adipose tissue or skeletal muscle. Rats fed >60% fructose for the same period also develop insulin resistance with hyperinsulinemia, hypertriglyceridemia, and interestingly, hypertension, but the fructose-induced hypertension is not associated with the hyperinsulinemia and hypertriglyceridemia (Hwang et al., 1987). Both the hamster and the rat models have been utilized to test anti-diabetic small molecules improving hepatic insulin sensitivity (Lee et al., 1994).

High fat fed (HFF) insulin resistance model

Rats fed a high fat diet (60% of calories as fat) develop insulin resistance with reduced basal glucose metabolism (Kraegen et al., 1986). The insulin resistance is exemplified by >50% reduction in net whole-body glucose utilization at physiological insulin levels and the failure to suppress liver glucose output (Storlien et al., 1986). The major suppressive effects on glucose transport are in oxidative skeletal muscle and brown adipose tissue (BAT), suggesting these tissues contribute mainly to the overall insulin resistance (Storlien et al., 1986). The HFF model is suitable for studies of mild insulin resistance.
because it is closer to normal animals than diabetic animals. If caloric intake is carefully
controlled to avoid obesity, this model does not exhibit hyperglycemia even after several
weeks on the diet (Kraegen et al., 1986). Insulin resistance develops within a few weeks,
with associated hyperinsulinemia and impaired glucose tolerance, but the development of
frank hyperglycemia takes much more time.

High-fat-diet mice also develop insulin resistance with glucose intolerance (Ahren
and Scheurink, 1998). The susceptibility to develop obesity and diabetes varies among
different rodent strains (Surwit et al., 1988). High-fat diets have been widely used to study
processes involved in the development of insulin resistance, in screening candidate anti-
obesity and anti-diabetic drugs, and as an environmental stressor to probe the roles of
specific genes in knockout and transgenic mice. For example, the HFF rat model is widely
used to test the anti-diabetic thiazolidinediones (Khouriheid et al., 1995). The mechanism of
dietary fat-induced insulin resistance is not clear. One hypothesis is that the fat induced
insulin resistance is acquired by increasing the flux of the hexosamine biosynthetic pathway,
but this was demonstrated at only maximally effective insulin levels (Hawkins et al., 1997).
At physiological insulin levels, the flux through the hexosamine biosynthetic pathway is not
affected by high-fat-diet or increased plasma free fatty acids (Choi et al., 2001). Recent
findings suggest that high fat feeding impairs glucose uptake in the peripheral tissues
(Hansen et al., 1998).

**TNF-α induced insulin resistance model**

Increased TNF-α mRNA and protein levels have been observed in rodent models of
obesity and diabetes. Evidence from the molecular level indicates that TNF-α inhibits the
signaling events mediated by the insulin receptor (Hotamisligil et al., 1993). In vivo studies
demonstrated that TNF-α caused insulin resistance (Lang et al., 1992) and neutralization of
TNF-α in obese fa/fa rats significantly improved insulin sensitivity in peripheral tissues
(Hotamisligil et al., 1993). On the basis of these findings, TNF-α infusion has been used to
create insulin resistance in rats.

In this model, rats are infused with a high level of TNF-α for 4-5 days. The infused
animas have higher basal plasma insulin and free fatty acids and develop peripheral insulin
resistance. This model has been used to test small molecule compounds with insulin
sensitizing activities (Miles et al., 1997). Evidence suggests that the TNF-α effect is
mediated by serine phosphorylation of IRS-1, which inhibits its tyrosine phosphorylation and
activation and insulin signaling. In addition, TNF-α also inhibits the phosphorylation of Akt,
a downstream kinase in the insulin signaling pathway (Sykiotis and Papavassiliou, 2001).

**Glucosamine-induced insulin resistance model**

Hyperglycemia is known to induce insulin resistance in vivo. Numerous in vitro
studies have demonstrated that high concentrations of glucose impair insulin-stimulated
glucose transport in rat adipocytes (Garvey et al., 1987). Additional studies suggested that an
increased flux through the hexosamine biosynthetic pathway might be the mechanism by
which hyperglycemia causes insulin resistance. Excess hexosamine flux has been shown to
cause insulin resistance in cultured cells (McClain and Crook, 1996). Over expression of
glutamine: fructose-6-phosphate amidotransferase (GFAT), the first and rate-limiting enzyme
in the hexosamine biosynthetic pathway, led to insulin resistance (Hresko et al., 1998). The
increased flux through the hexosamine pathway produces increased UDP-N-
acetylglucosamine, which serves as a substrate in the formation of glycoproteins and proteoglycans. On the basis of these findings, it was hypothesized that increasing the flux through the hexosamine biosynthetic pathway can generate an insulin resistant animal model (Rossetti et al., 1995). Because glucosamine enters the pathway downstream of the Rate-limiting step and mediates insulin desensitization, it was used to test the hypothesis in vivo and confirmed that it can induce insulin resistance in skeletal muscle of normoglycemic rats (Rossetti et al., 1995).

Exposure of rats to hyperglycemia or glucosamine in vivo, results in accumulation of hexosamine pathway end products in insulin sensitive tissues, with a time course that precedes the onset of insulin resistance (Rossetti et al., 1995; Hawkins et al., 1997a). This model can be used to test small molecule insulin sensitizers (Miles et al., 1998).

**Dexamethasone-induced insulin resistance model**

Glucocorticoid excess results in insulin resistance by blunting insulin's action to suppress hepatic glucose production and stimulate peripheral glucose utilization (Pagano et al., 1983). Glucocorticoids also have a direct inhibitory effect on glucose-induced insulin release in the β-cells (Delaunay et al., 1997). Glucocorticoids could potentially inhibit glucose uptake at one or more steps along the signalling pathway through which insulin stimulates glucose transport. The uptake of glucose in insulin sensitive cells like muscle and fat cells is to a great extent an insulin-regulated process, mediated primarily by the facilitative glucose transporter isoform GLUT4 (Czech and Corvera, 1999; Pessin et al., 1999).

Several situations of insulin resistance in man as well as in animal models have some degree of hypercortisolism, e.g. Cushing's syndrome and abdominal obesity. Pharmacological treatment with high doses of glucocorticoids leads to an impairment of insulin sensitivity. A study in 3T3-F442A adipocytes reported a dexamethasone-induced decrease in IRS-1 but increase in PI3-K protein levels, yielding a mild non-significant decrease in IRS-1 associated PI3-K activity (Saad et al., 1994).

Dexamethasone treatment impair glucose transport in fat cells (3T3-L1 adipocyte cell line) both at physiological and at abnormally high glucose concentrations, and the effects were also mainly independent of the concomitant insulin level (Buren et al., 2002). Single-dose dexamethasone induced whole-body insulin resistance and altered both cardiac fatty acid and carbohydrate metabolism in wistar rats (Qi et al., 2004).

**Genetic models**

**Ob/ob and db/db mice**

The obese (ob) gene is an autosomal recessive mutation that occurred in a non-inbred stock in the early 1950s and was later established and maintained in the C57BL/6J (BL/6) Strain (Coleman, 1978). Mice homozygous for the ob mutation on chromosome 6, known as ob/ob, develop mild diabetes with marked obesity, hyperphagia, and transient hyperglycemia (Coleman, 1978). The wild-type ob gene, also known as leptin, is a 167 amino acid protein highly expressed in adipose tissue. The expression of the ob gene is markedly reduced in ob/ob mice, which inhibits the leptin-mediated signaling pathway that stimulates energy expenditure (Campfield et al., 1998).

The diabetes (db) autosomal recessive mutation occurred in the C57BL/KsJ inbred strain (Coleman, 1978). The mutation is in the leptin receptor on chromosome which is
required for the leptin-mediated metabolic pathway for energy expenditure (Chua et al., 1996). Given the importance of the leptin-mediated pathway for energy consumption, both the ob and the db/db mutations result into reduced energy expenditure and lead to diabetes & obesity. When maintained on the same genetic background, both mutations exhibit identical syndromes from 3 weeks of age onward. However, the C57BL/KsJ background appears to enhance the severity of diabetes, possibly due to genetic interactions with the leptin pathway. The commonly used ob/ob strain is on the C57BL/6J background, whereas the db/db mouse is on the C57BL/KsJ background. Therefore, the db/db mouse, although displaying a degree of obesity at younger ages similar to that of the ob/ob, exhibits a more severe diabetic phenotype with marked hyperglycemia and hyperphagia (Coleman, 1978).

Both animal models are used for studies of diabetes and obesity but, depending upon the need for the severity of diabetes, a choice of the two can be made. Homozygous mutants of both sexes for either ob/ob or db/db are infertile, and obese homozygous mutants are obtained by mating known heterozygotes (Coleman, 1978).

**Obese Zucker Fatty (Fa/fa) rat**

Similar to the db/db mouse, the obese Zucker fatty rat harbours the fa autosomal recessive mutation in the leptin receptor (Ogawa et al., 1995) and is a rat obesity model. The Zucker fatty rat, homozygous for the fa mutation and known as fa/fa, develops massive obesity after weaning associated with hyperphagia, hyperinsulinemia, and hypertriglyceridemia. Additional metabolic abnormalities in the Zucker fatty rat include increased fatty acid synthesis in liver and adipose tissue and high fat-storage capacity. Unlike the db/db mouse, the obese Zucker fatty rat is not diabetic but has IGT, mild hyperglycemia, pronounced hyperinsulinemia, and marked reduction in insulin sensitivity (Terrettaz, and Jeannreaud, 1983). It is therefore widely used as a model for tests of glucose tolerance.

**Zucker Diabetic Fatty (ZDF) rat**

The zucker diabetic fatty (ZDF) rat harbours the same mutation in the leptin receptor as the Zucker fatty rat but, in addition, it has a defect in the pancreatic β-cells that affects insulin production, which later progresses to a state of insulin deficiency (Griffen et al., 2001). The ZDF rat develops overt diabetes with severe hyperglycemia, polyuria, and polydipsia, similar to human NIDDM (Upton et al., 1998). Therefore, the ZDF rat is a good type 2 diabetic model, and it has been used extensively for testing small molecule anti-diabetic compounds.

**KK and KKAγ mouse**

The inbred mouse strain KK was established in the 1960s in Japan. The KK mice have inherent glucose intolerance and insulin resistance mainly in the peripheral tissues. They become modestly obese with aging and further develop overt diabetes with frank hyperglycemia (Ross et al., 2004). The symptoms of glucose intolerance and insulin resistance are exacerbated in KKAγ mice, a congenic strain harboring the Aγ allele at the agouti locus (Suto et al., 1999). The Aγ allele facilitates the expression of the agouti peptide, which acts as an antagonist of the melanocortin-4 receptor (MC4-R), leading to maturity onset obesity (Fan et al., 1997).
Plasma triglyceride, total cholesterol, and free fatty acids are elevated in KK mice (Suto et al., 1999). The KK mouse has been used as a model for studies of progressive obesity and complications associated with diabetes.

**Obese Rhesus monkey**

Rhesus monkeys develop obesity and insulin resistance by aging when allowed free access to chow. As is the case with humans, a subset of monkeys progresses to frank diabetes with advancing age. The insulin resistance is partly due to defective glucose cycle caused by dysfunctional insulin activation of protein kinase C in skeletal muscle (Standaert et al., 2002). The spontaneous development of obesity and insulin resistance in Rhesus monkeys make them an attractive model for examining the sequence of metabolic changes associated with the development and onset of diabetes. In addition, these animals have increased plasma triglyceride, increased very low-density lipoprotein (VLDL), decreased high density lipoprotein (HDL), hypertension, and hyperinsulinemia, but they are normoglycemic in the prediabetic state (Hannah et al., 1991). These symptoms resemble the human metabolic syndrome X that eventually progresses in some individuals to overt type 2 diabetes.

Rhesus monkey is a good model to study the natural history of the development of type 2 diabetes from which a better understanding of the human disease progression can be gained. The rhesus monkey is a good primate model for syndrome X and suitable for studies of insulin resistance and dyslipidemia, especially for evaluation of leading small molecule drug candidates refined through screening of compounds in rodent models (Oliver et al., 2001).

### 3.3 COMPLICATIONS ASSOCIATED WITH DIABETES MELLITUS

The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction and failure of various organs, especially the eyes, kidneys and nerves but also the arteries. The classical long term complications are thus retinopathy, nephropathy, neuropathy, all of which are considered to be microvascular complications and macroangiopathy. These complications affect quality of life and/or life expectancy. Retinopathy may lead to severe retinal bleeding and has previously been the most common cause of blindness among young adults. Diabetic nephropathy (DN) can progress to renal failure and need for renal replacement therapy. A symmetric peripheral loss of sensibility and motor nerve function in the lower extremities are commonly early signs of neuropathy and increase the risk of developing foot ulcers. The combination of lower extremity arterial disease and neuropathy may contribute to an increased risk for gangrene and amputation. Autonomic neuropathy may lead to alterations in gastrointestinal, cardiovascular and urogenital function. Hyperglycemia is a common risk factor for all these complications but there are also other risk factors, some of which seem to have organ specific effects.

Before Banting and Best discovered insulin in 1921 the only therapy for diabetes was diet and more than 80% of patient died within the first ten years of type 1 diabetes. The most common cause of death was ketoacidosis. The first injection of insulin for treatment of juvenile diabetes was given in February 1922 and insulin therapy for general use was introduced a few years later. After the introduction of insulin treatment life expectancy increased and instead the problem with chronic complications evolved, cardiovascular disease and renal failure becoming the major causes of death among patients with diabetes (Paz-Guevara et al., 1975). Today, patients with diabetes still have an excess morbidity and mortality when compared with the general population, the major causes still being
cardiovascular disease and renal failure (Rossing et al., 1996; Morrish et al., 2001; Orchard et al., 2001). In a Danish cohort of patients diagnosed with type 1 diabetes between 1933-1952 and followed up until 1982, the increased mortality in patients with type 1 diabetes was predominantly accounted for by a poor prognosis for patients with DN. On the contrary, among patients who did not develop DN, mortality was only slightly elevated at all ages (Borch-Johnsen et al., 1985).

3.3.1 Diabetic Cardiomyopathy

Diabetes mellitus (DM) is a major public health problem in the current era. The prevalence of the disease in the general adult population was approximately 2.8% in 2000, which is anticipated to rise to 4.4% in 2030 (Wild et al., 2004). Up-to-date studies report that 195 million people currently suffer from DM and this number is expected to increase to 330 to 500 million in the year 2030 (Ryden et al., 2007). Cardiovascular complications are the primary causes of morbidity and mortality in patients with DM. The mortality of diabetic patients with cardiovascular disease is twofold to fourfold higher than that of non-diabetic patients. Poor prognosis of the diabetic patients is mainly attributed to the development of chronic heart failure (CHF) (Benjamin et al., 2003). The prevalence of DM amongst patients with heart failure is rising to high levels, with an estimated rate of 12% vs. only 3% for non-diabetic subjects (Thrainsdottir et al., 2005). Moreover, the Framingham Heart Study showed a twofold increased incidence of heart failure in diabetic men and a fivefold increase in diabetic women (Kannel et al., 1974). The development of CHF in diabetic patients has been traditionally attributed to (a) the concurrent hypertensive heart disease and other metabolic disturbances or (b) the progression of atherogenesis and the sequential presentation of coronary artery disease. The former mechanism arises from insulin resistance which is the primary pathophysiologic disorder and causes hyperinsulinemia, hyperglycemia, dyslipidemia and hypertension. In the metabolic syndrome, diabetes, together with other metabolic disturbances, provokes endothelial dysfunction, vascular remodeling, disorders in the balance of coagulation fibrinolysis and increases inflammation process. These disturbances can gradually lead to chronic complications such as nephropathy, neuropathy, retinopathy and diabetic heart disease. On the other hand, it is well established that DM is defined as a strong risk factor of atherosclerosis (Ryden et al., 2007). Not only does it stimulate the atherogenic progression but it also causes the destabilization of the atheromatic plaque leading to severe ischemic events and the progressive establishment of ischemic cardiomyopathy. The first description of the diabetic myocardial disease (DMD) appears in literature in 1972. It was used to describe the development of heart failure in diabetic subjects independently of the presence of additional causative factors (Rubler et al., 1972). The term was initially met with skepticism and until recently it used to be a medical term, which was defined as something between fiction and fact (Francis, 2001). Yet, nowadays, following certain scientific evidence it is more and more being considered as a self-existent nosologic entity, with the potential to lead to a progressive development of heart failure, without depending on the co-existence of arterial hypertension, other cardiovascular risk factors or coronary artery disease (Fang et al., 2004; Marwick, 2006). Thus, several papers with experimental, epidemiological, laboratory and clinical data have proven that even asymptomatic, healthy diabetic subjects, with normal left ventricular mass and absence of hypertension, coronary artery disease and metabolic syndrome, present with systolic and diastolic myocardial dysfunction (Zabalgoitia et al., 2001; Fang et al., 2003; Boyer et al., 2004; Fang et al., 2005).
3.3.1.1 Pathophysiology

The development of DMD is probably multi-factorial. The main pathophysiological mechanisms that are implicated are as follows.

**Brief Pathophysiologic mechanisms of diabetic myocardial disease**

(a) Metabolic disturbances
- Alteration in substrate supply and utilization
- Free fatty acid metabolism
- Abnormality in calcium homeostasis
- Advanced glycation end products accumulation
- Activation of PKC

(b) Insulin resistance
- Reduced insulin sensitivity
- Hyperinsulinemia

(c) Small cardiac vessels' disease
- Microvascular remodeling
- Impaired coronary flow reserve
- Endothelial dysfunction

(d) Disorders of the autonomous nerve system of the heart
- Sympathetic cardiac aponeurosis
- Hypercatecholaminemia

(e) Myocardial fibrosis, apoptosis, necrosis
- Activation of the neurohormonal axis
- Over-expression of the inflammatory process
- Oxidative stress

(a) Metabolic disturbances

Normally, heart utilizes FFA as its primary energy source during aerobic perfusion at normal workloads and increasingly relies on glycolysis and pyruvate oxidation during periods of ischaemia or increased work.

(i) Alteration in substrate supply and utilization: Metabolic disorders that are directly triggered by hyperglycemia (Rodrigues et al., 1998). The heart of a diabetic patient shows a primary defect in the stimulation of glycolysis and the oxidation of glucose (Mokuda et al., 1990). Altered substrate supply and utilization by cardiac myocytes could be the primary injury in the pathogenesis of diabetic cardiomyopathy (Radrigues et al., 1998). There is an increasingly reported evidence supporting that the primary cause of DMD pathogenesis is the deviation in the management of the glucose metabolic substrate by the myocardial cells either due to deficiency in GLUTs 1 and 4 of the cellular membrane (Garvey et al., 1993; Russell et al., 1998) or to the inhibitory action of the excessive increase in oxidized free fatty acids (Liedtke et al., 1988) and the concomitant carnitine deficiency (Malone et al., 1999).

(ii) Free fatty acid metabolism: In diabetes, there occurs elevation of circulating FFA because of enhanced adipose tissue lipolysis and hydrolysis of augmented myocardial triglyceride stores (Zhi et al., 2004). High circulating as well as cellular levels of FFA results in abnormally high oxygen requirements during FFA metabolism and intracellular
accumulation of potentially toxic intermediates of FFA, all of which can lead to impaired myocardial performance and severe morphological changes (Radrigues et al., 1998). The FFA induced impairment of glucose oxidation is a major factor in the development of diabetic cardiomyopathy, as described earlier. Furthermore, the availability of carnitine, an essential substance for myocardial FFA metabolism, is usually reduced in diabetics.

(iii) Abnormality in calcium homoeostasis: It has been experimentally shown that this disorder in the use of the metabolic substrate, combined to uncouple oxidative phosphorylation and myocardial oxygen demand, leads to malfunction of the contractile device of the heart. Myocyte bioenergetics are compromised by the concomitant homoeostatic disorders of calcium (reduced sodium–potassium and sodium–calcium ATPase) and myosin deficiency (Malhotra and Sanghi, 1997; Belke et al., 2000; Watts and Marwick, 2003) which reduces the rate of Ca++ removal from the cytoplasm in diastole (Liedtke et al., 1988). Such alterations may contribute to the increased diastolic stiffness characteristic of diabetic cardiomyopathy. Accumulation of toxic molecules such as long chain acylcarnitine and free radicals contribute to alteration of calcium sensitivity of regulatory protein.

(iv) Advanced glycation end products accumulation: The hyperglycaemia, characteristic of diabetes leads to non-enzymatic glycation of macromolecules. By complex reaction, the sugars linked to macromolecules are condensed into large heterocyclic derivatives. These structures are known as advanced glycation end products (AGEs). These AGEs accumulate in tissues and are implicated in morphological changes that occur in the diabetic heart. Accumulation of AGE-modified extra-cellular matrix results in inelasticity of the vessel wall and could interfere with the myocardial function. In diabetics, prolongation of isovolumic relaxation time, as assessed by Doppler echography, correlated with serum levels of AGEs after adjustment for age, diabetes duration, renal function, blood pressure and autonomic function parameters (Berg et al., 1999).

(v) Activation of protein kinase C: Hyperglycaemia induced up-regulation of PKC by diacylglycerol (DAG) has been proposed as a mechanism in the development of vascular complications in diabetes. PKC interferes with the contraction protein Troponin-T, Troponin-I, Troponintropomyosin complex (Xia et al., 1994). Increased PKC activity influences nuclear gene transcription by way of the mitogen-activated protein kinase (MAPK) cascade to induce the immediate early gene programme with subsequent stimulation of late genes that increase production of ACE, α-MHC and skeletal α-actin (Koj, 1996). ACE, in particular, may account for development of abnormalities that contribute to the development of diabetic cardiomyopathy (Giles and Sander, 2004).

(b) Insulin resistance

It is expressed as reduced sensitivity to insulin and hyperinsulinemia, is directly associated not only with diabetes (Bonora et al., 2002; Nakano et al., 2003), but also with hypertension (Eiro et al., 2003) and coronary artery disease (Hong et al., 2002). The linkage of myocardial disease with abnormal metabolism has been identified in patients with diabetes, in obese subjects as well as in those with the metabolic syndrome. Reduced insulin sensitivity can be found even when Type 2 diabetes is isolated and well controlled (Bonora et al., 2002). Endothelial dysfunction (Aso et al., 2001), changes in autonomic nervous system modulation (Nakano et al., 2003) and inflammatory process (TNF-α increment) may be involved in the
pathogenesis of insulin resistance (Fernandez-Real and Ricart, 1999; Fernandez-Real et al., 2002).

Changes in diastolic function have been widely reported in diabetic patients without evidence of heart disease caused by other factors (Raev, 1994; Lind et al., 1996). The LV ejection time is often reduced, and the length of the pre-ejection period and the ratio of pre-ejection period to LV ejection time are often increased (Zhi et al., 2004). Diastolic inflow patterns are frequently abnormal, reflecting underlying abnormalities in relaxation and/or reduced myocardial compliance. In 46 well-controlled Type-2 diabetic patients who had no evidence of diabetic complications, hypertension, CAD, CHF, or overt thyroid or renal disease, and no overt systolic dysfunction, LV diastolic dysfunction was present in 28 subjects (60%), of whom 13 (28%) had a pseudonormal filling pattern (reflecting raised filling pressure), and 15 (32%) had impaired relaxation (a milder form of diastolic dysfunction) (Poirier et al., 2001). Echocardiographic examination has made it easier to detect subtle abnormalities in LV diastolic dysfunction before its clinical appearance. Schwannwell et al. examined 87 young Type-1 diabetics and demonstrated reduced early peak mitral velocity, increased late peak mitral velocity, and prolonged deceleration time and isovolumetric relaxation time, despite having normal LV dimensions and systolic function (Schwannwell et al., 2002). Newer techniques like tissue doppler imaging have confirmed these findings (Fang et al., 2003).

Studies suggest that patients with Type-2 diabetes are more likely to have diastolic dysfunction than Type-1 diabetics. In 20 Type-1 and 10 Type-2 diabetic patients, ventricular filling was impaired more significantly in the latter group of patients (Astori et al., 1997). Its presence has been linked to left ventricular (LV) early diastolic abnormalities in hypertension (Guida et al., 2001) and obesity (Wong et al., 2004), while it has been associated with LV hypertrophy or increased LV mass even in non-diabetic subjects (Paternostro et al., 1999; Davis et al., 2002). Experimental and clinical studies have demonstrated that insulin resistance can alter the cardiac contractile function of diabetic patients at the myocyte level (Dutta et al., 2001; Hintz and Ren, 2002) and is associated with LV hypertrophy and diastolic dysfunction (Hirayama et al., 2001).

The fasting plasma insulin level was found to be the strongest independent predictor of cardiac mass, both in the normotensive and hypertensive diabetic subgroups (de Kreutzenberg et al., 2000), while in the Framingham Study insulin resistance was associated with increased LV mass mainly in women, but this relation was largely accounted for by obesity (Rutter et al., 2003). Several epidemiological and clinical studies have shown diabetes to be associated with systolic dysfunction also. There is a significant association of idiopathic dilated cardiomyopathy with diabetes mellitus (Coughlin et al., 1994). In a study of 40 Type-2 diabetics, 22 (55%) patients had systolic dysfunction, but only 3 (7.5%) had ECG changes compatible with cardiac ischaemia; 16(40%) patients were also found to have LV hypertrophy (Mbanya et al., 2001). Many diabetics may have normal LV systolic function at rest with abnormal systolic function only during exercise or dobutamine stress. In 30 diabetic men with normal resting LV ejection fraction and without coronary or any other cardiovascular disease, LV ejection fraction decreased after exercise in 5 of the 30 diabetics (17%), remained unchanged in 8 (27%) and increased normally in only 17 patients (Vered et al., 1984).

Although many studies have shown that diabetic patients have abnormal diastolic dysfunction but preserved systolic function, this may well be due to techniques used for systolic function evaluation. Techniques utilized for systolic function evaluation are less sensitive than those used for assessment of diastolic dysfunction. Newer techniques for systolic function assessment such as strain, strain rate, and myocardial tissue doppler velocity
can detect pre-clinical systolic dysfunction in diabetic patients. The parameters of peak strain, strain rate, and calibrated integrated backscatter were determined echocardiographically in 186 patients who had normal ejection fraction and no evidence of CAD (Fang et al., 2003). Forty-eight patients had diabetes mellitus (DM group); 45 patients had LV hypertrophy (LVH group) only; 45 patients had diabetes and LVH (DH group); and there were 48 controls. All patient groups (DM, DH, LVH) showed reduced LV systolic function, compared with controls as evidenced by lower peak strain (p < 0.001) and strain rate (p = 0.005). Calibrated integrated backscatter, which signifies myocardial reflectivity, was greater in each patient group than in controls (p < 0.05). Peak strain and strain rate were significantly lower in the DH group than in the DM (p < 0.03) or LVH (p = 0.01) groups. Diabetic patients who did not have overt heart disease demonstrated evidences of LV systolic dysfunction and increased myocardial reflectivity. Although the changes were similar to those that were caused by LVH, they were independent and incremental to the effects of LVH.

(c) Small cardiac vessels’ disease with both structural and functional disorders.

The former are manifested as a microvascular remodeling with glassy retrogression, thickening of the intima, formation of capillary microaneurysms and reduction of the transversal intersection of the cardiovascular plexus (Gherasim et al., 1985). Structural and functional alterations of the small vessels in diabetes have been incriminated in the development of diabetic cardiomyopathy, although this remains controversial. In a biopsy study, diabetic patients had normal or mildly depressed LV systolic function but significantly greater thickening of the capillary basement membrane, interstitial fibrosis and smaller myocytes compared with the control subjects (Kawaguchi et al., 1997). This suggests that alterations in capillaries due to diabetes may lead to myocardial cell injury and interstitial fibrosis and ultimately to diabetic cardiomyopathy.

Evidence for association of small vessel disease with myocardial disease is supported by an autopsy study of diabetic patients also in whom both the endothelial and subendothelial proliferation with fibrosis was observed in small coronary arteries (Hamby et al., 1974). Despite these findings, it has been proposed that such focal changes in microvessels are insufficient to account for diffuse myocardial degeneration with interstitial fibrosis in diabetic cardiomyopathy. No significant difference in the extent of small vessel disease or the density distribution of vessels of various size categories could be found between the diabetic patients and controls by Sunni et al. (1986). Indeed, there is no direct proof that microvasculopathy is an underlying cause of diabetic cardiomyopathy (Zhi et al., 2004). Endothelial dysfunction commonly occurs in coronary vasculature in the diabetic patients and may lead to abnormal control of blood flow. Diabetes with an otherwise low likelihood of atherosclerosis have impaired endothelium dependent dilatation in the epicardial coronary arteries (Nitenberg et al., 1993). Several mechanisms have been implicated in the abnormal endothelium dependent vasodilatation in diabetes. The half-life of nitric oxide is reduced due to increased oxidative stress. On the other hand, synthesis of vasoconstrictor prostanoids by the endothelium is increased, so that vasoconstriction is enhanced in diabetic patients (Tesfamariam et al., 1989).

As described already, protein Kinase C activity is increased in the presence of hyperglycaemia. This may also play a role in endothelial dysfunction in diabetes and subsequent development of diabetic cardiomyopathy. Functional changes that occur in the microvasculature are related to reduction of endogenous nitric oxide production and protein kinase C activation, both leading to endothelial dysfunction (Calles-Escandon and Cipolla,
These alterations in capillaries induce microvascular spasm, reduction of capillary density and increased permeability with consequent increases in extracellular volume. The consequent deficiency of the coronary reserve contributes to loss of contractile proteins and necrosis of myocardial cells, with reactive creation of perivascular focal interstitial fibrosis and hypertrophy, increased collagen deposition and development of DMD (Strauer et al., 1997).

(d) Disorders of the autonomic nervous system of the heart

These disorders lead to cardiac sympathetic aponeurosis and counterbalancing increase of the catecholamine levels in the cardiac tissue. This leads to the activation and over-enhancement of the apoptosis and oxidative stress processes (Iwai-Kanai et al., 1999; von Harsdorf and Dietz, 1999). The toxic effects on myocardium are immediate, promoting hypertrophy, interstitial fibrosis, suppressed contractility to myocytic level, accompanied by increased myocyte necrosis (Frustaci et al., 2000). Cardiac neuropathy is evidenced by attenuation of heart rate and blood pressure responses to breathing, Valsalva and posture, reduction of heart rate variability, and reduction of heart rate recovery after exercise. Evidence of denervation can be shown by modern technique applications such as single photon emission computed tomography and positron emission tomography (Marwick, 2006).

(e) Myocardial fibrosis and myocyte hypertrophy

They are the most frequently proposed mechanism to explain cardiac changes in diabetic cardiomyopathy. Hyperglycaemia results in the production of reactive oxygen and nitrogen species, which increases oxygen stress and causes abnormal gene expression, alters signal transduction, and activates the pathways leading to programmed cell death or apoptosis (Zhi et al., 2004). Hyperglycaemia can also directly induce myocyte necrosis in myocardium (Cai et al., 2002). While apoptosis does not cause significant interstitial collagen accumulation, myocyte necrosis results in increased deposition of collagen in a diffuse or scattered manner (Anversa et al., 1998). Collagen deposition in the diabetic myocardium can occur also due to impaired collagen degradation resulting from glycosylation of the lysine residues on collagen (Zhi et al., 2004). The functional abnormality in diabetic myocardium is considered to be associated with the structural changes and indeed, these structural changes might play a role in progressive deterioration of cardiac haemodynamics (Zhi et al., 2004). Myocardial fibrosis and myocyte hypertrophy which are directly associated with the activation of the neurohormonal axis (angiotensin II, endothelin) (Chen et al., 2000; Fiordaliso et al., 2000), the pre-inflammatory cytokines and the presence of oxidative stress (Diamant et al., 2005; Wold et al., 2005) as a reaction to the myocytic necrosis and apoptosis (Frustaci et al., 2000).

Experimental studies have shown that diabetes causes defects in cellular calcium transport, defects in myocardial contractile proteins and an increase in collagen formation, which result in anatomic and physiological changes in the myocardium (Giacomelli et al., 1979; Regan et al., 1981; Ganguly et al., 1983). The co-existence of arterial hypertension and/or the myocardial ischemia in diabetic patients helps the initially “subclinical” (silent) DMD to break out, which at first is clinically expressed as a clear diastolic and/or systolic cardiac dysfunction. It has been suggested that the reduced coronary reserve due to diabetic microvascular disease in addition to coronary epicardial stenoses leads to reduction of the threshold of myocardial ischemia (Bauters et al., 2003; Fang, Prins, et al., 2004). Thus, repeated ischemic incidents lead to focal myocytic necrosis and reactive development of
fibrosis and hypertrophy, contributing to further rapid evolution of DMD. On the other hand, DM with concomitant arterial hypertension obviously leads to more severe structural and functional cardiac lesions, promoting thus the abolition of myocardial cells and the interstitial fibrosis mainly due to neurohormonal hyperactivation, inflammatory response and oxidative stress (van Hoeven and Factor, 1990; Frustaci et al., 2000). Nevertheless, in daily clinical practice, special attention is required in order to diagnose “depurated” DMD and to exclude the possibility of concomitant silent ischemia and/or arterial hypertension.

Pathologic Anatomy

The pathologic findings of DMD, as they are detected in both heart biopsy and necropsy studies of diabetic patients, are the progressive abolition of muscular fibrils, the reactive myocardial hypertrophy, the increased synthesis of intercellular substance and connective tissue collagen, and, eventually, the interstitial fibrosis. Moreover, the occurrence of microvascular disease is pointed out with the presence of thickened and sclerotic small coronary vessels (Nunoda et al., 1985; Kawaguchi et al., 1997; Fang et al., 2004).

3.3.1.2 Treatment

There is no standard specific therapeutic strategy to recommend for DMD. However, the aforementioned pathophysiological mechanisms considered to be liable for the development of DMD indicate that different therapeutic approaches may be effective for the prevention, delay in progress and, eventually, treatment of the emerging heart failure.

Since hyperglycemia increases the free fatty acid levels, stimulates the oxidative stress, activates the growth factors and disturbs the calcium homoeostasis and lipid metabolism, it is more than obvious why the accurate regulation of the blood glucose levels is considered to be of major importance. It has been shown that the strict control of DM with intensive treatment with insulin can suspend up to a certain point the progress of DMD (von Bibra et al., 2004).

Insulin resistance and hyperinsulinemia deriving from a combination of environmental and genetic factors can be dealt with a change in lifestyle, physical practice, body weight control and certain medications, which increase insulin sensitivity [metformin, thiazolidinediones (TZDs) and ACE-I]. The perspective of modification in the use of the metabolic substrate of the diabetic heart seems to be very attractive. Special metabolically active medications promise the suspension of free fatty acid oxidation and the stimulation of glucose usage by the myocardium as a main metabolic source of energy.

Etomoxir, a carnitine palmitoyltransferase inhibitor and dichloroacetate (stimulator of pyruvate dehydrogenase activity), has exhibited conflicting results (Lewis et al., 1998; Schmidt-Schweda and Holubarsch, 2000). More recently, trimetazidine, a stimulator of the lactate dehydrogenase, according to the first results seems to improve the ventricular function of the diabetic heart, while its action appears to be in favor of the endothelial function by promoting the expression of cGMP and reducing the levels of endothelin-1 (Monti et al., 2006). Regarding sulindac, a second drug in this category (aldoze reductase inhibitor), it was announced that it has a cardioprotective activity in experimental models, suspending thus the evolution of DMD (Krishna et al., 2005).

Another study reported that diabetic patients with autonomous neuropathy and confirmed cardiac dysfunction may be stabilized and partially suspend the evolution of DMD, when treated with another reductase inhibitor (zopolrestat) (Johnson et al., 2004). Currently in experimental stages also are glycation end product cross-link breakers which
reduce the collagen storages of diabetic myocardium. Besides, they promise to have a therapeutic role in diabetic patients by improving cardiac function while reducing aortic stiffness, amending LV elasticity and, finally, inhibiting DM progression to clinically obvious heart failure (Liu et al., 2003). Regulation of the lipidemic profile with a change in lifestyle but mainly with the use of statins is a useful therapeutic tool at the physician's disposal. The severe effect of DM with concomitant dyslipidemia in the evolution and pathology of atherosclerosis is a well-known fact. These last years, this situation has led to the recognition of DM as an equivalent of coronary artery disease and to the standardization of the respective guidelines for aggressive confrontation of the concomitant dyslipidemia (Ryden et al., 2007).

In the case of DMD, the treatment of dyslipidemia must start in the initial stages, due to the fact that the lipidemic profile disorder along with the atherosclerosis is present very early and, potentially through microvascular disease and repeated ischemic incidents, they obviously aggravate its evolution. The cardioprotective activity of statins is attributed to both the reduction of lipid levels in blood and their well-studied pleiotropic activities (endothelium protective activity, stabilization of atheromatic plaque, anti-inflammatory activity). Treatment of hypertension represents an essential aspect in the management of diabetic patients, as it has been proved that for every 10 mmHg lowering of blood pressure there is a 15% reduction in the total mortality (UKPDS 38, 1998).

Despite the lack of specific data concerning the effect of optimal control of hypertension on myocardial function, there is no doubt that the latter must be a priority in the overall treatment of DM. Apart from the aforementioned regulation of metabolic homoeostasis (glucose, lipids, hypertension), physical exercise seems to have additional beneficial results: it improves the endothelial dysfunction and the function of cardiac and peripheral skeletal musculature; it reduces the activity of the excessively stimulated neurohormonal systems; it inhibits the prethrombotic environment and promotes fibrinolysis (Merritt and Foody, 2004).

As already mentioned, oxidative stress, i.e., the imbalance amongst the inherently derived products of oxidation and their neutralization by inherent anti-oxidatives, may trigger the initiation and promote the evolution of DMD. Therefore, it is normal for the scientific interest to intensively turn to the research and implication of an anti-oxidative therapy on the primary stages of the disease. So far, the research underlines the fact that anti-oxidatives (vitamin E, carnitine, $\alpha$-lipoic acid) are very promising, mainly regarding the delay in progress of the autonomous cardiac neuropathy and also the occurrence of complications in the diabetic heart (Manzella et al., 2001; Lo et al., 2002; Wold et al., 2005).

Once CHF is evident in patients with DMD (expressed either as left ventricular dysfunction detected in echocardiography or as a clinical syndrome), the same applicable therapeutic intervention and goals intended for non-diabetic patients are indicated: relief from congestion, delay in progress of the disease and eventual improvement of the patient prognosis. As long as there are no existing special data for the therapy of diabetic patients with CHF, evidence derived from large clinical trials referring to the subgroup of diabetic patients is used in everyday practice. However, the diabetic patients' response to the pharmaceutical treatment of CHF is similar to but not better than that of non-diabetic patients (Nesto, 2004).

Yet, the administration of ACE inhibitors to diabetic patients with CHF has proven that, it does not only delay the progress to more severe stages and reduce mortality, but it may also be used as a primary prevention of imminent CHF development. This fact is attributed to the competence of ACE inhibitors to facilitate blood flow through microcirculation into both skeletal and cardiac muscles; to improve insulin activity and use
of the glucose metabolic substrate in myocytic level; to inhibit the neurohormonal hyperactivation, and eventually to delay or to prevent the development of interstitial myocardial fibrosis and cardiovascular remodeling. An alternative choice and equally reliable solution are as it is already known angiotensin II receptor antagonists, which also block the rennin angiotensin aldosterone axis effectively (Swedberg et al., 2005).

Aldosterone receptor antagonists, medications with potent action against interstitial fibrosis and cardiovascular remodeling improve the endothelial dysfunction and promote the balance of the autonomous nerve system. Therefore, they should probably take part in the early treatment of DMD taking into consideration the status of renal function which is often seriously affected in diabetic subjects (Swedberg et al., 2005; Ryden et al., 2007). Despite the unquestionable evidence of the benefit of beta-adrenergic blockers when administered for CHF, according to great contemporary studies, they are still insufficiently prescribed to diabetic patients with CHF (probably due to the fact that they weaken the response to potential incidents of hypoglycaemia, affect the lipidemic profile and inhibit insulin expression in Type 2 DM) (Nesto, 2004). Perhaps, carvedilol must be the beta-blocker of choice for diabetic patients, due to the fact that it has beneficial effect on insulin tolerance and neutral activity on lipidemic profile, causes peripheral vasodilatation and, furthermore, has an anti-oxidative and anti-apoptotic activity, promoting the reverse cardiovascular remodelling (Giugliano et al., 1997). Other beta-blockers that are indicated in CHF are bisoprolol, metoprolol and nebivolol (Swedberg et al., 2005). The latter with extra beneficial activity on endothelial dysfunction (which is obviously observed in DMD) potentially has its own special role in the treatment of the disease (Kaiser et al., 2006; Peter et al., 2006).

According to the aforementioned reports and the fact that diabetic patients obviously need a more aggressive management, it is easy to come to the conclusion that the use of beta-blockers must by all means be recommended in DMD, except, of course, in cases where it is contraindicated. Intracellular retention of calcium in diabetes is associated with the depletion of high-energy phosphate stores and a derangement of ultrastructure and cardiac dysfunction. Calcium channel blockers can reverse the intracellular calcium defects and prevent diabetes induced myocardial changes. Verapamil has been shown to significantly improve the depressed rate of contraction and rate of relaxation and lower peak LV systolic pressure (Afzal et al., 1988). Verapamil can also improve the altered myofibrillar ATPase activity, myosin ATPase and sarcoplasmic reticular Ca++ pump activities (Afzal et al., 1989).

3.3.2 Diabetic Nephropathy

3.3.2.1 Pathophysiology

The association of proteinuria with diabetes mellitus was first recognized in eighteenth century but it was Kimmelstiel and Wilson in 1936 that defined the condition by describing the lesions of nodular glomerulosclerosis and the association with proteinuria and hypertension in type 2 diabetes. These features represent a late stage in the progression of the condition. Subsequent work, mainly on type 1 diabetes led to the definition of several distinct phases in the evolution of the disease (Mogensen 1984; Foggensteiner et al., 2001). The first morphological signs, glomerular basement membrane (GBM) thickening and mesangial expansion, can be seen within two years of diabetes on examination with electron microscopy. Later on, nodular or diffuse glomerular sclerosis, exudative lesions in glomeruli, atrophic tubular lesions develop and can be seen in kidney biopsies from patients with Diabetic Nephropathy (DN) (Osterby et al., 1983).
DN is typically defined by either microalbuminuria that is, a urinary albumin excretion of greater than 300mg in a 24 hour collection or by abnormal renal function as represented by abnormality in serum creatinine, calculated creatinine clearance or glomerular filtration rate (GFR). The common progression from microalbuminuria to overt nephropathy has led many to consider microalbuminuria to define early or incipient nephropathy. Renal disease is suspected to be secondary to diabetes in the clinical setting of long standing diabetes. Clinically DN is characterized by a progressive increase in proteinuria and decline in GFR, hypertension, decline in renal function and high risk of cardiovascular morbidity and mortality in patient with diabetes mellitus (Augustine and Vidt, 2004).

Diabetes has become the number one cause of ESRD in the US, and incidence of diabetes mellitus continue to grow both in the US and worldwide. DN may develop in 30% - 40% patients with diabetes mellitus (Andersen et al., 1983); however, recent studies suggest that the incidence in this group is declining (Bojestig et al., 1994). But still approximately 20% to 30% of all diabetics will develop evidence of nephropathy, although a higher percentage of type 1 progresses to ESRD. Approximately 45% of new patients entering dialysis in US are diabetics (Augustine and Vidt, 2004). DN is the leading cause of ESRD in developed countries and leads to a heavy burden of dialysis and transplantation. The risk of premature death in patients with DN is increased by the factor of 40 to 100, and other complications such as retinopathy and neuropathy cluster in these patients. Early studies suggested that the cumulative death rate of diabetic subjects with nephropathy was around 70% at 10 years (Borch-Johnsen et al., 1985). Although type 1 (insulin dependent) and type 2 (non-insulin dependent) diabetes are etiologically and epidemiologically different conditions affecting different segments of population, no major difference has been identified between the nepthropathies seen in these conditions, either pathophysiological or in terms of management. However, the patients with type 2 diabetes tend to be older and more hypertensive, thus more likely to have concomitant hypertensive and renovascular disease (Foggensteiner et al., 2001).

The natural history of DN is a process that progresses gradually over years. Early diabetes is heralded by glomerular hyperfiltration and an increase in GFR. This is thought to be related to increase cell growth and expansion in the kidney, possibly mediated by hyperglycemia itself. Microalbuminuria typically occurs after 5 years in type 1 diabetes. Overt nephropathy, with urinary protein excretion greater than 300mg/day, often develops after 10 to 15 years. ESRD develops in 50% of type 1 diabetic, with overt nephropathy within 10 years’ time (Augustine and Vidt, 2004).

In animals, streptozotocin (STZ)-induced model of type 1 diabetes mellitus in the rat has been the most widely studied model to demonstrate the hemodynamic and structural alterations of the diabetic state on the kidney. Although STZ-induced model of type 2 diabetes is relatively less studied for diabetes related changes in kidney, scattered reports are available in this direction. Progression of DN can be described in the following stages:

i. Renal hemodynamic alterations (hyperfiltration) and structural changes
ii. Microalbuminuria
iii. Overt nephropathy (proteinuria)

**Clinical stages of nephropathy**

From studies performed in the 1980’s Mogensen et al., (1983) defined stages in the development of renal alterations that can be used in clinical classification. (Fig. 2)
In stage 1, glomerular hyperfiltration and renal hypertrophy may be seen at onset of diabetes and shortly thereafter. With insulin treatment these changes are, at least, partly reversible. Stage 2 is a clinically “silent” period of progression with normal albumin excretion rate (AER) but subtle morphological lesions, e.g. GBM thickening and mesangial expansion, are seen in kidney biopsies. Occasionally, in stress situations such as very poor metabolic control or during physical exercise, AER may increase, but this is a reversible phenomenon. Transition to incipient nephropathy, stage 3, occurs in 2-4% of cases per year and this is associated with poor glycemic control and high levels within the normal range of urine albumin excretion (Mogensen et al., 1995). Stage 3, is characterized by a persistent and usually slowly increasing microalbuminuria (AER 20-199 μg/min). Patients with incipient nephropathy have a high risk of progression to overt nephropathy. Intervention, e.g. optimized glycemic control (DCCT study group, 2000) as well as antihypertensive treatment (Rudberg et al., 1999), may change the natural history and reverse or stabilize functional and may be even structural changes. Overt or manifest nephropathy, stage 4, is characterized by persistent macroalbuminuria (AER ≥200 μg/min), increasing blood pressure and a declining glomerular filtration (Rossing et al., 1994). Finally, in stage 5, kidney function becomes severely impaired and uremia, i.e. ESRD, evolves.

Renal Hemodynamic Alterations

Far from being bystanders in DN, changes in the proximal tubule are important for the development of progressive diabetic kidney disease. The proximal tubule is uniquely susceptible to a variety of metabolic and hemodynamic factors associated with diabetes. Renal function and prognosis correlate better with structural lesions in the tubuli and cortical
interstitium than with classical glomerular changes of DN. The proximal tubules show a variety of poorly characterized changes, which have led to the notion that tubular damage, represents a "final common pathway" for proteinuric renal injury. However, tubular hypertrophy, reduced organic ion transport, and other tubular changes reviewed, are already apparent before the onset of proteinuria in diabetes. Indeed, increased tubuloglomerular feedback and defective uptake and lysosomal processing may independently contribute to hyperfiltration and urinary protein loss, respectively. This finding does not mean that glomerular or vascular dysfunction does not contribute to progressive nephropathy. However, although subdividing the nephron for the purposes of analysis and scientific discovery may be useful, the interactions between tubule, glomerulus, and interstitium are likely key to the understanding of complex disorders such as DN. From this "holonephric" point of view, an understanding of the changes in the diabetic tubule forms an important component to the understanding of kidney disease in diabetes (Thomas et al., 2005).

Early studies showed that systemic hypertension accelerates renal injury in diabetes (Mogensen et al., 1983) and that the rate of progression of renal disease is slow in normotensive patients with type 1 diabetes (Jacobsen et al., 1999). An increase in the intraglomerular pressure has been suggested to promote progressive renal injury early in DN (Hostetter et al., 1982). This hypothesis is supported by the clinical findings that a high GFR early in diabetes is a risk factor for later development of DN as shown both in cross-sectional (Mogensen, 1984) and longitudinal studies(Rudberg et al., 1992; Bangstad et al., 2002).

The ability of the kidney to maintain a constant GFR over a range of renal perfusion pressures is called autoregulation, and some data suggest that an impaired autoregulation is present in overt DN (Parving et al., 1984). There is an association between nephropathy, proliferative retinopathy and autonomic neuropathy (Spallone et al., 1994; Malik et al., 2000) and this could be due to the coexistence of two or more diabetic complications. On the other hand, autonomic neuropathy could possibly have an effect of its own to cause renal injury via higher blood pressure, renal vascular dilatation and an increased intraglomerular pressure, all of which could be caused by an impaired vascular autoregulation (Sundkvist and Lilja, 1993; Spallone et al., 1994). Studies have shown that microalbuminuria and autonomic neuropathy coexist in patients with type 1 diabetes (Berglund et al., 1991; Clarke et al., 1999) and among patients without nephropathy the prevalence of autonomic neuropathy is low (Meinhold et al., 2001).

Glomerular hyperfiltration is a common diagnosis of diabetes mellitus and is often accompanied by renal hypertrophy and increased renal plasma flow. Adenosine reuptake inhibition corrects glomerular hyperfiltration and microalbuminuria in incipient type1 DN (Heyne et al., 2004). Unilateral renal artery stenosis protects the affected kidney from the development of diabetic glomerulosclerosis, while nephrectomy leads to increased risk of glomerulosclerosis in the remaining kidney. Intraglomerular hypertension is consequent upon hyperfiltration that leads to mesangial expansion and basement membrane thickening. These features can be the hallmarks of diabetic glomerulosclerosis.

A wide variety of growth factors and cytokines have been implicated in the etiology of glomerular hyperfiltration. An early abnormality seen in diabetes is increased levels of in insulin-like growth factor-1 (IGF-1) (Flyberg et al., 1988). IGF-1 may have a role in renal hypertrophy by virtue of its mitogenic activity on mesangial and vascular muscle cells. Inhibition of IGF-1 with octreotide reverses renal hypertrophy in experimental animals (Flyvberg et al., 1989). TGF-β may also play a part in the development of glomerular hyperfiltration by promoting synthesis of extracellular matrix proteins and modulating cell growth. Increases in TGF-β mRNA are seen in renal tissue exposed to hyperglycemia (Wolf
Atrial natriuretic peptide (ANP) may also have a role in the development of DN. Infusion of ANP in non-diabetic rats induces similar glomerular changes to those seen in diabetic rats (Dunn et al., 1986), whereas, inhibition of ANP reverses the hyperfiltration observed in rodent diabetes (Zhang et al., 1994). Furthermore, longitudinal observation of urinary ANP levels in humans has shown a good correlation with changes in albumin excretion (Jungmann et al., 1993). ANP mediates glomerular hyperfiltration in patients with normoalbuminuric type 1 diabetes. Primary event in diabetic glomerular hyperfiltration is an increase in proximal tubular sodium reabsorption. Sodium reabsorption was not due to systemic volume expansion but changes in tubular sodium handling most probably influence tubuloglomerular feedback (Vervoort et al., 2005). Alteration in blood rheology may also influence the onset of nephropathy. Reduction in fibrinolysis and increases factor VIII levels has been noted in DN (Fuller et al., 1979). Raised levels of thromboxane A2 have also been noted in diabetes along with reduction in prostaglandin I2 (Dollery et al., 1979). Nitric oxide (NO) appears to be an attractive mediator of glomerular hyperfiltration as it has a predominant action of decreasing preglomerular vascular tone (Deng and Baylis, 1993) and increased NO metabolites in the urine of STZ diabetic rats has been described (Bank and Aynedjian, 1993; Tolins et al., 1993). Inhibition of intrarenal NOS with L-arginine analogues leads to increased afferent arteriolar resistance and a decrease in hydraulic glomerular capillary ultrafiltration coefficient (Kf) (Deng and Baylis, 1993). Raloxifene which lacks the harmful effects of estrogen is useful for the treatment of DN (Chin et al., 2005).

The most intensively studied factor that may underline the altered renal hemodynamics in STZ diabetic rats is AT II as this peptide has potent constrictor effects preferentially on the efferent arteriole. Relative efferent arteriolar constriction may be postulated to be due to increased intraglomerular AT II production. It is demonstrated that ACE staining is increased in the glomerular endothelial cells of diabetic rats (Anderson et al., 1993) suggesting increased local production of AT II. It may also be postulated that the afferent arteriole from STZ diabetic rats has reduced responsiveness accounting for afferent vasodilatation. However, conflicting reports are available about the state of RAS in diabetic kidney. It has been shown that in the early course of STZ diabetes in rats, there is a reduction in the density of glomerular AT II receptors (Bellermann et al., 1984; Wilkes, 1987). Another group of investigators found an increased density of glomerular AT II receptors but impaired contractile responsiveness of diabetic glomeruli to AT II (Kikkawa et al., 1986).

Glomerular Structural Changes

DN in humans presents several structural changes that are characterized by early hypertrophy of both glomerular and tubuloepithelial elements, thickening of the GBM, progressive accumulation of extracellular matrix components in the glomerular mesangium, and less well recognized lesions such as tubulointerstitial fibrosis and renal arteriosclerosis (Mauer et al., 1984; Osterby et al., 1988; Ziyadeh et al., 1989; Steffs et al., 1992).

Glomerular hypertrophy

Progressive increase in kidney weight and glomerular volume in moderately hyperglycemic STZ diabetic rats during a period of six months of diabetes has been reported (Rasch, 1979). In severely hyperglycemic rats without insulin treatment the glomerular...
volume is reported to be continuously increased during the first eight months of diabetes (Hirose et al., 1982).

**Mesangial matrix expansion**

Progressive expansion of the glomerular mesangial matrix is considered to be the most important lesion for the development of chronic renal failure in the diabetic population. Diffuse intracapillary sclerosis correlates closely with the progressive decline in the glomerular capillary surface area available for filtration; hence it is the structural counterpart of reduced GFR (Steffses et al., 1989). Mesangial expansion is predominantly due to an overabundance of normal structural components of the mesangial extracellular matrix including collagen Type IV, laminin and fibronectin. It is reported that whole glomeruli from STZ diabetic rats demonstrate a significant increase in the steady state mRNA levels encoding the α1 chain of Type IV collagen, fibronectin, and laminin B1 and B2 (Fukui et al., 1992). Yamamoto et al. (1993) have showed that glomerular staining for fibronectin and tenasin is also increased in long term STZ diabetic rats. The phenotypic expression of mesangial extracellular matrix such as the appearance of interstitial collagen Type III may also be altered in STZ diabetic rats (Abrass et al., 1988). Appreciable induction of gene expression for interstitial collagen type III and I is also demonstrated (Scheinaman et al., 1978). These fibrillar collagens are distinctly absent in non-diabetic rats.

**GBM thickening and increased permeability**

The exact biochemical and ultrastructural basis for the thickening of GBM and the increased permeability for macromolecules across the filtration barrier of the glomerular capillary wall remains to be completely understood. It has been suggested that increased synthesis and/or decreased degradation of collagens and laminins are responsible for increased GBM thickness (Ziyadeh, 1993). The thickness of basement membrane is reversible by strict blood glucose control with insulin (Ziyadeh, 1995).

**Metabolic factors**

Hyperglycemia is necessary for the development of microvascular diabetic complications and it is associated with both onset of incipient and overt DN, i.e. micro and macroalbuminuria (Krolewski et al., 1995) and the progression of overt DN to renal failure (Nyberg et al., 1987). The level of hyperglycemia seems to be quantitatively linked to the risk of developing renal lesions (Krolewski et al., 1995). Hyperglycemia enhances the non enzymatic glycosylation of proteins and AGE are formed. AGE are stable and resistant to degradation by enzymes and they injure cells by structural rearrangement of proteins. They accumulate on long lived tissue proteins, such as collagen, and have been implicated in diabetic complications (Brownlee et al., 1988). Administration of (Advanced glycosylation end products) AGEs to non-diabetic animals can induce similar vascular changes to those seen in diabetes (Vlassara et al., 1994) and, in particular, can induce gene for growth factors, such as TGF-β, and extracellular matrix protein deposition (Vlassara et al., 1988).

Increased serum levels of AGE seem to predict changes in kidney morphology such as expansion of mesangial cell matrix and GBM thickening (Berg et al., 1997). AGE may also affect the charge selectivity on the GBM, altering the filtering capacity (Vlassara et al., 1986). AGE levels are well correlated to the degree of long term glycemic control (Chiarelli et al., 1999) and, in addition, they are increased when renal function declines (Makita et al., 1997).
Importantly, the level of these products do not return to normal when hyperglycemia is corrected since they may accumulate in the blood vessel wall and remain there during the life time of the proteins. In renal failure the AGE concentration declines moderately after treatment with hemodialysis and markedly after kidney transplantation (Makita et al., 1991). Aminoguanidine, a compound that inhibits the formation of AGE, has both in animal studies (Brownlee et al., 1988; Dunlop, 2000) and in clinical trials demonstrated promising effects to limit the progression of DN (Appel et al., 1999).

Hyperglycemia also enhances formation of sorbitol via the polylol pathway, and this is facilitated by the enzyme aldose reductase. The net result is an increase in sorbitol and a decrease in myoinositol content in cells. An accumulation of sorbitol in cells is toxic and can cause both functional and structural changes. Although this pathway has been most extensively investigated in neuropathy, some studies also support a role in DN (Dunlop, 2000). In human DN, treatment with aldose reductase inhibition has slightly reduced hyperfiltration (Pedersen et al., 1991), but until now the results of clinical trials have been disappointing mainly due to toxic side effects.

Diabetic glomerulopathy is associated with changes in the amount and composition of extracellular matrix proteins, such as heparin sulfate proteoglycan (HSPG). HSPG is a major determinant of permselectivity in the GBM by virtue of its negative charge (Deckert et al., 1989). Renal biopsy studies show a significant reduction in HSPG in the GBM of patients with DN (Shimomura and Spiro, 1987; Tamsma et al., 1994). This may, in part, explain the loss of selectivity of biosynthesis of HSPG has been demonstrated in humans with DN with the use of [3H] glucosamine (Deckert et al., 1991) and in diabetic rats by mRNA analysis (Fukui et al., 1992). A genetic polymorphism in HSPG has also been associated with DN (Hansen et al., 1997). From these data it has been postulated that deficient regulation of HSPG biosynthesis results in a lower content of HSPG in the GBM. Diabetic subjects with defects in this biosynthetic pathway may be more susceptible to the development of nephropathy. Interestingly, treatment of proteinuric insulin dependent diabetic patients with heparin appears to attenuate protein excretion, although the mechanism of this effect is uncertain (Myrup et al., 1995).

### Microalbuminuria

The normal urinary protein excretion rate is up to 300 mg/23 hr, of which about 10% is albumin, equivalent to an AER of 20 μg/min. AER of 20-200 μg/min, equivalent to a urine albumin creatinine ratio (ACR) of 10-25 mg/mmol, are defined (also called as incipient nephropathy) as these levels are not detectable by conventional urine dipstick analysis. The onset of microalbuminuria is highly significant since its presence predicts the development of overt renal disease in both type 1 and type 2 diabetes (Viberti et al., 1982; Mogensen 1984). Furthermore, microalbuminuria is associated with an increased risk of cardiovascular and microvascular complications as well as increase in mortality, especially in type 2 diabetes (Dinneen and Gerstein, 1997). Renal histology at this stage reveals typical glomerulosclerosis. Once microalbuminuria is established the trend is one of increasing proteinuria until overt nephropathy develops.

### Clinical criteria for microalbuminuria and DN

The following criteria have been proposed for the diagnosis of renal involvement for type 1 diabetes and they have subsequently been used in many studies (Mogensen et al., 1985). Incipient nephropathy is defined as microalbuminuria, i.e. AER 20-199 μg/min and...
overt nephropathy as macroalbuminuria, i.e. AER ≥200 μg/min, found in at least two out of three consecutive urine samples, preferably collected over a period of six months. Urine should be sterile. Other causes of increased excretion rate such as other renal or urogenital diseases, physical activity or fever should be ruled out. Particularly, when diabetes duration is less than 6 years, other causes should be considered. In clinical practice, 24 h or overnight urine collections may be difficult to achieve and spot collections in early morning samples can be performed as recommended by the American Diabetes Association; microalbuminuria, i.e. 3-29 mg/μmol creatinine and macroalbuminuria, i.e. ≥30 mg/μmol creatinine (American diabetes association, 2002).

Different studies have reported that the progression from normoalbuminuria to micro or macroalbuminuria in patients with type 1 diabetes during ten years is 7-17% (Parving et al., 1982; Mogensen and Christensen, 1984; Forsblom et al., 1992; Caramori et al., 2000). Albumin excretion in the upper normal range indicates a greater risk of progression to microalbuminuria (MC study group, 1999). Early studies in patients with type 1 diabetes showed that ~80% progressed from microalbuminuria to overt proteinuria within ~10 years (Mogensen, 1984) This led to a broad acceptance of microalbuminuria as a useful clinical predictor of the development of overt DN (Mogensen et al., 1985).

More recent studies have observed only about a 30-45% risk of progression of microalbuminuria to macroalbuminuria within 10 years in patients with type 1 diabetes, whereas about 30% became normoalbuminuric and the rest remained microalbuminuric (Forsblom et al., 1992; Rudberg et al., 1992; Caramori et al., 2000). It is unclear whether this is due to changes in the natural history of DN resulting from improved glycemic control and early intensive blood pressure treatment or whether there were overestimates in the original studies. To increase the complexity, some normoalbuminuric longstanding type 1 diabetic patients have morphological renal lesions typical for DN (Osterby, 1992; Berg et al., 1998).

In addition, some patients with persistent microalbuminuria have quite advanced renal lesions (Bangstad et al., 1993) and, thus, in these patients microalbuminuria may be a marker rather than a predictor of established structural renal damage. In type 2 diabetes, progression from normoalbuminuria to microalbuminuria has been found in 15-30%, from microalbuminuria to macroalbuminuria in 20-50% and from normoalbuminuria to macroalbuminuria in 0-8% during 6-9 years of follow-up (Forsblom et al., 1998; Ravid et al., 1998). However, these studies have a rather short follow-up time, less than 10 years, and probably more patients develop micro and macroalbuminuria with time.

It is important from the clinical point of view to identify patients at risk of developing nephropathy early as this allows intervention before advanced renal damage is established. AER still remains the best available non invasive predictor of risk of developing overt DN and should regularly be measured according to established guidelines. Currently 24 h collections (mg/24 h), timed collections (μg/min) and spot collections expressed in relation to urine creatinine are recommended by the American Diabetes Association in screening for microalbuminuria (American diabetes association, 2002). However, new technologies for screening are now being validated and may change the screening procedure in the future (Caramori et al., 2000).

Microalbuminuria is a predictor for development of renal failure, but also a powerful independent risk factor for cardiovascular disease and death in patients with diabetes and in non-diabetics (Mogensen, 1984; Messent et al., 1992). In addition, microalbuminuria has been linked to insulin resistance both in patients with diabetes and in non-diabetics (Yip et al., 1993; Ekstrand et al., 1998; Mykkanen et al., 1998). However, there are other studies suggesting that there is no direct relationship between microalbuminuria and insulin resistance (Nielsen and Jensen, 1999; Toft et al., 2002). Microalbuminuria has also been
considered to be a key factor in the development of glomerulosclerosis in both diabetic and non-diabetic renal disease and this can occur via so-called protein trafficking. Proteins filtered by the glomerulus may, due to the excessive reabsorption, cause injury to the tubular interstitium leading to renal dysfunction (Remuzzi and Bertani, 1998).

**Associations with microalbuminuria**

- Development of overt nephropathy and ESRD
- Increased cardiovascular risk
- Blood pressure changes:
  - Loss of nocturnal dip in blood pressure
  - Rise in blood pressure (mean 3 mmHg per year)
- Other microvascular complications of diabetes:
  - Proliferative diabetic retinopathy
  - Macular edema
  - Neuropathy
- Dyslipidemia
- Insulin Resistance

**Overt Nephropathy**

Proteinuria is generally regarded as a marker for the degree of glomerular damage. The levels of proteinuria correlate well with the prognosis of renal function, and interventions that retard the progression of diabetic renal disease also reduce proteinuria. However, it is still unknown whether the flux of protein across the GBM is causally implicated in the evolution of diabetic renal disease or simply reflects glomerular damage (Remuzzi and Bertani, 1990). AER above 200 μg/min or 300 mg/day (equivalent to an ACR of >25 mg/mmol) are dipstick positive and defined as overt nephropathy. This is usually associated with a relentless loss of GFR (by 1-24 ml/min per year) until end stage renal failure necessitates dialysis or renal transplantation. The rate of progression of microalbuminuria and overt nephropathy is heavily influenced by blood pressure control, glycemic control and the use of ACE-I. Overt DN is characterized by macroalbuminuria, hypertension and a variable decline, median 12 ml/min/year, in GFR, if left untreated (Mogensen, 1982; Ritz and Orth, 1999) until GFR <10 ml/min when ESRD evolves. ESRD, independent of its causes, e.g. diabetes, is characterized by several perturbations such as hypertension, accumulation of uremic toxins, hyperkalemia, hyperphosphatemia and anemia due to erythropoietin deficiency. Secondary hyperparathyroidism and alterations in D-vitamin metabolism, together with metabolic acidosis, are considered to be responsible for the osteodystrophy seen in uremic patients. Several risk factors for mortality among patients with uremia have been identified, and age, protein energy malnutrition and low serum albumin, commonly considered to be an index of malnutrition, appear to be strong predictors of mortality (Lowrie and Lew, 1990). Uremia due to diabetes is associated with a higher mortality when compared to non-diabetic renal diseases. Many factors may contribute to malnutrition and low serum albumin in uremia and they include low protein and energy intake. Other concomitant diseases such as heart failure and infections, inflammation, catabolic effects of acidosis, reduced physical inactivity and loss of protein and amino acid during dialysis treatment may contribute (Bergstrom, 1995). Chronic inflammation appears to be involved and aggravate malnutrition and progressive atherosclerotic disease by several pathogenic mechanisms. Available data
suggest that inflammation, reflected by high levels of cytokines such as TNF-α and IL-6, plays a central role in the development of both malnutrition and cardiovascular disease in ESRD (Stenvinkel and Alvestrand, 2002).

Table 14: Definitions of diabetic renal disease

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<th>Microalbuminuria</th>
<th>Clinical</th>
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<td>24 hr urinary albumin (mg/day)</td>
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<td>30-300</td>
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<td>Urine albumin secretion rate (μg/min)</td>
<td>&lt;20</td>
<td>20-200</td>
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<tr>
<td>Urine albumin/creatinine ratio (mg/mmol)</td>
<td>&lt;2.5 M</td>
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Role of RAS

A beneficial effect of ACE-I has been shown with respect to progression of both incipient (Parving et al., 1989) and overt DN (Mulec et al., 1998) and this includes reversal of structural changes (Rudberg et al., 1999). This supports that the RAS, and particularly AT II, play a central role in the intrarenal hemodynamic changes and in promoting structural changes in DN. AT II modulates glomerular filtration both by influencing afferent and efferent glomerular arteriolar tone and by a direct effect on mesangial cells and may also influence glomerular permeability (Burns, 2000). It also promotes renal sodium reabsorption by effects in the proximal tubuli and via stimulation of aldosterone secretion(Aguilera, 1993). In addition, AT II induces ECM accumulation (Singh et al., 1999). AT II interact with two specific receptors, angiotensin type 1 (ATR 1) and angiotensin type 2 receptor (ATR 2) and most of its actions seem to be mediated via the ATR 1 (Burns, 2000). Measurements of the different components of the RAS in plasma (Leehey et al., 2000) and within the kidney (Anderson et al., 1993) have revealed low or normal levels in diabetes but in vitro studies have shown an increased angiotensinogen expression in proximal tubular cells in a diabetic animal model (Zhang et al., 1999). In overt DN there is an activation of the RAS, due to a declining GFR, with an increased generation of AT II. This results in both systemic and intraglomerular hypertension, which accelerate the renal injury (Remuzzi and Bertani, 1998).

Increasing number of diabetic patients develop different stages of renal failure. However, often an inappropriate parameter, the serum creatinine is measured as a marker of glomerular function. Calculated GFR or endogenous creatinine clearance are suggested to be used for the estimation of the glomerular function. Important structures preventing proteinuria in the kidney are GBM, podocytes and proximal tubular cells. In diabetes mellitus loss of nephrin of podocytes can play a role in the development of microalbuminuria, and podocyte desquamation may result in the progression to proteinuria. In diabetes mellitus there is an increased formation of AGE, of which the only elimination organ is the kidney. The AGE induces proteinuria and atherosclerosis. Therefore, in diabetes mellitus a vicious circle develops due to proteinuria, nephron loss and accumulation of AGE, which play a role in the initiation and progression of DN and atherosclerosis. ACE-I and ARBs having antiproteinuric effect may decrease the risk of DN and atherosclerosis. Improvement of carbohydrate metabolism with a consequential decrease in the formation of AGE is an important contributor to the prevention and treatment of DN and atherosclerosis (Wittmann et al., 2005).
Glucose can bind irreversibly to protein in the kidney and circulation to form so called AGEs can complex crosslinks over years of hyperglycemia and can contribute to renal damage by stimulation of growth and fibrotic factors via receptors to AGEs (Augustine and Vidt 2004). It is postulated that ACE inhibition reduces the accumulation of AGEs in diabetes partly by increasing the production and secretion of receptor for advance glycation end products (sRAGE) into plasma (Forbes et al., 2005). Olmesartan medoxomil a newly developed AT II type 1 receptor antagonist could be a valuable drug for the treatment of DN by blocking the deleterious effects of AGEs (Yamagishi et al., 2005)

AT II itself contributes to the progression of DN. AT-II is stimulated in diabetes despite the high volume state typically seen with the disease, and intra renal AT-II is typically high, even in the face of lower systemic concentrations. AT-II preferentially constricts the efferent arteriole in the glomerulus, leading to higher glomerulus capillary pressures. In addition to its hemodynamic effects, AT-II also stimulates renal growth and fibrosis through AT-II type 1 receptors, which secondarily upregulate TGF-β and other growth factors (Augustine and Vidt 2004). Aldosteron synthetase inhibitor FAO 286 ameliorates AT II induce end organ damage (Fiebeler et al., 2005).

Spironolactone in addition to ACE inhibition reduces proteinuria in patient with chronic renal disease (Chrysostomou and Becker, 2001). The mechanisms involved in development of cardiovascular complications associated with diabetes mellitus are not well elucidated. Among the vasoactive factors that may play a role in development of these complications are AT II and thromboxane B2 (TXB2). They hypothesized that diabetes increases renal production of TXB2 through stimulation of angiotensin type-1 receptor. These results demonstrate that diabetes mellitus is accompanied by increased renal production of AT II and TXB2. The increase in TXB2 is mediated through stimulation of angiotensin type-1 receptor. Glomerulosclerosis and albuminuria in transgenic rats occur due to AT1 receptor over expression in podocytes (Kriz et al., 2004). Losartan reduces of urinary Connective tissue growth factor (CTGF) by type 1 patient with DN. CTGF is an important profibrotic cytokine implicated in development of diabetic glomerulosclerosis. Urinary CTGF is reported to be significantly increased in patients with DN. The present study aimed to investigate the short and long term effects of AT II receptor blockade by Losartan on urinary CTGF levels in hypertensive type 1 diabetic patients with DN. Their 3-year study demonstrates that Losartan persistently reduces urinary CTGF excretion, which is associated with a slower rate of decline in GFR (Anderson et al., 2005).

In contrary to above finding, complete knockout of renal and vascular ETB receptors in male rats with STZ-induced diabetes mellitus results in severe hypertension. The pronounced structural and functional renal pathology in this group is probably secondary to hypertension. The strong rise of blood pressure is not caused by suppression of the NO system. Possible mechanisms altered vascular response to hyperglycemia or altered renal response to osmotic hyperfiltration remain to be elucidated. These findings suggested ETB receptor antagonists might be harmful for diabetic individual at least when using that blocks the majority of the receptors (Pfab et al., 2004).

In clinical trials in high risk vascular patients (after myocardial infarction), patients with heart failure and patients with nephropathy show the benefits of ACE inhibition. ARBs likely have similar benefits as ACE-I when used after myocardial infarction, in patients with heart failure and for management of DN. However, ARBs generally remain a second line treatment because it has been more difficult to demonstrate that ARBs prevent acute vascular events, such as myocardial infarction, together with the greater clinical trial evidence for ACE inhibition. The primary application of ACE-I over ARBs is reflected in the Canadian clinical
guidelines for the management of patients with diabetes, hypertension, heart failure and following myocardial infarction. (Fitchett, 2005)

Treatment with heparin and ACE-I lowers proteinuria in DN. Besides its hemodynamic effects, AT II inhibits synthesis of glomerular HSPG. In vitro, heparins modulate AT II signaling in cultured glomerular cells. It has also been described that heparin inhibit aldosteron synthesis. It is not known, if the in vivo effects of heparin on proteinuria are mediated via the RAAS. In vivo findings show, that the antiproteinuric effect of heparin cannot be explained via interaction with the RAAS. The absence of hemodynamic changes together with a significant decrease in proteinuria can only be explained by intrinsic alterations in glomerular filter after heparin therapy. The effect could only be demonstrated in DN, not in IgA or membranous glomerulonephritis (Benck et al., 2004).

In one study, the role of interaction of polymorphisms in the RAS with ACE or angiotensin receptor (AGTR1) inhibitors (RAS inhibitors) is unknown, as is the role of such therapy in ESRD patients. So, they determined survival specific for allelic variants of the ACE (insertion/deletion), Angiotensinogen (M235T) and AGTR1 (A1166C) genes. The effect of therapy with RAS inhibitors at study inclusion was determined for the allelic variants of each gene. The primary end point was all cause mortality (ACM). For all polymorphisms, and for therapy with RAS inhibitors there was no significant effect on survival in the complete collective (n = 445), though there was an insignificant trend for improved survival in patients on AGTR1 antagonists. Increased ACM risk was associated with treatment with RAS inhibitors only in patients homozygous for the wild type AGTR1 1166A allele (HR 1.65, p = 0.01). For all other polymorphisms, therapy with RAS inhibitors had no significant effect on ACM, irrespective of genotype. Similar results were obtained in patients with systolic ventricular dysfunction. Their data do not show a survival advantage for type 2 diabetes hemodialysis patients receiving RAS inhibiting therapy. In addition, their data indicate that allelic variation in RAS genes and pharmacogenetic interaction with RAS inhibition does not affect mortality risk in diabetic hemodialysis patients. (Boger et al., 2005)

DN is the leading cause of chronic renal failure in westernized countries. The polymorphism in ACE, which leads to higher than normal levels of this enzyme, is a predictor of nephropathy in patients with diabetes. As increasing the levels of ACE by approximately 50% in this polymorphism is only calculated to increase the levels of AT II by < 5%, whereas the levels of bradykinin will decrease by 20%, bradykinin may be nephroprotective. In diabetic mice without bradykinin B2 receptors, the only parameter that is altered compared with the diabetic mouse is that the nephropathy is worse. Thus, in diabetic mice without a bradykinin receptor (Bdrb2 (+/-) Ins2 (+/C96Y)), compared with diabetic mice (Bdrb2 (+/+)/Ins2 (+/C96Y)), there is a greater kidney weight, increased urinary albumin output, and glomeruli mesangial sclerosis. In addition to reducing the levels of AT II, vasopeptidase inhibitors increase the level of bradykinin. A vasopeptidase inhibitor (AVE7688) has been shown to prevent nephropathy developing and to ameliorate it once it has developed in Zucker diabetic rats. The nephroprotective effects (reduced albumin secretion and reduced kidney damage) of AVE7688 in Zucker diabetic rats were partially prevented by the bradykinin B2 receptor antagonist icatibant. These data establish that stimulation of bradykinin B2 receptors is a target in DN (Doggrell, 2005).

There is increased tubular organic ion clearance following chronic ACE inhibition in patients with type 1 diabetes. The tubular excretion of creatinine significantly contributes to its clearance. Administration of an ACE-I is associated with increased organic ion clearance in experimental diabetes. This is consistent with experimental models showing increased ion transporter expression and improved tubular blood flow, following blockade of the RAS. These findings may have implications for the interpretation of creatinine based indices in
Review of Literature

patients with diabetes (Thomas et al., 2005). Renoprotective effect of benazepril in diabetic rats may be partly related to the inhibition of AT II - P42/44MAPK pathway (Ly et al., 2005).

Role of inflammatory mediators and growth factors

In addition to components of the RAS, there are several vasoactive peptides that have been postulated to influence renal injury, e.g. vasoconstrictors such as endothelin and vasopressin, and vasodilatators such as bradykinin, ANF, prostaglandins and NO (Cooper, 2001).

Ongoing research has led to further understanding of the complex Pathophysiology in DN. Increased glomerular capillary pressure occurs early in diabetes and is associated with hyperfiltration at the glomerulus. The glomerular messangium expands, initially by cell proliferation and then by cell hypertrophy. Increased mesangial stretch and pressure can stimulate this expansion, as can high glucose levels. Mediators of proliferation and expansion include platelet derived growth factor and TGF-β is particularly important in the mediation of expansion and later fibrosis via the stimulation of collagen and fibronectin.

Glucose can bind irreversibly to protein in the kidney and circulation to form so-called AGEs can complex crosslinks over years of hyperglycemia and can contribute to renal damage by stimulation of growth and fibrotic factors via receptors to AGEs (Augustine and Vidt, 2004). Agents that directly inhibit or degrade TGF-β or inhibit AGEs have shown success in animals and are being developed for clinical human trials. One such agent, currently known as ALT-711, breaks crosslinks in AGE complexes. It has been shown to improve vascular endothelial function in diabetes and may have promise in DN (Vasan et al., 2001).

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Thus, early expansion and proliferation, with high glomerular pressures and hyperglycemia, herald the development of DN. After years of expansion, fibrosis begins to develop TGF-β upregulation leads to secondary collagen and fibronectin production. Inflammatory cells cause cellular damage and scarring through release of cytokines and oxygen radicals, and ultimately renal cells themselves may transform into fibroblasts and cause late tubulointerstitial fibrosis. DN is also an inflammatory process with evidence of macrophage infiltration in glomeruli with early diabetic sclerosis. There is upregulation of Monocyte chemoattractant protein-1 (MCP-1) in diabetes, which stimulates macrophage infiltration both in the glomeruli and in the renal interstitium. Macrophage enters from the circulation and release inflammatory mediators and cytokines. MCP-1 is stimulated by AT2 and can be down regulated by renin angiotensin blockage in experimental models (Augustine and Vidt 2004).

Determination of urinary MCP-1 level is beneficial to know the degree of kidney inflammation in DN patients. Triptolide (TL) can inhibit inflammatory reaction to decrease the level of urinary MCP-1, and thus improve the renal function (Song et al., 2005).

Sodium ferulate (SF) can decrease urinary albumin excretion rate (UAER), serum endothelin (ET) and blood urea nitrogen (BUN) in DN patients, the mechanism may relate
with the decreasing of ET production and antagonizing to the binding of ET with its receptors (Zheng et al., 2005).

Renal dysfunction and platelet activation are present in type 2 diabetes with microvascular complications, and the synchronized changes between renal dysfunction and platelet activation indicate the involvement of elevated PAF in vascular injury (Fang et al., 2005).

In contrast to patients with peripheral arterial disease stage IV, patients with less severe atherosclerosis and elevated (C reactive protein) CRP are, if any, at less risk for cardiovascular mortality, possibly due to the difference in extent of affected vasculature and thus activated platelets and coagulation. Before judging the predictive value of CRP for mortality, peripheral vessel status should be determined (Boger et al., 2005).

There is an influence of IL-10 gene G-1082A polymorphism on progression of primary glomerulonephritis (Bantis et al., 2004). Since IL-10 has met the criteria for an anti-inflammatory and an immunosuppressive cytokine, its activity may be important for clinical outcome of DN. The aim of their study was to analyze the level of circulating IL-10 and relates it to the grade of albuminuria in patients with DN due to type 1 diabetes mellitus (DM). IL-10 appeared to be the strongest independent predictor of albuminuria, followed by HbA(1)c, diastolic blood pressure and DN duration. There was a positive correlation between the values of IL-10 and albuminuria in DM patients with DN. The patients in the fourth quartile of albuminuria had a distinctly higher concentration of IL-10 than those in the lower quartiles. The increased concentration of IL-10 in the serum samples from DM patients with DN seems to depend on the severity of the nephropathy. The excessive IL-10 production may indirectly contribute towards DN progression. On the other hand, it may explain the relatively long course of DN (Mysliwska et al., 2005).

The changes of sPLA2-IIA expression under increased glucose concentrations as well as in diabetic rat kidneys suggest a function of this enzyme as an acute phase protein providing lipid autacoids that may contribute to early changes in the course of DN (Vlachojannis et al., 2005).

Macrophage infiltration in kidney is one of the most important events for the progression of DN. Mycophenolate mofetil (MMF), an anti-inflammatory agent, has been shown to suppress macrophage infiltration and to improve renal injury in streptozotocin-induced diabetic kidneys. They examined whether mizoribine, which acts through immunosuppressive mechanisms similar to MMF, inhibits progression of DN in non-insulin-dependent diabetic rats. Mizoribine inhibited renal macrophage accumulation and prevented the progression of glomerulosclerosis and interstitial fibrosis in non-insulin-dependent diabetic kidneys. In addition to standard treatments, antiinflammatory agents may be useful for management of non-insulin-dependent DN (Kikuchi et al., 2005).

Tubulointerstitial fibrosis is seen in advanced stages of DN and is better predictor of renal failure than glomerular sclerosis. Hyperglycemia, AT2, TGF-β, and likely proteinuria itself all play a role in stimulating this fibrosis. There is an epithelial mesenchymal transition that takes place in the tubules, with proximal tubular cell conversion to fibroblast-like cells. These cells can then migrate into the interstitium and produce collagen and fibronectin (Augustine and Vidt, 2004).

The hexosamine pathway, through which glucose is converted into glucosamines, may also be involved in the development of diabetic complications. An increased flux through this pathway is associated with PKC activation, increased TGF-β expression and ECM production, all of which are associated with the development of DN (Schleicher and Weigert, 2000). In addition, a direct glucose induced rise in renal TGF-β expression has been shown in the kidney (Wang et al., 2002) and may be responsible for some of the structural...
renal changes in DN. TGF-β closely interacts with the RAS and PKC activity and their interplay could be central in the development of DN (Flyberg, 2000).

Some investigators consider oxidative stress as a major mechanism in the development of diabetic complications (Baynes and Thorpe, 1999). It may be a critical link between hyperglycemia and chronic complications (Evans et al., 2003) or a consequence of some other pathogenetic mechanism, for example AGE-formation (Scivittaro et al., 2000).

An increased exposure of mesangial cells to lipoproteins and incorporation of lipids enhances proliferation of the mesangium. Lipoproteins bind to the poly anionic glucoseaminoglycanes in the ECM and GBM and may alter charges of the GBM (Klahr et al., 1988). Alterations in lipid metabolism are seen already in incipient DN (Jensen et al., 1988) and may perhaps, via TGF-β, accelerate structural injuries and kidney complications (Bonnet and Cooper, 2000).

Tubulointerstitial macrophage accumulation is an important marker of prognosis that correlates closely with declining renal function in a range of human and experimental diseases, including DN. These inflammatory cells are rich in the profibrotic growth factor TGF-β such that their presence in areas of injury is frequently associated with tissue fibrosis. The migration of macrophages occurs in response to the site specific production of chemokines, with osteopontin closely associated with their trafficking into the tubulointerstitium of the kidney. Although cell culture studies indicate that PKC mediates the expression of osteopontin, its role in the in vivo setting is unknown. Accordingly, Ren-2 control and diabetic rats that were treated with or without the specific PKC-β isoform inhibitor ruboxistaurin (10 mg/kg per d) were examined. After 12 wk, diabetic rats showed increases in osteopontin expression in tubular epithelial cells of the cortex in association with macrophage infiltration, interstitial fibrosis, and activity of TGF-β as indicated by the expression of its receptor activated protein phospho-Smad2 (P < 0.05 for all parameters). Ruboxistaurin treatment significantly attenuated these parameters (P < 0.05) in diabetic rats without affecting either BP or glycemic control. These findings suggest that osteopontin and macrophage accumulation may play a role in the tubulointerstitial injury in DN and that inhibition of osteopontin expression may be one of the mechanisms by which inhibition of the β-isofrom of PKC confers a renoprotective effect (Kelly et al., 2005).

Even with this regimen, GFR was significantly impaired from week 2. This regimen was accompanied by progressive histological changes, including tubular and glomerular hypertrophy, mesangial area expansion, as well as interstitial macrophage, CD4+ and CD8+ T-cell accumulation. By careful optimization of STZ dose, a stable and reproducible diabetic murine model was established. However, even in this optimized model, renal functional impairment was observed. The frequency of ATN and functional impairment casts doubt on conclusions about experimental DN drawn from reports in which ATN has not been excluded rigorously (Tay et al., 2005).

Activation of a transcription factor, nuclear factor-kappa β (NF-kappa β), is a key step in the pathogenesis of DN. In this study, they investigated the role of P-selectin, a platelet-derived adhesion molecule, in DN by examining the activation status of NF-kappa β in the renal cortex of streptozotocin-treated rats. Treatment with ammonium pyrrolidinedithiocarbamate, an antioxidant and inhibitor of NF-kappa β, inhibited the activation of NF-kappa β in STZ-treated rats and decreased P-selectin in the renal cortical tissue. Their results indicate that expression of the P-selectin gene is induced through the activation of NF-kappa β and that P-selectin may be involved in the pathogenesis of DN (Iwamoto et al., 2005).

It was hypothesized already in the 1970’s that GH and IGF-1 influence the development of diabetic complications. Possibly, chronic hyperglycemia and/or lack of insulin in diabetes lead to impairment in the hepatic IGF-1 formation and a lower serum IGF-
1 level, inducing GH hypersecretion by feedback. The increase in GH concentration, in turn, might stimulate local IGF-1 production in the kidneys. In fact, all components of the GH/IGF-1 system, i.e. IGF-1, IGF-2, the six IGF-binding proteins and the two specific IGF-receptors are expressed locally in the kidney. Both experimental animal studies (Gronbaek et al., 1996) and treatment studies on diabetic retinopathy in humans with hypophysectomy (Ray et al., 1968) as well as long acting somatostatin analogues (Boehm and Lustig, 2002) have suggested beneficial effects on microvascular complications following inhibition of the IGF/GH system. Additionally, some studies have shown that puberty, partly due to the hormonal changes, e.g. high levels of growth and sex hormones together with a concomitant deterioration in glycemic control, may promote the development of microvascular complications (Dahlquist et al., 1987; Kordonouri et al., 1998). In vivo and in-vitro studies demonstrated a relevant role of vascular endothelial growth factor (VEGF) in experimental diabetic renal disease, whereas inhibition of VEGF ameliorated development of experimental DN. VEGF and VEGF-receptor system is differently regulated during diabetic glomerulopathy. VEGF-receptor bound VEGF representing endothelial cells actively undergoing VEGF-induced angiogenesis are markedly increased in early stages of diabetic glomerulopathy, while later stages are associated with a decreased activity of VEGF-mediated angiogenesis (Hohenstein et al., 2004).

Poor glycemic control and low portal insulin levels in type 1 diabetes is associated with elevated GH levels and elevated levels of IGFs and their high affinity binding proteins (IGFBP-1) as well as reduced levels of free IGF-1 (Janssen et al., 1997). In particular, this is observed in patients with microvascular complications and microalbuminuria. Circulating GH levels may be elevated in patients with renal failure due to both increased secretion and impaired degradation (Tonshoff et al., 1995). This may by a compensation that occurs in response to a relative tissue resistance to the effects of GH, which in turn may be due to an altered GH-receptor function. Potentially, uremic toxins and IGFBP may act as IGF-1 inhibitors and the increase in IGFBP also results in a lower free fraction of IGF-1, promoting GH release (Tonshoff et al., 1995).

Mesangial cells are critical for glomerular filtration. Mesangial cell dysfunction, the hallmark of DN, results from disordered mesangial growth induced by cytokines, abnormal hemodynamic influence, and metabolic factors associated with chronic hyperglycemia. IGFBPs exert major actions on mesangial cell survival, but their underlying mechanisms remain unclear. In light of emerging IGF-independent roles for IGFBP-3, they investigated IGFBP-3 actions during mesangial cell apoptosis induced by cytokine or high glucose concentration. Quantified by DNA fragmentation ELISA and Annexin V flow cytometry, apoptosis occurred in rat mesangial cells (RMC) exposed to 2μg/mL IGFBP-3 for 24h under high ambient or standard glucose. Anti-sense IGFBP-3 oligo at 10μg/mL significantly inhibited apoptosis induced by 100ng/mL TNF-alpha, serum free conditions, or high (25mM) glucose. Increased IGFBP-3 release associated with high ambient glucose or TNF-alpha was inhibited by pre treatment with anti-sense oligo. Under serum free conditions, recombinant human IGFBP-3 blocked phosphorylation at threonine 308 (pThr308), whereas anti-sense oligo treatment was associated with enhanced pThr308 activity. In summary, these data support a novel mechanism for TNF-alpha-induced mesangial cell apoptosis mediated by IGFBP-3 and present regulation of pThr308 activity as a novel mechanism underlying IGFBP-3 action (Vasylyeva et al., 2005).

TGF-β drives fibrosis in diseases such as DN. (CTGF; CCN2) has also been implicated in this, but the molecular mechanism is unknown. They show that CTGF enhances the TGF-β/Smad signaling pathway by transcriptional suppression of Smad 7 following rapid and sustained induction of the transcription factor TIEG-1. Smad 7 is a
known antagonist of TGF-β signaling and TIEG-1 is a known repressor of Smad 7 transcription. CTGF enhanced TGF-β-induced phosphorylation and nuclear translocation of Smad 2 and Smad 3 in mesangial cells. Antisense oligonucleotides directed against TIEG-1 prevented CTGF-induced down regulation of Smad 7. CTGF enhanced TGF-β-stimulated transcription of the SBE4-Luc reporter gene and this was markedly reduced by TIEG-1 antisense oligonucleotides. Expression of the TGF-β-responsive genes PAI-1 and Col III over 48 h was maximally stimulated by TGF-β + CTGF compared to TGF-β alone, while CTGF alone had no significant effect. TGF-β-stimulated expression of these genes was markedly reduced by both CTGF and TIEG-1 antisense oligonucleotides, consistent with the endogenous induction of CTGF by TGF-β. We propose that under pathological conditions, where CTGF expression is elevated, CTGF blocks the negative feedback loop provided by Smad 7, allowing continued activation of the TGF-β signaling pathway (Abdel et al., 2005).

DN (DN) is the most common cause of ESRD in western society. While glomerulosclerosis is the pathological hallmark of DN, tubulo interstitial fibrosis (TIF) is being increasingly recognized as a major predictor of progressive renal failure. The molecular basis for TIF remains incompletely understood. In aggregate these results suggest that sustained recapitulation of developmental programs induced by high glucose and/or its downstream effectors may be an important contributor to TIF in human DN (Sadlier et al., 2004).

Developmental gene gremlin reemerges in the context of tubulointerstitial fibrosis in DN and suggests a role for TFG-β as inducers of gremlin (bone morphogenetic protein antagonist expression), in human DN in this context (Dolan et al., 2005).

The expression of α smooth muscle actin (α-SMA), a marker of myofibroblast in kidney of diabetic rats and its role in pathogenesis of DN. At early stage of diabetes, increased (α-SMA) in kidney suggests the formation of myofibroblast, which may be involved in accumulation of C-IV and contributed to DN (Zhejiang et al., 2005).

They report the case of a 46 year old man with a 16 year history of type I diabetes mellitus that developed rapid onset of nephrotic syndrome. Renal biopsy revealed DN, characterized by thickened GBM, mild nodular glomerulosclerosis, and focal arteriolar hyalinization. Immunofluorescent (IF) studies showed strong granular IgM staining along glomerular loops, with subepithelial and intramembranous immune complex deposits along glomerular capillary loops demonstrated by electron microscopy (EM). These findings are consistent with membranous glomerulopathy with IgM as the predominant immunoglobulin. In addition, there were large aggregates of electron dense material composed of numerous ring or spherical particles, ranging from 200 to 400 nm, in Bowman's space, which corresponded to eosinophilic aggregates on light microscopy (LM) and strong IgM stained materials by IF studies (Zhang et al., 2005).

Renal interstitial and in particular glomerular collagen VI protein expression was significantly up regulated in experimental models of type 1 and type 2 mellitus as well as in human DN. The data and the expression pattern of collagen support a specific up regulation of the microfibrillar collagen type VI in diffuse diabetic glomerulosclerosis (Kuhlmann et al., 2004).

Type 8 collagen is a non fibrillar short chain collagen, which was first described in vascular and corneal endothelial cells. Two distinct chains, α1 (8) and α2 (8), have been identified. In healthy kidney α1 (8) collagen has been localized to the glomerular capillary loops, the messangium and to the subendothelium and the tunica media of large intrarenal arteries. DN leads to glomerular matrix accumulation, progressive tubular atrophy and interstitial fibrosis. The precise composition of these matrices and the pathogenetic
mechanism of the processes are far from understood. To investigate the potential role of type 8 collagen in DN, they investigated the expression profile of Col8a1 and Col8a2 mRNA in mouse mesangial, tubular and glomerular cells in response to high glucose. Their data suggest that high glucose up regulates Col8a1 in the kidney. Since it has been previously shown that type8 collagen regulates growth of smooth muscle cells during vascular remodeling, similar pathophysiological changes may contribute to the pathogenesis of DN (Hopfer et al., 2004).

Glomerular hyperfiltration has been shown a leading cause and promoter of albuminuria and progressive renal impairment in type 1 diabetes mellitus. Adenosine reuptake inhibition is novel therapeutic concept in controlling glomerular hyperfiltration in DN. In the kidney, the adenosine reuptake inhibitor dipyridamole induces selective preglomerular vasoconstriction and attenuates glomerular hyperfiltration and urinary albumin excretion in experimental diabetes (Heyne et al., 2004).

Tubular atrophy is a feature of DN and is associated with loss of renal function. The isoform of the PKC family are activated in response to hyperglycemia and might be important in the hyperglycemia mediated tubular apoptosis (Menne et al., 2004).

DN is a serious complication of diabetes associated with poor prognosis and progress to ESRD. Increased urinary excretion of protein and albumin are early clinical markers for diabetic renal disease and an increased risk for cardiovascular disease. Diabetes causes activation of the renal endothelin system inducing renal damage. They analyzed the effects of SLV306, an inhibitor of both neutralendopeptidase and endothelin converting enzyme on the progression of DN. SLV306 decreases renal matrix protein content as well as protein and albumin excretion in diabetic rats in a blood pressure independent manner similarly to ACE-inhibition (Simon et al., 2004).

Multiple regression analysis showed that CCS is significantly associated with urinary AER as well as age, duration of diabetes and serum creatinine (R(2) = 0.31), while Aortic calcification score(ACS) is strongly associated with age, smoking, serum creatinine, systolic blood pressure and low density lipoprotein cholesterol level (R(2) = 0.29). Increased urinary albumin excretion is associated with coronary arterial calcification in diabetic patients (Yamagami et al., 2005).

To discover whether islet sympathetic nerves are damaged during the autoimmune destruction of islet β cells, they immunostained sections of pancreas from BB diabetic rats, using antibodies against vesicular monoamine transporter 2 (VMAT2), marker of sympathetic nerve terminals. They found a marked decrease in VMAT2-positive fiber area in the islets of BB rats that had been diabetic for only 1-2 weeks compared with their non diabetic controls, in contrast, there was no decrease in the VMAT2–positive fiber area in the exocrine pancreas in these early diabetic BB rats (Early selective and marked loss of sympathetic nerves from the islets of biobreeder diabetic rats) (Mei et al., 2002).

In patients with DN, anaemia is more severe and is seen earlier than in patients with non-diabetic renal disease. Use of recombinant human erythropoietin (rhEPO; epoetin) for the treatment of anaemia is well established (Ritz, 2005).

Role of nitrosive and oxidative stress

The plasma levels of NO in diabetic rats were low compared to those in control rats. The hippocampal NO levels in both diabetic rats were almost same as those in control rats, while the levels of 5-HT and dopamine were low in diabetics. A sudden decrease in the plasma glucose level due to insulin administration reduced the NO level as well as enhanced the 5-HT level in the diabetic hippocampus, a finding consistent with the result of 7 days
administration of insulin. Taken together, these findings suggest that changes in the plasma glucose level cause, at least in part, the changes in the levels of NO and monoamines in the diabetic brain (Kino et al., 2004). In early DN the blood flow is increased in both the superficial and deep cortices, and NO plays an important role in regulating the Cortical Blood Flow during the development of DN (Nakanishi et al., 2005). NO-PKG pathway inhibits AGE-induced proliferation by suppressing activation of JAK2-STAT5 and cyclin D1/cdk4 and induction of p21 (Waf1/Cip1) (Huang et al., 2005).

In contrary to above findings, NO appears to be an attractive mediator of glomerular hyperfiltration as it has a predominant action of decreasing preglomerular vascular tone (Deng and Baylis, 1993) and increased NO metabolites in the urine of STZ diabetic rats has been described (Bank and Aynedjian, 1993; Tolins et al., 1993). Inhibition of intrarenal NO synthase (NOSes) with L-arginine analogues leads to increased afferent arteriolar resistance and a decrease in $K_e$ (Deng and Baylis, 1993). Increased renal NO production and/or activity have been associated with glomerular hyperfiltration, vascular permeability and modulation of glomerulosclerosis and tubulointerstitial fibrosis. Several studies demonstrated regulated expression of NOSes in experimental DN. However, the regulation of NOSes and its clinical relationship in DN in man is still not yet specified. This study demonstrates significantly increased eNOS expression in kidney biopsies from patients with histologically proven DN. Expression of eNOS as well as iNOS was in addition related to clinical risk factors of disease progression (Hohenstein et al., 2004).

Chronic treatment with diltiazem in diabetic rats significantly attenuated both renal dysfunction and markedly prevented diabetic-induced renal morphological alterations. The present study suggests that oxidative stress/nitrosative stress is increased in the diabetic kidney and calcium channel blockage can prevent these changes. The results also suggest that in STZ-induced diabetic rats, the protective action of diltiazem might be mediated, at least in part, by its effect on tissue oxidant/antioxidant status (Anjaneyulu and Choprae, 2005).

The principal intracellular reductant is NADPH whose production is mainly dependent on glucose-6-phosphate dehydrogenase (G6PD) activity. In cultured cells previously showed that high glucose caused activation of PKA and subsequent phosphorylation and inhibition of G6PD activity and hence decreased NADPH. Chronic hyperglycemia caused inhibition of G6PD activity via decreased expression and increased phosphorylation of G6PD, which therefore led to increased oxidative stress. PKA activity and serine phosphorylation of G6PD were significantly increased. NADPH levels and glutathione levels were significantly decreased in diabetic kidneys as compared to control. Lipid peroxidation was significantly increased which correlated with decreased G6PD activity ($r=0.48$). G6PD expression was significantly reduced which correlated with decreased G6PD activity ($r=0.72$) (Xu et al., 2005).

Glutathione peroxidase concentrations are decreased in plasma, urine, and glomeruli of patients and experimental rats with DN (Chiu et al., 2005). Their data indicates that hemodialysis patients with type 2 DN may benefit from statin therapy irrespective of baseline LDL-cholesterol level. Patients with LDL > 100-mg/dl benefit most when treated with HMG-CoA-reductase inhibitor (CSEI) (Gotz et al., 2005). Increased lipid peroxidation and decreased antioxidant enzymes in plasma may play a role in the progression of DN. Simvastatin may ameliorate these changes to protect kidney from oxidative lesion in diabetes even in the absence of lipid abnormalities (Karger et al., 2005). CSEI is already recommended for tight control in diabetics because of the high cardiac risk in these patients. Statins may have additional unique benefits independent of lipid lowering. In animal models of DN, statin treatment blocks intracellular signaling and decreases the mRNA expression of...
TGFB (Yokota et al., 1999). Statin treatment reduces glomerulus inflammation and injury in hypertensive nephrosclerosis (Klanke et al., 2004).

Diabetes mellitus is associated with altered iron homeostasis in both human and animal diabetic models. Iron is a metal oxidant capable of generating reactive oxygen species (ROS) and has been postulated to contribute to DN. Two proteins involved in iron metabolism that are expressed in the kidney are the divalent metal transporter, DMT1 (Slc11a2), and the Transferrin Receptor (TfR). Thus, they investigated whether renal DMT1 or TfR expression is altered in diabetes, as this could potentially affect ROS generation and contribute to DN. Together these data demonstrate renal DMT1 downregulation and TfR upregulation in STZ-diabetes. Whilst the consequence of altered DMT1 expression on renal iron handling and oxidant damage remains to be determined, the attenuation of the putative lysosomal iron exit pathway in proximal tubules could potentially explain lysosomal iron accumulation reported in human diabetes and STZ-diabetic animals (Ward et al., 2005).

In many diseases, including progressive renal disorders, tissue injury and pathological intracellular signaling events are dependent on oxidative stress. Glutathione peroxidase-1 (Gpx1) is an antioxidant enzyme, which is highly expressed in the kidney and removes peroxides and peroxynitrite that can cause renal damage. Therefore, they examined whether this abundant renal antioxidant enzyme limits renal damage during the development of type 1 DN. Contrary to expectations, this study has shown that the high level of Gpx1 expressed in the kidney is not protective against the development of renal oxidative stress and nephropathy in a model of type 1 diabetes. (De haan et al., 2005)

**Role of PPAR**

PPARgamma nuclear receptors are expressed in renal glomerular tissue and in vascular walls, thus participating through various and complex mechanisms, to glomerular and vascular sclerosis and to nephropathy development and progression. Glitazones, pharmacological agonists of nuclear receptor PPARgamma, in experimental models, either in vitro, or in vivo in animal models, have demonstrated their favorable effects on arterial blood pressure and on prevention and/or progression of DN (Deray et al., 2005).

DN progresses relentlessly to ESRD. Animal experiments have found that peroxisome proliferator activated receptor-gamma (PPAR-gamma)-based therapy can have a glucose independent effect on renal protection. They hypothesized that PPAR-gamma-based anti-diabetic therapy would result in greater reduction in proteinuria compared to sulfonylurea-based therapy. The glipizide group had an adjusted mean increase in proteinuria of 6.1% (95% CI -11.7%, 23.8%), whereas the pioglitazone group had a reduction of 7.2% (95% CI -24.9%, 10.6%). The adjusted reduction with pioglitazone of 13.2% (95% CI -38.4%, 11.9%) was not statistically significant (P= 0.294). Baseline proteinuria, diastolic ambulatory blood pressure, and serum albumin concentration were independent predictors of reduction in proteinuria. The frequency and patterns of adverse events were similar in the two groups. In patients with advanced DN, we found no reduction in proteinuria over 4 months. These data are useful to design larger studies with longer duration of follow up to demonstrate renal protection of PPAR-gamma agonists (Agarwal et al., 2005).

PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors. Three PPAR isoforms, designated PPARalpha, -beta/delta, and gamma, have been identified and attracted enormous attention due to the key role these receptors play in regulating adipogeneses, lipid metabolism, insulin sensitivity, inflammation and blood pressure. Growing evidence points to a causative relationship between PPAR activity and the metabolic syndrome, including insulin resistance, glucose intolerance or type II diabetes,
Review of Literature

obesity, dyslipidemia, hypertension, atherosclerosis, and albuminuria. Importantly, both PPARalpha activators such as fibric acid class of hypolipidemic drugs and PPARgamma agonists including antidiabetic TZDs have been proved to be effective for improving metabolic syndrome. All three PPAR isoforms appear to play important roles in the development of type II diabetes and DN. Accumulating data has begun to emerge suggesting PPARs may serve as potential therapeutic targets for treating the metabolic syndrome and its related complications. Here we review the literature pertaining to the action, ligand selectivity and physiological role of PPARs. Particular emphasis is placed on their pathogenic roles in the metabolic syndrome and the therapeutic utility of PPAR modulators in the treatment of type II diabetes. (Zhang et al., 2005)

Proteomic analysis with CE-MS coupling permits fast and accurate identification and differentiation of polypeptide patterns in the urine of patients with IgAN, allowing differentiation from healthy controls and, probably, other renal diseases. (Haubitz et al., 2005).

Environmental factors

Low birth weight has been associated with retardation of renal development, a reduced number of nephrons and an increased risk of systemic and intraglomerular hypertension (Brenner and Chertow, 1994). Low birth weight and intrauterine growth retardation have also been linked to cardiovascular risk factors and an increased risk of developing DN (Rossing et al., 1995; Rudberg et al., 1998). These findings support that perinatal factors may contribute to the development of DN. Physical activity may have a beneficial effect in preventing or delaying macrovascular diabetic complications (LaPorte et al., 1986). Moreover, an impaired aerobic work capacity has been shown in patients with type 1 diabetes with incipient as well as overt nephropathy compared to those with normal renal function (Jensen et al., 1998). Other potential environmental factors include diet and tobacco use and they are described in the section on clinical management.

Genetic Factors

Diabetes remains the number one cause of ESRD worldwide. Only about one third of diabetic patients develop nephropathy, and the risk appears to be, in part, genetically determined. In this article, we review clinical and genetic markers for the development and progression of DN. Microalbuminuria remains the best available predictor of the subsequent development of nephropathy, even though in recent years it has become clear that less than 50% of individuals with type 1 diabetes progress to overt proteinuria over a period of less than 10 years. It is of great interest for early recognition of risk of nephropathy that small elevations in night time blood pressure predict microalbuminuria in type 1 diabetes (William et al., 2005).

A number of observations suggest that there may be an important hereditary component in the etiopathogenesis of DN. Epidemiological studies have shown that the incidence of DN peaks in the second decade of disease and after 25 years the risk rapidly declines, which is in contrast to other diabetic complications. The concept of a genetic predisposition for development of DN has therefore been postulated (Krolewski et al., 1985). This is supported by studies that have shown familial clustering in different populations (Seaquist et al., 1983; Borch-Johnsen et al., 1992; Quinn et al., 1996) suggesting a fourfold increase in risk of development of nephropathy in type 1 diabetic subjects whose type 1 diabetic siblings have nephropathy. A similar phenomenon has been observed among Pima
Indians and African-Americans with type 2 diabetes, but this could also be a result of shared environmental factors. These patterns of incidence suggest that nonmetabolic factors are influential in the development of nephropathy. Some studies have demonstrated that a predisposition to hypertension (Barzilay et al., 1992; Fagerudd et al., 1998) and a family history of cardiovascular disease and type 2 diabetes (Rudberg et al., 1998) may be associated with increased risk of developing DN. Parental history of hypertension and coronary heart disease are found more commonly in patients with DN than in diabetic subjects who do not develop this complication (Viberti et al., 1985).

This also suggests a genetic association to the metabolic syndrome. Ethnic differences in the risk of developing diabetic renal disease have also been described (Cowie et al., 1989; Burden et al., 1992) supporting that genetic background has an impact. Genetic loci involved in susceptibility to these conditions are, therefore, possible determinants of inherited susceptibility to nephropathy. Genetic markers for DN have not been conclusively identified. Genes involved in the regulation of blood pressure, glucose and lipid metabolism as well as in renal embryonic development have been regarded as potential candidate genes in DN. The most extensively examined gene polymorphism in the RAS is the insertion/deletion (I/D) polymorphism in the ACE gene. The results of previous studies on DN have been somewhat conflicting, but the progression of nephropathy may be faster in the group homozygous for the D allele (Parving et al., 1996) (Fujisawa et al., 1998). Other polymorphisms in RAS genes have also been investigated and a mutation in the angiotensinogen (M235T) and in the AT II receptor (A1166C) genes may slightly increase risk of developing DN (Doria et al., 1997).

A polymorphism in the aldose reductase gene, that may be important in the regulation of the polyl pathway, has been associated with an increased risk of DN (Heesom et al., 1997). There are also potential candidate genes involved in the metabolism of AGEs that are being investigated in ongoing studies (Doria et al., 1998).

An emerging new paradigm is the role of genes involved in organ development, i.e. oncogenic genes. Normal renal growth and development depend on a precise, complex and tightly regulated interplay between a number of regulatory growth promoters and inhibitors. These genes may also play a role in the development and repair of tissue injury, for example in DN (Lappin et al., 2000).

The occurrence of renal events in diabetic patients, however, appears to be influenced by the ACE genotype, with a dominant deleterious effect of the D allele (D/D or I/D) versus I/I genotype. Some patients with the DD genotype also appear less susceptible to the renoprotective effects of conventional doses of ACE-I, suggesting that ACE genotyping might be useful in selecting those patients that could benefit from higher doses of ACE-I and more aggressive treatment to prevent or delay disease progression. Predicting the development of DN and its progression (William et al., 2005). Genes controlling the expression of the RAS have received particular attention in this respect. Disturbance of the RS is observed in DN with increased levels of plasma rennin, prorenin, ACE and AT II. An insertion (I)/ deletion (D) polymorphism in the ACE gene has been identifies that is strongly associated with raised circulating ACE levels and with increased risk of coronary artery disease in non-diabetic individuals. Some studies have found the DD genotype to be associated with an increased risk of DN and a rapid decline of GFR in both type 1 and type 2 diabetes (Yoshida et al., 1996). The clinical implications are yet to be explored. Other genetic loci that may be involved include the sodium-lithium exchanger and the sodium-hydrogen antiporter genes (Foggensteiner et al., 2001).

The ACE insertion/deletion polymorphism has been examined for association with DN over the past decade with conflicting results. To clarify this situation, we conducted a
comprehensive meta analysis encompassing all relevant studies that were published between 1994 and 2004 and investigated this potential genetic association. The results of our meta analysis support a genetic association of the ACE Ins/Del polymorphism with DN. These findings may have implications for the management of DN using ACE-I especially among type 2 diabetic (Ng et al., 2005).

In kidney, Alpha8 integrin chain is expressed in glomerular messenger cells. Deletion of alpha8 gene in mice developed more severe glomerular lesions and causes deposition of collagen 4 after onset of STZ diabetes than wild types. Thus alpha8 seems to play a protective role for the maintenance of functional and structural integrity of the glomerulus during the development of DN (Hartner et al., 2004).

Apolipoprotein E (APOE) genetic variation has been implicated in DN. APOE allelic associations with chronic kidney disease beyond DN are unknown. Epsilon4 decreased risk of ESRD while Epsilon2 was associated with a decline in renal function. The epsilon 2 allele increase and the epsilon4 allele decrease risk. APOE variation predicts chronic kidney disease progression, independent of diabetes, race, lipid, and nonlipid risk factors. Their study suggests that nonlipid mediated pathways, such as cellular mechanisms of kidney remodeling, may be involved in the association of APOE alleles and progression of chronic kidney disease (Hsu et al., 2005).

In this study, variations in the frequencies of the apo epsilon4 allele and the apo E3/E4 and E4/E4 genotypes were found in patients with NIDDM, GN and ADPKD. This result may be a consequence of the effects of the apo epsilon4 and epsilon2 alleles on P-cholesterol and remnant lipoprotein levels. The decreased frequency of apo E3/E4 found in patients with NIDDM may be explained by the fact that the epsilon4 allele gives renoprotection against DN by lowering plasma remnant lipoprotein levels. Conversely, there may be an association between the apo E3/E4 genotype and the epsilon4 allele in patients with ADPKD, due to the effect of the epsilon4 allele in elevating P-cholesterol levels. The most plausible explanation for the absence of the apo E4/E4 genotype and the lower prevalence of the epsilon4 allele in patients with GN, which is known to result in a higher P-cholesterol compared to the epsilon2 and epsilon3 alleles, ought to be an increase in cardiovascular morbidity, which is known to be associated with a higher P-cholesterol level (Roussos et al., 2004).

Accumulating evidence supports intrinsic genetic susceptibility as an important variable in the progression of DN in people. Mice provide an experimental platform of unparalleled power for dissecting the genetics of mammalian diseases; however, phenotypic analysis of diabetic mice lags behind that already established for humans. Standardized benchmarks of hyperglycemia, albuminuria, and measurements of renal failure remain to be developed for different inbred strains of mice. The most glaring deficiency has been the lack of a diabetic mouse model that develops progressively worsening renal insufficiency, the since DN in humans. Differences in susceptibility of these inbred strains to complications of diabetes mellitus provide a possible avenue to dissect the genetic basis of DN; however, the identification of those strains and/or mutants most susceptible to renal injury from diabetes mellitus is lacking. Identification of a mouse model that faithfully mirrors the pathogenesis of DN in humans will undoubtedly facilitate the development of new diagnostic and therapeutic interventions (Breyer et al., 2005).

Diabetes is the leading cause of ESRD because DN develops in 30 to 40% of patients. DN does not develop in the absence of hyperglycemia, even in the presence of a genetic predisposition. Multigenetic predisposition contributes in the development of DN, thus supporting that many factors are involved in the pathogenesis of the disease. Hyperglycemia induces renal damage directly or through hemodynamic modifications. It induces activation
of protein kinase C, increased production of AGEs, and diacylglycerol synthesis. In addition, it is responsible for hemodynamic alterations such as glomerular hyperfiltration, shear stress, and microalbuminuria. These alterations contribute to an abnormal stimulation of resident renal cells that produce more TGF-β. This growth factor up regulates GLUT-1, which induces an increased intracellular glucose transport and D-glucose uptake. TGF-β causes augmented extracellular matrix protein deposition (collagen types I, IV, V, and VI; fibronectin, and laminin) at the glomerular level, thus inducing mesangial expansion and GBM thickening. However, low enzymatic degradation of extracellular matrix contributes to an excessive accumulation. Because hyperglycemia is the principal factor responsible for structural alterations at the renal level, glycemic control remains the main target of the therapy, whereas pancreas transplantation is the best approach for reducing the renal lesions (Schena and Gesualdo, 2005).

Metabolic consequences

Most studies on the metabolic and endocrine alterations during the development of renal failure have been performed in non-diabetic patients rather than those with DN. However, most of the findings can probably be generalized also to patients with DN, but further investigations on the interaction between diabetes and early renal impairment and the uremic state, respectively, are needed.

Insulin clearance

Approximately 40-50% of the insulin secreted by the pancreatic β-cells is removed by the liver during its first passage (Ferrannini et al., 1983). The kidneys degrade 30-40% of the insulin entering the systemic circulation while the remainder is cleared via degradation in various tissues, mainly muscle and adipose tissue. In total, renal degradation accounts for ~20% of whole body insulin removal (Rubenstein et al., 1975). Insulin is freely filtered by the glomerulus and ~60% is eliminated by glomerular filtration and the remaining ~40% by uptake from the peritubular capillaries into tubular cells where insulin is degraded (Ferrannini and De Fronzo, 1984). Insulin present in the tubular lumen is reabsorbed and also degraded in the tubular cells. Only 1% of the filtered insulin is excreted with the urine. Renal plasma clearance of insulin is about 200 ml/min. In renal failure, the reduction in GFR can be compensated by an increase in the uptake from the peritubular capillaries and until GFR falls to 15-20 ml/min, no reduction in insulin clearance has been detected. At lower GFR, renal clearance of insulin and probably also liver clearance, become drastically reduced (Rabkin et al., 1970).

Insulin action on glucose metabolism

It is considered that peripheral insulin resistance, with respect to glucose utilization, is mainly located in skeletal muscle (DeFronzo and Alvestrand, 1980). It has been reported that in renal failure it occurs when GFR becomes markedly reduced, i.e. <40 ml/min/1.73((DeFronzo and Alvestrand, 1980; DeFronzo et al., 1981; Eidemak et al., 1995). A decreased maximal response to insulin in vivo, suggesting a defect at the post insulin-receptor level, has been shown in patients with severe renal failure (Schmitz et al., 1985). Similar results were seen in studies in adipocytes from uremic subjects (Pedersen et al., 1985). The mechanisms for insulin resistance in severe renal failure are probably multifactorial. In addition to the accumulation of uremic toxins, there are several other
abnormalities, such as impaired physical fitness, altered body composition, metabolic acidosis, hyperosmolality and medication that may contribute (Eidemak et al., 1995; Alvestrand, 1997). Abnormalities in circulating hormones or neuroendocrine pathways and markers of inflammation, e.g. cytokines, may also promote insulin resistance (Hotamisligil et al., 1995). Anemia can also be a factor contributing to insulin resistance, as patients with chronic anemia have been shown to be insulin resistant and one study (Mak, 1996) has shown beneficial effects on insulin resistance following erythropoietin treatment and correction of anemia in patients with renal failure.

Interestingly, insulin resistance has also been found in patients with polycystic kidney disease despite normal GFR. Even though the mechanism leading to insulin resistance in these patients remain unclear, it is suggested that it may be directly related to the structural protein and membrane alterations occurring in this disease (Tong et al., 1995; Vareesangthip et al., 1997). Many studies in patients with diabetes have demonstrated that chronic hyperglycemia, per se, may induce glucotoxicity and impair insulin sensitivity (Yki-Jarvinen et al., 1984; Leslie et al., 1997). Near normoglycemic diabetes control and intensive treatment with insulin improve but do not fully restore insulin action (Yki-Jarvinen and Koivisto, 1984). In human diabetes an elevated endogenous glucose production is mainly due to a high rate of gluconeogenesis which occurs in the liver (Gerich, 1988). However, studies have shown that the kidneys play a role in glucose counter regulation (Meyer et al., 1999) as they have capacity for both glycogen formation and gluconeogenesis, and this may be compromised in renal failure. This observation may be clinically relevant since patients with advanced DN seem to be more prone to develop severe hypoglycemia (Arem, 1989). The kidneys also account for a small proportion, less than 10%, of the overall insulin-mediated glucose metabolism, but it is not known whether this is impaired in renal failure (Meyer et al., 1999).

**Lipid metabolism**

In both type 1 and type 2 diabetes with renal involvement, even in the earlier stages of renal disease, various abnormalities in plasma concentrations of lipoproteins have been described. DN is associated with an altered lipid profile characterized by elevated triglyceride rich lipoproteins, in particular very low density lipoprotein (VLDL), but also low density lipoprotein (LDL) and thus, plasma triglycerides are high. The levels of high density lipoprotein (HDL) are low as a secondary phenomenon (Attman et al., 1998). Elevated plasma concentrations of apolipoprotein B, apoC-III and lipoprotein (a) have also been reported (Groop et al., 1996; Attman et al., 1998). However, there still seems to be uncertainty on the underlying mechanisms, but changes in lipoprotein lipase (LPL) and hepatic lipase (HL) have been suggested. An increased HL-activity and a reduced post heparin plasma LPL/HL ratio have been reported (Groop et al., 1996). These multiple lipoprotein alterations become more accentuated with declining renal function and increasing urinary albumin excretion (Jensen et al., 1988a). When compared with non-diabetic patients with renal failure, the lipid abnormalities are more marked in DN probably reflecting an additional effect of the diabetic state and in particular the level of glycemia and the relative insulin deficiency (Attman et al., 1992). The lipid disorders seen in chronic renal failure resemble those seen in the metabolic syndrome and it has also been speculated that the insulin resistance seen in uremic patients may also be associated with some of the observed lipid disorders (Rigalleau et al., 1997).

The total and LDL cholesterol levels usually are normal or slightly elevated in renal failure and the diameter of LDL particles has been reported to be smaller, i.e. small dense
LDL, in patients with both incipient and overt DN. Small dense LDL are more readily oxidized and glycosylated (Bonnet and Cooper, 2000) and they are also more deleterious to vessel walls than normal, larger LDL-particles. LDL cholesterol seems to have a similar effect on glomerular mesangial cells as on endothelial cells. Mesangial cells are closely related to vascular smooth muscle cells and possess binding sites for LDL and oxidized LDL. They help recruit macrophages and then secrete proliferative factors inducing glomerulosclerosis, i.e. a process similar to the role of endothelial cells in the process of atherosclerosis (Schlondorff, 1993). Finally, the lipid lowering agents, statins, appear to have a beneficial effect on mesangial cells independent of their cholesterol lowering effect (Grone et al., 1994). Another factor that may reduce insulin sensitivity is an elevated level of NEFA (Boden, 1996). However, NEFA levels are not generally elevated in chronic renal failure (Schmitz et al., 1985) and the antilipolytic effects of insulin in uremic patients are comparable with those in healthy subjects (Schmitz, 1988).

### Hormones

Alterations in the levels of insulin-antagonistic hormones and cytokines during the development of DN and renal failure could potentially contribute to insulin resistance and to the changes in glucose and lipid metabolism. There are several potential pathogenic mechanisms leading to endocrine perturbations during the development of renal failure. There can be a decreased renal and extrarenal hormones production of, i.e. erythropoietin, vitamin D₃ and testosterone, but also alterations in the conversion of prohormones into active hormones, e.g. reduced conversion of triiodothyronine (T₃) into tetraiodothyronine (T₄) and activation of vitamin D₃. An increase in hormone production can be a result of an adaptive response to homeostatic signals, for example the increase in parathyroid hormone (PTH) in response to hypocalcemia. An increased hormone level may also be the result of an impaired degradation both in renal and extrarenal tissue, which could be true for insulin. Changes in the activity of hormones may be due to alterations in isohormon profile, glycosylation, alterations in the levels of binding proteins, e.g. IGFBP, occurrence of inhibitors, altered receptor function (Tonshoff et al., 1995), and there may also be post receptor defects as for insulin. Moreover, there may also be alterations in circadian rhythm of hormone profiles in plasma (Schmitz, 1984).

### Glucagon

Elevated levels of glucagon are detected in uremic subjects (Schmitz, 1988). However in patients with long standing type 1 diabetes a reduction in the secretion of glucagon has been found and the glucacon response to hypoglycemia is subnormal or absent (Bolli et al., 1983). This may partly be due to a high level of circulating insulin (Liu et al., 1992) and the glucagon response could, although not completely, be restored by continuous intraperitoneal insulin infusion (Oskarsson et al., 2000). Insulin resistance was not altered in uremic patients when using somatostatin to suppress endogenous insulin, glucagon and GH release thus implying that insulin resistance in uremia is not likely to be due to acute effects of glucagon or GH (Alvestrand et al., 1989). The fact that insulin sensitivity is improved by dialysis treatment also argues against a major role of GH or glucagon, since dialysis therapy does not alter the levels of these hormones (Schmitz et al., 1985).
Parathyroid hormone

Early in renal failure there is an increase in PTH levels, i.e. secondary hyperparathyroidism due to both slight hypocalcemia and phosphate retention but also a decline in conversion of vitamin D₃ into its active forms. High PTH has therefore been suggested as a mechanism producing insulin resistance (DeFronzo et al., 1973). Experimental data indicated that PTH can impair insulin secretion but it had no effect on insulin sensitivity assessed with the glucose clamp technique (Fliser et al., 1997). On the other hand, intravenous administration of vitamin D, that can reduce PTH levels, improved both insulin secretion and insulin sensitivity in patients on hemodialysis (Mak and DeFronzo, 1992). Both animal and human studies have suggested that D-vitamin per se may influence insulin sensitivity but the findings have been conflicting (Rudnicki and Molsted-Pedersen, 1997; Taylor and Wise, 1998; Huang et al., 2002) and the role of vitamin D is not yet established.

Glucocorticoids

Cortisol is filtered by the glomeruli and nearly totally reabsorbed by the kidney and less than 1% of filtered cortisol appears intact in the urine. Instead, cortisol undergoes both conversion and conjugation in the liver resulting in the excretion of cortisol and cortisone metabolites in the urine. Cortisol is also inactivated in the kidney by conversion to cortisone via the action of the type 2 isoform of 11β-hydroxysteroid dehydrogenase. Patients with renal failure have reduced levels of urine cortisol, but both normal and elevated circulating levels of cortisol have been reported (Heaton et al., 1985 Schmitz et al., 1985). The diurnal variation, however, seems to be preserved (Heaton et al., 1985). Dexamethasone-induced suppression of plasma cortisol was reported to be normal (Barbour and Sevier, 1974) or blunted (Wallace et al., 1980). An essentially normal response to ACTH stimulation has been found both using a standard (250 μg) and a low-dose (1μg) ACTH test in patients with renal failure (Clodi et al., 1998). It can not be ruled out that a tissue specific dysregulation of cortisol metabolism that is linked to insulin resistance may be present in renal failure, as was previously reported in obesity (Rask et al., 2001).

Sex hormones

In male patients with renal failure, plasma testosterone levels are decreased or in the low normal range, despite an increase in gonadotropins, i.e. luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Prem et al., 1996). This may be the result of a combined abnormality including a decrease in the strength of the gonadotropin releasing hormone (GnRH)-pulse, altered feedback mechanism at the hypothalamic-pituitary level and a peripheral Leydig-cell resistance with moderately decreased testosterone production despite elevated gonadotropin levels (Veldhuis et al., 1993). Low levels of androgens in men are associated with cardiovascular risk factors and also insulin resistance (Marin et al., 1992; Jansson et al., 2002). There is also a decreased metabolic clearance of GnRH, LH, FSH and prolactin. Hyperprolactinemia in renal failure is partly induced by a decreased metabolic clearance but also by autonomic overproduction and can contribute to the reduction in testosterone levels (Palmer, 1999). Similar changes, i.e. elevated levels of LH, FSH and prolactin and reduced levels of oestradiol, have also been found in girls with renal failure (Ferraris et al., 1987).
The autonomic nervous system and catecholamines

Increased concentrations of circulating catecholamines have been demonstrated in patients with advanced renal failure (Darwish et al., 1984). Plasma norepinephrine levels have also been reported to be elevated (Ziegler et al., 1990), however, levels of epinephrine do not appear to be generally increased (Cuche et al., 1986). Autonomic neuropathy is also a prevalent finding in uremic patients. Studies have shown that it can be improved by long term dialysis treatment or kidney transplantation (Vita et al., 1996; Yildiz et al., 1998). Autonomic neuropathy also is a well known complication among patients with diabetes and these abnormalities may be accentuated when renal failure evolves. An imbalance between sympathetic and parasympathetic nervous activity may potentially contribute to insulin resistance and perhaps a relative inactivity in the parasympathetic nervous system is the most important factor (Laitinen et al., 1999; Lindmark et al., 2003).

Adipose tissue hormones and cytokines

Leptin is a hormone that is produced and released by the adipose tissue and leptin levels are associated with obesity and insulin resistance (Girard, 1997). Similar to several other peptide hormones, leptin becomes elevated with renal impairment probably due to a decrease in renal clearance occurring via glomerular filtration followed by metabolic degradation in the renal tubular cells (Cumin et al., 1996). This is supported by the finding that leptin levels in renal failure are elevated despite a lower leptin gene (ob gene) expression (Nordfors et al., 1998). Leptin is associated with plasma insulin levels, sympathetic nervous activation as well as obesity and may through different pathways be associated with insulin resistance (Stenvinkel et al., 1999).

Adiponectin and resistin are two other hormones, secreted from adipose tissue, both of which may affect insulin resistance (Yamauchi et al., 2001; Steppan et al., 2001). Resistin seems to impair insulin action and induce glucose intolerance. Adiponectin levels, on the other hand, seem to be associated with high insulin sensitivity and the hormone may have a protective role on the vascular endothelium but it has also been reported to be elevated in patients with severe renal failure (Zoccali et al., 2002). Serum adiponectin concentrations are increased in type 1 diabetic patients with nephropathy and levels are further associated with renal insufficiency (Saraheimo et al., 2005).

Positive correlations between urinary adiponectin levels and albumin to creatinine ratios were observed in IgA-nephropathy and DN patients, but not in control subjects. These findings suggested that the increase of urinary adiponectin levels partly results from enhanced filtration of circulating adiponectin through the changes of glomerular permselectivity and intraglomerular hydraulic pressure (Shimotoma et al., 2005). The 94T/G polymorphism in the adiponectin gene is not associated with diabetic complications. The significance of a higher prevalence of the G allele in type 2, compared to type 1 diabetes remains to be clarified (Rudofsky et al., 2005).

Several cytokines are also secreted by the adipose tissue, for example TNF-α, IL such as IL-6 and IL-1. Previous studies have shown an association between TNF-α and insulin resistance (Hotamisligil et al., 1995). Elevated cytokine activity, e.g of IL-1 β, TNF-α and their specific inhibitors, has also been found in patients with renal failure and may be correlated to the serum creatinine level (Pereira et al., 1994). An interesting finding in this context is that TNF-α may be suppressed by ACE inhibition as shown both in vitro and in vivo (Stenvinkel et al., 2002). IL-6 could also potentially contribute to insulin resistance. IL-6 is also interesting since it may affect nutritional status, is proatherogenic and associated
with poor outcome in hemodialysis (Stenvinkel et al., 1999). Other endocrine alterations in nephropathy that potentially could influence insulin sensitivity include erythropoietin deficiency (Mak, 1996) and activation of the RAS (Stenvinkel et al., 1999; Pedersen-Bjergaard et al., 2001; Nordfeldt and Samuelsson, 2003), but at present their role is not clear. The mentioned endocrine perturbations seem to only partly be influenced by renal replacement therapy. Chronic hemodialysis or continuous peritoneal dialysis significantly improves insulin action in subjects with renal failure. This is not explained by changes in the levels of GH and glucagon, since they are not altered. Dialysis also increases insulin clearance at the hepatic level (Schmitz, 1985). In patients with kidney transplants, immunosuppressive medication exhibit adverse metabolic effects on glucose tolerance and lipid metabolism and this can counteract the beneficial metabolic effects of the improved renal function.

Most of the studies on endocrine perturbations in development of renal failure have been performed in patients with severe renal failure, ESRD or on renal replacement therapy. There are so far very few studies on endocrine alterations in patients with early renal disease.

3.3.2.2 Clinical management

Strategies for therapeutic interventions include primary prevention, in patients with diabetes and normoalbuminuria; secondary prevention, in patients with signs of incipient nephropathy, i.e. microalbuminuria; and tertiary prevention, in overt nephropathy to halt the development of end stage renal failure.

Glycemic control

Improved glycemic control has been shown to prevent the development of microalbuminuria in both type 1 and type 2 diabetes (Reichard et al., 1993; UKPDS 33, 1998; DCCT Research group, 1993) and it can also stabilize or normalize established microalbuminuria (Feldt-Rasmussen et al., 1986; Dahl-Jorgensen et al., 1988). Glycemic level is also important for the rate of progression of overt DN (Nyberg et al., 1987; Mulec et al., 1998). Some previous studies (Krolewski et al., 1995; Reichard, 1995) have suggested a glycemic threshold for the development of DN. However, no evidence for such a threshold was found in the DCCT, although the magnitude of the absolute risk reduction is greatest with reductions in HbA1c from a high level. Thus, there is still a clinically relevant risk reduction when HbA1c is reduced further towards the normal range (DCCT research group, 1996). On the other hand, an intensified glycemic control also increases the risk of hypoglycemia, particularly in type 1 diabetes. However, in the Diabetes Control and Complications Trial (DCCT) it was concluded that the problem of severe hypoglycemia with intensive insulin therapy is clearly outweighed by the reduction in microvascular complications (DCCT Research group, 1993).

A clinical observation in patients with DN is that glycemic control tends to deteriorate during the progression of nephropathy. To understand the pathophysiology with respect to glucose homeostasis, insulin action and kinetics is therefore important in order to optimize therapy. Some studies have suggested that rapid acting insulin analogues may be beneficial in patients with overt nephropathy (Rave et al., 2001) and in patients on hemodialysis treatment (Aisenpreis et al., 1999). In patients treated with continuous peritoneal dialysis, intraperitoneal insulin can be administered together with the dialysis fluid. Insulin may be delivered into the circulation in a physiological way with high levels in the portal system and lower peripheral insulin levels (Taylor et al., 1996). However, one problem is that the risk of infectious
peritonitis may be slightly increased (Wikdahl et al., 1997; Quellhorst, 2002). In patients with DN and dialysis treatment intraperitoneal insulin therapy may offer a better glycemic control and insulin sensitivity as compared to subcutaneous insulin. On the other hand, it was reported to reduce HDL levels, increase the LDL/HDL ratio significantly, and thus aggravate dyslipidemia (Nevalainen et al., 1997) and to predispose patients to hepatic steatosis (Nevalainen et al., 2000). Taken together, the present available results can not definitely prove whether insulin delivery via the subcutaneous or the intraperitoneal route is superior with respect to overall outcome.

Treatment of hypertension and microalbuminuria

Early in the 1980’s the progression of DN was shown to be postponed upon long term treatment with blood pressure lowering agents (Mogenson, 1982). A close correlation between arterial blood pressure and the rate of decline in GFR has also been shown in overt DN. Statistical analyses did not reveal a lowest threshold for the adverse effect of high systemic blood pressure on the fall in GFR (Rossing et al., 1993). Hemodynamic factors regulated by the RAS and AT II seem to play a central role in the development of DN, and hence ACE-I have been used in many trials. A meta analysis of trials with ACE-I in DN showed that these agents lead to a reduction in the risk of progression from microalbuminuria to overt nephropathy and they seem to be superior, in terms of renal protection, to other antihypertensive agents not targeting the RAS. Treatment with ACE-I also seemed to have long term effects with preservation of kidney function, i.e. GFR, over at least eight years (Mathiesen et al., 1999). Early intervention with ACE-I and blood pressure reduction already in normotensive patients with microalbuminuria has been shown to be beneficial both in type 1 and type 2 diabetes (Ravid et al., 1993; The EUCLID Study Group, 1997; Ravid et al., 1998).

During the last years, several clinical trials using ARBs in patients with diabetes have shown similar renoprotective effects as ACE-I, both in type 1 (Andersen et al., 2000) and, in particular, in type 2 diabetes (Lewis et al., 2001; Parving et al., 2001). The use of ACE-I and ARB can lead to renal protection in DN but still nephropathy may continue to progress but at a slower rate. A more effective blockade of the effects of AT II by reducing both synthesis and its binding to the AT-II type 1 receptor, i.e. a combination therapy with ACE-I and ARB has shown an additive effect on blood pressure and markers of renal function such as albuminuria (Mogensen et al., 2000). One of the reasons for employing “dual blockade” is that multiple pathways can generate AT II. It is debated whether the beneficial effects seen with ACE-I and ARB are due to blood pressure lowering, or if other effects of RAS inhibition are important in patients with DN. Clinical trials have also indicated that ACE-I and ARB may be more effective than traditional antihypertensive treatment in reducing the progression towards ESRD. These differences are not explained by different effects on blood pressure (Deferrari et al., 2002). It is nevertheless clear that treatment of hypertension is an important task in preventing and postponing the development of DN. The choice of antihypertensive agent(s) should be based on several factors including patient compliance, plausible side effects and other concomitant diseases. In the UKPDS trial more than 2/3 of the patients with type 2 diabetes and hypertension needed at least two antihypertensive agents to achieve a mean blood pressure of 144/82 (UKPDS 38, 1998). A combination of blood pressure lowering agents will thus be required in most hypertensive patients with type 2 diabetes.

In the Swedish national guidelines for diabetes care, ACE-I has been recommended as a first choice in patients with type 1 diabetes and signs of incipient or overt DN. A blood
pressure target $\leq 130/80$, or perhaps even lower, is recommended in these patients (SNBHW, 1999; Molitch et al., 2003).

The rate of synthesis for AT II seems to play an important role in initiation and progression of DN by affecting hemodynamic and non-hemodynamic mechanisms. In the future perhaps determination of I/D polymorphisms of the ACE gene or other candidate gene polymorphisms within the RAS may improve the evaluation of the individual risk profile and help us to individualize antihypertensive treatment.

**Dietary proteins**

It has long been known that GFR is influenced by protein intake and that a high protein intake worsens the clinical manifestations of ESRD. Several experimental studies in the last decades have also indicated a potentially harmful effect of dietary protein on renal function and structure in animals with renal lesions (Klahr et al., 1988) but it has been difficult to clinically demonstrate a beneficial action of protein restriction in the diet. However, a reduction of protein intake decreases GFR in hyperfiltrating young patients with type 1 diabetes (Rudberg et al., 1998). In 1996, a meta-analysis of clinical studies in diabetic and non-diabetic patients with chronic renal disease indicated that a low-protein diet (0.4-0.6 g/kg body weight) had beneficial effects on the reduction in GFR (Pedrini et al., 1996) and slowed the progression of both diabetic and non-diabetic renal disease. This was supported by a recent study (Hansen et al., 2002) where a moderate dietary protein restriction improved the renal prognosis in type 1 diabetic patients with progressive DN. In addition, it has been shown that a low-protein diet has beneficial effects on the alterations in insulin sensitivity and clearance in patients with chronic renal failure (Gin et al., 1989; Rigalleau et al., 1997). Because of the reduction in protein intake, the proportion of carbohydrates or fat must be increased. Interestingly, studies have shown that an increase in carbohydrates in the diet of patients with DN also was accompanied by an improved insulin action (Gin et al., 1989). However, when a low-protein diet is prescribed, the nutritional status of the patient must be carefully monitored and the glycemic control in patients with diabetes should be adequate since absolute or relative insulin deficiency leads to catabolism. Another interesting observation is that not only the amount of proteins but also the type of protein (Mollsten et al., 2001) may be of importance in the development of microalbuminuria, and, if confirmed, this might have implications for dietary advice in the future. Today a low-protein diet is only recommended in symptomatic uremia.

**Smoking and tobacco use**

Studies have shown that smoking is a risk factor for initiation (Christiansen, 1978) and also for the progression of DN (Sawicki et al., 1994; Ritz et al., 2000). Despite this, 35% of men and 29% of women, were smokers in a large European study on diabetic complications in patients with type 1 diabetes (Chaturvedi et al., 1995). Smoking patients with type 1 diabetes have higher insulin requirements, triglyceride levels and blood pressure, all features of insulin resistance, when compared with non-smoking patients (Madsbad et al., 1980; Poulsen et al., 1994). Cessation of smoking should be particularly important in patients with diabetes due to the high risk of developing both micro and macrovascular complications.
Lipid lowering treatment

Hypercholesterolemia plays an important role in aggravating renal damage in experimental diabetes in animals and this can be prevented by lipid lowering therapies (Kasiske et al., 1990). An independent association between hypercholesterolemia and rapid loss of renal function in patients with type 1 diabetes and DN has been demonstrated (Mulec et al., 1990; Hovind et al., 2001). Similar results are seen in patients with type 2 diabetes (Ravid et al., 1988). Intervention with the cholesterol lowering agents, statins, over a long period in type 2 diabetes slowed the decline in GFR and reduced albumin excretion (Lam et al., 1995; Tonolo et al., 1997). There are few trials in type 1 diabetes but in one trial lipid lowering treatment tended to have a beneficial effect on microalbuminuria (Fried et al., 2001).

Erythropoietin (EPO) treatment Anemia due to a relative erythropoietin deficiency may occur early in some patients with DN. The mechanisms for this have not been elucidated but an association with autonomic neuropathy (Bosman et al., 2001) or early renal interstitial damage has been suggested. Anemia in chronic renal failure is normally not observed until GFR drops to 20-40 ml/min. Many of these patients are also treated with ACE-I, which may cause a small decrease in serum erythropoietin level as a side effect (Kamper and Nielsen, 1990). Early EPO substitution is probably beneficial as anemia may contribute to insulin resistance (Mak, 1996) and left ventricular hypertrophy (Levin et al., 1999), both of which are strong risk factors for cardiovascular disease, and maybe also to the progression of renal disease (Rossert et al., 2002).

Vitamins

No alterations in vitamin D metabolism have been shown in patients with diabetes and normal renal function (Storm et al., 1983) even though an early loss of bone density has been observed in type 1 diabetes (Levin et al., 1976). A more pronounced bone loss is seen in uremic patients with diabetes compared to non-diabetic patients (Andress et al., 1987). In chronic renal failure an early elevation of PTH, partly due to hyperphosphatemia and hypocalcemia is seen (Reichel et al., 1991). The activation of 1-α hydroxylation of 25(OH) D-vitamin is reduced in renal failure, and hence, early substitution with active D-vitamin and dietary phosphate restriction in early chronic renal failure may be recommended, particularly in patients with diabetes. Vitamin E has been suggested to prevent microvascular complications patients with type 1 diabetes (Bursell et al., 1999). On the other hand, no beneficial effect of E-vitamin supplementation on cardiovascular events could be confirmed in a large clinical trial, the HOPE study (Lonn et al., 2002). Elevated levels of homocystein are observed in more than 90% of patients with ESRD (Bostom and Culleton, 1999), but they do not seem to be altered by diabetes (Wollesen et al., 1999) or isolated microalbuminuria (Agardh et al., 1994). High levels of homocystein have been linked to atherosclerosis, but not to the rate of progression of renal impairment (Samuelsson et al., 1999). It is speculated that a lowering of the homocystein levels, using a folic acid and B-vitamin regimen in renal disease, could reduce the excess incidence of cardiovascular disease and this is explored in ongoing trials (Bostom and Culleton, 1999; De Vriese et al., 2002).

ASA (acetylsalicylic acid)

Due to the elevated risk of cardiovascular disease, treatment with low dose (75-325 mg) aspirin (acetylsalicylic acid, ASA) is recommended as primary and secondary prevention
in adult patients with DN (Hansen et al., 2000). No adverse or beneficial short term effects on AER or GFR have been shown in patients with type 1 diabetes and microalbuminuria (American diabetes association, 2002).

Renal replacement therapy

In the first decade after the introduction of hemodialysis, diabetes was considered an absolute or a very strong relative contraindication to dialysis treatment due to poor survival. Since then, the treatment modalities have improved considerably but the survival of patients with diabetes in hemodialysis is still poor. Among some nephrologists, continuous ambulatory peritoneal dialysis (CAPD) is a preferred as treatment in patients with diabetes and renal failure (Passadakis et al., 2000). This may partly be motivated by less weight gain between the dialysis treatments, a risk factor for mortality due to cardiovascular problems, but also by the possibility of intraperitoneal insulin administration and fewer hypoglycemic episodes. On the other hand, it is not known if survival is better in CAPD compared to hemodialysis in diabetic patients since it has been difficult to perform randomized studies (Miskulin et al., 2002).

Today the best treatment in patients with DN and ESRD is transplantation with a kidney from a living donor or a combined kidney pancreas transplant (Tyden et al., 2000; Reddy et al., 2003). However, the availability of organs for transplantation is limited and a selection of eligible patients must therefore be performed. This process is mainly based on identification of certain risk factors, i.e. cardiovascular disease and high recipient age, predicting peri and postoperative outcome (Nyberg and Svensson, 2001). Hence, patients placed on the waiting list for transplantation are relatively healthier but long term survival is better even better among those who eventually undergo transplantation (Wolfe et al., 1999), arguing that this treatment is truly beneficial. It is noteworthy that survival after kidney transplantation is still worse in diabetics as compared with non-diabetics (Revanur et al., 2001).

3.4 PHYTOPHARMACOLOGY OF Lagerstroemia speciosa L. (L. speciosa)

From the time immemorial, man has depended on plants as medicine. From a historical perspective, it is evident that the fascination with plants is as old as mankind itself. Herbs have provided us some of the very important life saving drugs used in the armamentarium of modern medicine. The plant kingdom represents a rich storehouse of organic compounds, many of which have been used for medicinal and other purpose. There exists a plethora of knowledge and information and benefits of herbal drugs in our ancient literature of ayurvedic and unani medicine. Ethanomedicines are replete with descriptions of plant medicines and the grandmother’s pouch that has been called for years of medical wisdom is excellent proof of efficacy of these experimental medicines.

Traditional medicines for the treatment of diabetes mellitus are probably based mainly on treatment of its obvious symptoms of pronounced thirst and polyuria. Even glucosuria was recognized as a symptom of diabetes in ancient ayurvedic medical texts such as the shushruta samhita and charaka samhita.
**L. speciosa (Lythraceae)**

**Family**

Lythraceae

**Distribution**

It is distributed more or less throughout India, especially in Assam, Bengal and Deccan Peninsula.

**Common names**

In India the plant is known by different names in different languages like jarul in Hindi, jarool, ajar in Bengali, taman, mota-bondara in Marathi, varagou in Telugu, kadali, pumaruthu in Tamil, hole-dasavala, challa in Kannada, manimaruthu in Malayalam, patoli in Oriya. It has different local names in different parts of India like jarul in Punjab, ajar, thing-dou thlado in Assam, jarul, pyinma in Trade.

**3.4.1 Plant Description**

![Figure 3(A). L. speciosa plant with flower (Tree)](image-url)
A medium sized to large deciduous tree with a rounded crown, Bark smooth, greyish, exfoliating in irregular flakes; leaves oblong-lanceolate or elliptic; flowers in large panicles, mauve to purple; capsule ellipsoid or sub-globose; seeds pale brown.

Jarul is one of the well known ornamental trees and is widely cultivated in gardens and as an avenue tree. In favorable situations it attains a height of 24 m. and girth 3.6 m. with a clean bole of 12 m.; it is usually much smaller. It grows best on rich deep alluvial loam and it is typically found along banks of rivers and streams and in low-lying swampy areas, through it is not always confined to such places. The tree is a moderate light-demander and becomes suppressed under heavy shade. (Shastri, 1962)

A large tree 9-18 m high; branches widely spreading; bark pale, smooth, flaking off in irregular pieces. Leaves 10-20 by 3.8-7.5 cm, oblong-lanceolate or elliptic, subacute, glabrous and finely reticulate on both surfaces, pale beneath, base acute or rounded, main nerves 10-13 pairs, prominent curving upwards, petioles 6-10 mm long, stout, flowers 5-7.5 cm across, in large panicles some times reaching 30 cm. long; pedicels, stout, pubescent, thickened upwards and articulated below the middle Calyx turbinate, 1.6 cm long, covered with white or terruginous tomentum, ribbed with 12-14 prominent stout ridges, those opposite the calyx-teeth broader; teeth 6-7, triangular, acute spreading, 6 mm long, thickened at the edges petals 6-7, purple 2.5-3.8 cm long, suborbicular or rotund-ovate, clamed, much undulate and crumpled, spreading stamens all equal, shorter than the style. Capsules ellipsoid or subglobose, 2-3.2 by 1.6-2.5 cm, minutely apiculate. Seeds (including the wing) 1.25-1.45 cm by 6-4.5 mm, glabrous, pale brown.
The root is prescribed as an astringent. It is considered stimulant and febrifuge. The bark and leaves are purgative (Kirtikar and Basu, 1995).

3.4.2 Chemical constituents

Leaves of *L. speciosa* afforded three active ellagitannins, lagerstroemin, flosin B and reginin A identified (Hayashi *et al.*, 2002).

The new ellagitannin lagerstannins A, B, C was isolated from the aqueous acetone extract of the leaves of *L. speciosa* (Tanaka *et al.*, 1992).

Sixteen amino acids, pyrogallol tannins and lipids are reported for the first time from the leaves of *L. speciosa*. (Manalo *et al.*, 1993).

Triterpene Corosolic acid (Yamasaki, 1996; Okada *et al.*, 2003) and maslinic acid (Murakami *et al.*, 1993), Virgatic acid (Okada *et al.*, 2003) were isolated from *L. speciosa*.

The hexane extract of the *L. speciosa* leaves yielded sitosterol (Desai *et al.*, 1975).

Lipids like Arachidic acid, behenic acid, daturic acid and quinolizidine alkaloid named Lasubine II isolated from leaves of *L. speciosa* (Takahashi *et al.*, 1979).

Daucosterol steroid isolated from leaves of *L. speciosa* (Okada *et al.*, 2003).

Lagerstanin (Takahashi *et al.*, 1976) and Valoneic acid lactone were the coumarins isolated from leaves of *L. speciosa* (Unno *et al.*, 2004).

3.4.3 Pharmacology

**Anti-inflammatory and diuretic activity**

Various dosage forms prepared from *L. speciosa* leaves and pharmacochemical as well as pharmacological studies were done on them. TLC of all dosage forms showed the presence of B-sitosterol except 50 percent ethyl alcohol extract. The petroleum ether extract and ethyl ether extract showed anti-inflammatory activity while ethyl alcohol extract showed diuretic activity in mice (Garcia *et al.*, 1987).

Corosolic acid (CRA), a constituent of banaba leaves, has been reported to have anti-inflammatory and hypoglycemic activities. The aim of this study was to determine the effects of CRA on metabolic risk factors including obesity, hypertension, hyperinsulinemia, hyperglycemia, and hyperlipidemia together with oxidative stress and inflammation, all of which are characteristic of the SHR/NDmc-rcp (cp/cp) (SHR-cp) rat, an animal model of metabolic syndrome. Six-week-old male SHR-cp rats were fed a high fat diet containing 0.072% CRA for 14 weeks. Treatment with CRA lowered blood pressure, which was elevated in control animals, by 10% or after 8 weeks, and serum free fatty acids by 21% after 2 weeks. CRA treatment resulted in decreases in the levels of the oxidative stress markers thiobarbituric acid-reactive substances and 8-hydroxy-deoxyguanosine by 27% and 59%, respectively, after two weeks. CRA treatment also reduced the level of myeloperoxidase markers, 3-nitrotyrosine and 3-chlorotyrosine by 38% and 39%, respectively, after 10 weeks, and tended to decrease the level of high sensitivity C-reactive protein, a marker of
inflammation, after 6 weeks. However, CRA had no effect on weight gain and hyperglycemia. These results demonstrate that CRA can ameliorate hypertension, abnormal lipid metabolism and oxidative stress as well as the inflammatory state in SHR-cp rats. This implies that CRA can be beneficial for preventing atherosclerosis-related diseases that are an increasing health care problem worldwide (Yamaguchi et al., 2006).

Antifungal and antiyeast activity

Antifungal activity against Aspergillus niger and antiyeast activity against Candida vaginalis (Saksena and Tripathi, 1985).

Anticancer

Corosolic acid isolated from the fruit of Cratoegus pinnatifida var. psilosa was tested for anticancer activity. Corosolic acid displayed about the same potent cytotoxic activity as ursolic acid against several human cancer cell lines. In addition, the compound displayed antagonistic activity against the phorbol ester-induced morphological modification of K-562 leukemic cells, indicating the suppression of PKC activity by the cytotoxic compound. Corosolic acid showed PKC inhibition with dose-dependent pattern in an in vitro PKC assay (Ahn et al., 1998).

Antihyperglycemic activity

The Leaf extract of the tropical plant *L. speciosa* (known as banaba in the Philippine Tagalog dialect) had been used in folk medicine for the treatment of diabetes and kidney diseases. The extract from *L. speciosa* significantly reduced blood glucose and insulin levels in type 2 KK-AY diabetic mice and displayed anti-adipogenic activity by effectively reducing weight gain and parametrial adipose tissue in female diabetic mice (Liu et al. 2001). This was achieved by the reduction of PPAR-γ2 mRNA (a sub-isoform of PPAR-γ) and GLUT4 protein in cells induced from preadipocytes with 3-isobutyl-1-methylxanthine and dexamethasone. This combination of effect seems advantageous for the extract, since thiazolidinediones are known to up-regulate both glucose transport and lipid biosynthesis in adipocytes and weight gain is a frequent side effect of insulin therapy in type 2 diabetes mellitus patients (Gurnell et al. 2003). Therefore, drugs with glucose-lowering activity, but lacking adipogenic activity, are highly beneficial.

Banaba in the Tagalog name, *L. speciosa*, has been used as a folk medicine for a long time among diabetics in the Philippines. Extracts from banaba leaves have been reported to reduce diabetic symptoms in genetically diabetic mice (Type II, KK-Ay). In the present study, female mice of the same strain showing remarkable body weight gain were used to examine the antiobesity effect of dietary banaba extract. Five-week-old female KK-Ay mice were fed a control diet or test diet containing 5% of a hot-water extract from banaba leaves instead of cellulose for 12 wk. Neither group showed any changes in diet intake during the experimental period. Body weight gain and parametrial adipose tissue weight were lowered significantly in the banaba diet group. Blood glucose levels were not suppressed in the banaba diet group, but hemoglobin A1C was found to be suppressed at the end of the experiment. No effects on the serum lipids were observed, but the mice fed banaba extract showed a significant decrease, to 65% of the control level in total hepatic lipid contents. This decrease was due to a reduction in the accumulation of triglyceride. These results suggest that banaba had a beneficial effect on obese female KK-Ay mice (Suzuki et al., 1999).
The hypoglycemic effects of *L. speciosa*, known by the Tagalog name of banaba in the Phillipines, were studied using hereditary diabetic mice (Type II, KK-AY/Ta Jcl). The mice were fed a test diet containing 5% of the hot-water extract (HWE) from banaba leaves, 3% of the water eluent of the partial fraction unadsorbed onto HP-20 resin of HWE (HPWE), and 2% of the methanol eluent of the partial fraction adsorbed onto HP-20 resin of it (HPME) for a feeding period of 5 weeks. The elevation of blood plasma glucose level in non-insulin dependent diabetic mice fed the cellulose as control (CEL) diet were almost entirely suppressed by addition of either HWE or HPME in place of cellulose in the CEL diet. Water intakes were inclined to increase gradually in the group fed either CEL or HPWE, but lower in the mice fed either HWE or HPME than in the animals given either CEL or HPME. The level of serum insulin and the amount of urinary excreted glucose were also lowered in mice fed HWE. Plasma total cholesterol level was also lowered in mice fed the either HWE or HPME. It is suggested that HWE, especially HPME, obtained from banaba leaves have beneficial effects on control of the level of plasma glucose in non-insulin dependent diabetes mellitus (Kakuda et al., 1996).

The antidiabetic effects of corosolic acid (CA) were investigated in KK-Ay mice, an animal model of type 2 diabetes. CA (2 mg/kg body weight) reduced the blood glucose levels of KK-Ay mice 4 h after a single oral dose. CA (2 mg/kg) reduced the blood glucose levels in KK-Ay mice 2 weeks after a single oral dose and also significantly lowered plasma insulin levels were in KK-Ay mice under similar conditions. CA-treated KK-Ay mouse blood glucose significantly decreased in an insulin tolerance test. These results support the hypothesis that CA improves glucose metabolism by reducing insulin resistance. Therefore CA may be useful for the treatment of type 2 diabetes (Miura et al., 2006).

The effect of corosolic acid on blood glucose was studied in KK-Ay mice, an animal model of type 2 diabetes. Corosolic acid (10 mg/kg) reduced the blood glucose (p<0.05) of KK-Ay mice 4 h after single oral administration when compared with the control group. However, corosolic acid did not change the plasma insulin. The muscle facilitative glucose transporter isoform 4 (GLUT4) translocation from low-density microsomal membrane to plasma membrane was significantly increased in the orally Corosolic acid -treated mice when compared with that of the controls. These results suggest that the hypoglycemic effect of corosolic acid is derived, at least in part, from an increase in GLUT4 translocation in muscle. Therefore, it may be that corosolic acid has beneficial effects on hyperglycemia in type 2 diabetes (Miura et al., 2004).

The effects of extracts isolated from *L. speciosa* (banaba) on glucose transport and adipocyte differentiation in 3T3-L1 cells were studied. Glucose uptake–inducing activity of banaba extract (BE) was investigated in differentiated adipocytes using a radioactive assay, and the ability of BE to induce differentiation in preadipocytes was examined by Northern and Western blot analyses. The hot water BE and the banaba methanol eluent (BME) stimulated glucose uptake in 3T3-L1 adipocytes with an induction time and a dose-dependent response similar to those of insulin. Furthermore, there were no additive or synergistic effects found between BE and insulin on glucose uptake, and the glucose uptake activity of insulin could be reduced to basal levels by adding increasing amounts of BE. Unlike insulin, BE did not induce adipocyte differentiation in the presence of 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (DEX). BE inhibited the adipocyte differentiation induced by insulin plus IBMX and DEX (IS-IBMX-DEX) of 3T3-L1 preadipocytes in a dose-dependent manner. The differences in the glucose uptake and differentiation inhibitory activities between untreated cells and those treated with BE were significant (P < 0.01). The inhibitory activity was further demonstrated by drastic reductions of peroxisome proliferator-activated receptor 2 (PPAR2) mRNA and GLUT4 protein in cells induced from preadipocytes with IS-IBMX-
DEX in the presence of BE. The unique combination of a glucose uptake stimulatory activity, the absence of adipocyte differentiation activity and effective inhibition of adipocyte differentiation induced by IS-IBMX-DEX in 3T3-L1 cells suggest that BE may be useful for prevention and treatment of hyperglycemia and obesity in type II diabetics (Fang et al., 2001).

Obesity is a major risk factor for Syndrome X and type II diabetes (T2D). However, most antidiabetic drugs that are hypoglycemic also promote weight gain, thus alleviating one symptom of T2D while aggravating a major risk factor that leads to T2D. Adipogenesis, the differentiation and proliferation of adipocytes, is a major mechanism leading to weight gain and obesity. It is highly desirable to develop pharmaceuticals and treatments for T2D that reduce blood glucose levels without inducing adipogenesis in patients. Previously, we reported that an extract from *L. speciosa* (banaba) possessed activities that both stimulated glucose transport and inhibited adipocyte differentiation in 3T3-L1 cells. Using glucose uptake assays and Western/Northern blot analyses as major tools and 3T3-L1 cells as a model, we showed that the banaba extract (BE) with tannin removed was devoid of the 2 activities, and tannic acid (TA), a major component of tannins, had the same 2 activities as BE. Inhibitors known to abolish insulin-induced glucose transport also blocked TA-induced glucose transport. We further detected that TA induced phosphorylation of the insulin receptor (IR) and Akt, as well as translocation of GLUT 4, the protein factors involved in the signaling pathway of insulin-mediated glucose transport. We also demonstrated that TA inhibited the expression of key genes for adipogenesis. Differences between samples with or without TA in all of the quantitative assays were significant (*P*<0.05). These results suggest that TA may be useful for the prevention and treatment of T2D and its associated obesity. TA may have the potential to become the lead compound in the development of new types of antidiabetic pharmaceuticals that are able to reduce blood glucose levels without increasing adiposity (Liu et al., 2005).

The antidiabetic activity of an extract from the leaves of *L. speciosa* standardized to 1% corosolic acid (Glucosol) has been demonstrated in a randomized clinical trial involving Type II diabetics (non-insulin-dependent diabetes mellitus, NIDDM). Subjects received a daily oral dose of Glucosol and blood glucose levels were measured. Glucosol at daily dosages of 32 and 48mg for 2 weeks showed a significant reduction in the blood glucose levels. Glucosol in a soft gel capsule formulation showed a 30% decrease in blood glucose levels compared to a 20% drop seen with dry-powder filled hard gelatin capsule formulation (*P*<0.001), suggesting that the soft gel formulation has a better bioavailability than a dry-powder formulation (Judy et al., 2003).

In this study, 31 subjects were orally administered 10mg corosolic acid or a placebo, on different occasions, in a capsule 5min before the 75-g OGTT in a double-blind and cross-over design. Nineteen subjects had diabetes, seven had impaired glucose tolerance, one had impaired fasting glucose, and four had normal glucose tolerance according to the 1998 WHO criteria. There were no significant differences in plasma glucose levels before and 30min after the administration. Corosolic acid treatment subjects showed lower glucose levels from 60min until 120min and reached statistical significance at 90min. In this study, we have shown for the first time that corosolic acid has a lowering effect on postchallenge plasma glucose levels in vivo in humans (Fukushima et al., 2006).

Banaba (*L. speciosa*) has been used as a folk medicine for diabetes in the Philippines. Using bioassay-guided separation, valoneaic acid dilactone (1) was isolated from the leaves as a potent alpha-amylase inhibitor. A simple and efficient method for the quantitative determination of valoneaic acid and its derivatives in Banaba extract was established. Valoneaic acid exists as the structural parts of the polyphenols, which like flosin A, reginin
A, and lagerstroemin, are characteristic constituents of Banaba. These derivatives were hydrolyzed to valoneic acid by HCl and extracted with 2-butanone. This extract was subjected to HPLC analysis, and the contents of valoneic acid determined as the whole valoneic acid contents. Using this method, the whole valoneic acid contents were measured in eight Banaba leaf decoctions. The alpha-amylase-inhibiting activities of the decoctions were dependent on the whole valoneic acid contents. In addition, a strong linear correlation was observed between the whole valoneic acid contents and total polyphenol contents. This analytical procedure is applicable to the chemical evaluation of Banaba (Hosoyama et al., 2003).

Lagerstroemin, an ellagitannin isolated from the leaves of *L. speciosa* was examined for its biological activities. In rat adipocytes, the compound increased the rate of glucose uptake and decreased the isoproterenol-induced glycerol release. In Chinese hamster ovary cells expressing human insulin receptors, it increased the Erk activity. These insulin-like actions were accompanied by the increased tyrosine-phosphorylation of the α-subunit of the insulin receptors. Tryptic digestion of the extracellular sites of the insulin receptors markedly increased the effective concentrations of insulin without changing those of lagerstroemin. Thus lagerstroemin was considered to cause its insulin-like actions by a mechanism different from that employed by insulin (Katsuji et al., 2003).

Glucose transport enhancers were searched for in Lagerstroemia speciosa, a Philippine local herbal medicine used for diabetes mellitus. Bioassay-guided fractionation of the aqueous acetone extract of the leaves afforded three active ellagitannins, lagerstroemin, flosin B and reginin A, identified by NMR and optical rotation. These compounds increased glucose uptake of rat adipocytes, and could be responsible for lowering the blood glucose level (Hayashi et al., 2002).

The semi-synthesis, in vitro and in vivo biological evaluation of corosolic acid (1) and maslinic acid (2) are described. Compound 1 and 2 represent a new class of inhibitors of glycogen phosphorylases. Both 1 and 2 inhibit the increase of fasted plasma glucose of diabetic mice induced by adrenaline. It is therefore proposed that naturally occurring pentacyclic triterpenes 1 and 2 might reduce blood glucose, at least in part, through inhibiting hepatic glycogen degradation (Wen et al., 2005).

**Treatment of hyperuricemia**

Xanthine oxidase (XOD) is key enzyme playing a role in hyperuricemia, catalyzing the oxidation of hypoxanthine to xanthine and then to uric acid. This study aimed to identify the XOD inhibitors from the leaves of *L. speciosa*, which was traditionally used as a folk medicine in Philippines. Using a bioassay-guided fractionation technique, two active compounds were isolated from the aqueous extracts of the *L. speciosa* leaves, namely valoneic acid dilactone (VAD) and ellagic acid (EA). The result non-competitive mode for the enzyme with respect to xanthine as the substrate. These results may explain and support the dietary use of the aqueous extracts from Lagerstroemia speciosa leaves for the prevention and treatment of hyperuricemia (Unno et al., 2004).