Chapter 2

Literature Review
Chapter 2. Literature Review

2.1. Diabetes definition and classification

2.1.1. 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, fat 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 Chakrata 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 dependant diabetes. Greek physician Aretaeus 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, a German medical student, Paul Langerhans, noted that the pancreas contains two distinct groups of cells, the acinar cells which secrete digestive enzymes and a 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 first 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.

2.1.2. 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 dependant diabetes mellitus (type 1) and non-insulin dependant 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 shown in Table 1. Changes to diagnostic criteria were also proposed. The main features of new classifications are elimination of the terms insulin-dependant diabetes mellitus and non-insulin dependant 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 in 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</td>
</tr>
</tbody>
</table>
Carboxypeptidase-I

There are some proteins identified which are targeted by the immune system; insulin, a neuroendocrine enzyme glutamic acid decarboxylase (GAD), membrane granule proteins with homology to tyrosine phosphatases (termed as islet cell antibody (ICA)512/IA-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 CD8+ lymphocytes are likely to be involved in the pathogenesis (Santamaria et al., 1995).

Given the complexity and chronicity of type 1A diabetes mellitus, multiple interventions are able to prevent the disorder in animal models. One of the most specific preventive modalities utilizes peptides of insulin. Single injection of the 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 such interventions in different animal models to prevent the diabetes.
2.2.1. Pathophysiology of Type 1 diabetes mellitus

The trigger for the process of destruction of β-cells is poorly characterized but is either an 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. The 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 IA 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 IA 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 IA 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 IA diabetes mellitus

<table>
<thead>
<tr>
<th>Antigens</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>
</tbody>
</table>
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 90% to 95% of the diabetes syndrome.

Other specific types of diabetes
Other types 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.

Gestational diabetes
Caused by insulin resistance and relative insulin deficiency associated with pregnancy. Occurs approximately 3% to 5% of all pregnancies.

2.2. 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 dependant 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).
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><strong>Immune suppression</strong></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 treatment</td>
</tr>
<tr>
<td><strong>Monoclonal anti CD4⁺</strong></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><strong>Anti-CD3</strong></td>
<td>T Cells</td>
<td>NOD</td>
<td>Treatment at diabetes onset (only at onset), long term remission</td>
</tr>
<tr>
<td><strong>IL-2 toxin</strong></td>
<td>Activated T-cells</td>
<td>NOD</td>
<td>Short term efficacy</td>
</tr>
<tr>
<td><strong>CTLA4-lg</strong></td>
<td>Accessory T cell molecule</td>
<td>NOD</td>
<td>Prevention of diabetes by blocking accession of molecules</td>
</tr>
<tr>
<td><strong>Transplantation</strong></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><strong>Immune stimulation</strong></td>
<td>Unknown</td>
<td>NOD</td>
<td>Intervention ranging from injection of monoclonal antibodies, Freund’s adjuvant, BCG vaccine, or even attenuated mycobacterial organisms might have this effect</td>
</tr>
<tr>
<td><strong>Oral/nasal tolerance</strong></td>
<td>Insulin/GAD</td>
<td>NOD</td>
<td>Mice fed insulin or receiving</td>
</tr>
</tbody>
</table>
intranasal insulin or GAD have delayed or reduced onset of diabetes; no effect on BB rat

<table>
<thead>
<tr>
<th>Diet and nutritional therapies</th>
<th>Unknown</th>
<th>NOD, BB</th>
<th>These diets can be divided into those with a hypothesized mechanism such as radical scavenging and those that are nutritionally special (e.g., eliminating proteins)</th>
</tr>
</thead>
<tbody>
<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
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>

DZ-dizygotic; MZ-monozygotic.

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 ICA512 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 H 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 dual-parameter model has been described recently for the prediction of type 1A DM that
Stage 5, overt DM
Stage 6, total or near total \(\beta\)-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 21.3). 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 1DM, 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 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.
takes into account both first-phase insulin release and levels of antiinsulin 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 patients 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), tumour necrosis factors (TNFs) and colony stimulation factors (CSFs). Both the production of cytokines by cells and the action of cytokines on cells are 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 neighbouring cells (paracrine)
c) on the cell that secretes cytokine (autocrine)

In some cases macrophage derived inflammatory cytokines, such as IL-1, IL-6 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. Th1 cells and their cytokine products IL-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 humoral immune response that is immunoglobulin production, especially immunoglobulin E by B cells. Furthermore, responses of Th1 and Th2 cells are mutually inhibitory. Thus, Th1 cytokine IFN-γ inhibits the production of the Th2 cytokines 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) expressing cytokine genes transgenically in β-cells

iii) 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

Effects 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 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-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 or benign insulitis

<table>
<thead>
<tr>
<th>Proinflammatory cytokines</th>
<th>Type 1 cytokines</th>
<th>Type 2 cytokines</th>
<th>Type 3 cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<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>
</tr>
<tr>
<td>BB Rats</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Humans</td>
<td>0</td>
<td>nd</td>
<td>+</td>
</tr>
</tbody>
</table>

17
IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor;

+, cytokine presence correlates with β-cell destructive insulitis;
-, cytokine presence correlates with benign insulitis;
0, 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 responses. Transgenic expression of TNF-α, IL-4, IL-6 and TGF-β by β-cells in autoimmune DM-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>IL-1</td>
<td>TNF-α</td>
<td>IFN-α</td>
<td>IL-12</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>To islets/β-cells in vitro (rodent and human)</td>
<td>Toxic</td>
<td>Toxic</td>
<td>0</td>
<td>?</td>
</tr>
<tr>
<td>Systemic (parenteral) administration to:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) NOD mice</td>
<td>+ (&lt;3 wks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) BB rats</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>?</td>
</tr>
</tbody>
</table>

IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor;
+, cytokine presence correlates with β-cell destructive insulitis;
-, cytokine presence correlates with benign insulitis;
0, 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-β have been reported in DM-prone NOD mice and/or BB rats. Systemic administration of cytokines however, produces a 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 on 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, 1998). 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 blocking more than one cytokine. Alternatively, a pathogenic mechanism common to the diabetogenic cytokines may be identified.
Table 7. Effects of cytokine deletions in NOD mice and BB rats

<table>
<thead>
<tr>
<th>Cytokine deletions</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 α</td>
<td>IFN-γ 12 α</td>
<td>IL-12 β</td>
<td>H-4</td>
</tr>
<tr>
<td>By knockout of gene for cytokines/receptor in NOD mice</td>
<td>?</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>By neutralization of cytokine, blockade of receptor, or deletion of receptor-positive cells in a) NOD mice</td>
<td>- (&lt;3 wks)</td>
<td>-</td>
<td>-</td>
<td>- Insulitis (&lt;3 wks)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>b) BB rats</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor;
+, cytokine presence correlates with β-cell destructive insulitis;
-, cytokine presence correlates with benign insulitis;
0, 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 in 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), Kilham rat virus (KRV) (Guberski et al., 1991), rubella virus (Rayfield et al., 1986), retrovirus (Suenaga and Yoon, 1988), bovine viral diarrhea-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. The 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 pancreata 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.

Coxsackievirus 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 pancreata 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). These 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 found 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 DM 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 recognize 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.

2.2.2. 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. Nondiabetic 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 mice 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

<table>
<thead>
<tr>
<th>Features</th>
<th>Human</th>
<th>Spontaneously diabetic animals</th>
<th>Animals with induced diabetes mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NOD Mouse</td>
<td>DP-BB Rat</td>
</tr>
<tr>
<td>Onset</td>
<td>Spontaneous</td>
<td>Spontaneous</td>
<td>Inducible</td>
</tr>
<tr>
<td>Latency</td>
<td>Severe</td>
<td>Up to 6 months</td>
<td>1-2 months</td>
</tr>
<tr>
<td>Ketosis</td>
<td>Severe</td>
<td>Mild</td>
<td>Severe</td>
</tr>
<tr>
<td>Insulin deficiency</td>
<td>Absolute</td>
<td>Mild to severe</td>
<td>Severe</td>
</tr>
<tr>
<td>Associated autoimmune diseases</td>
<td>Thyroiditis, adrenalinities</td>
<td>Sialodinitis, thyroiditis</td>
<td>Thyroiditis</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td>Insulin, GAD, ICA, islet cell surface antibody, carboxypeptidase H, IA-2</td>
<td>Insulin, GAD, ICA</td>
<td>ICA, GAD, insulin, Gastric parietal cells</td>
</tr>
<tr>
<td>MHC genes</td>
<td>HLA-DQ</td>
<td>Unique I-A</td>
<td>RTI</td>
</tr>
<tr>
<td>Gender effect</td>
<td>Male = female</td>
<td>Female &gt; male</td>
<td>Male = female</td>
</tr>
<tr>
<td>Response to general immunosuppression</td>
<td>Cyclosporine prolongs endogenous insulin production</td>
<td>Cyclosporine prevents diabetes</td>
<td>Cyclosporine prevents 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 non-responsive. Co-stimulatory blockade prevents DM but not insulitis in NOD mice (Balasa et al., 1997).

Additional data indicate that B-lymphocytes, functioning as antigen-presenting cells, are critical to the development of DM in NOD mice. They may act by trapping islet antigens 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 has 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 achromogenes* 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-tumour and oncogenic properties (Rakietan, 1971). One 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 dose 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 automodified 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 synthesises 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 co-stimulatory 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 pseudopregnanant foster mothers. This germine 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 or 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>Cytokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Increase</td>
<td>Increase</td>
<td>Allison <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>IL-6</td>
<td>Increase</td>
<td>Decrease</td>
<td>DiCosmo <em>et al.</em>,</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 way. 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 altered 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., 1998</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>↑</td>
<td>↓</td>
<td>Hultgren et al., 1996</td>
</tr>
<tr>
<td>IFN-γR</td>
<td>↓</td>
<td>↓</td>
<td>Wang et al., 1997</td>
</tr>
<tr>
<td>Tumour necrosis factor receptor 1</td>
<td>↑</td>
<td>↓</td>
<td>Kagi et al., 1999</td>
</tr>
</tbody>
</table>

→ No change; ↓ Decrease

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 unexpected 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.
2.3. 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 both of them are 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).

2.3.1. Risk Factors

Genetic factors

Type 2 DM results 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 expatriate 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, only a small proportion of obese people contract the disease. Among obese subjects with a 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., 1998). 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).

2.3.2. Pathophysiology of Type 2 Diabetes Mellitus

The type 2 DM is preceded by hyperinsulinemia and impaired glucose tolerance (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 can not 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
Flow chart showing pathophysiologic development of obesity and type 2 diabetes mellitus
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 failure to increase 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 hypothalamic-pituitary-adrenal axis via adrenocorticotropic hormone (ACTH) that is released by pituitary. Glucocorticoids play a key role in regulating salt and water homeostasis, 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 normalised 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 noradrenaline, 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 mobilisation, 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 fat, 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 mobilises 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 increase in sympathetic Vs parasympathetic activity following standardised stress or following 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 (Nyholm 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 plasma free fatty acid concentrations can lead to an attenuated effect of insulin to stimulate 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**

The renin-angiotensin system (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 (Limura *et al.*, 1995) and prevent the development of type 2 diabetes (Lindholm *et al.*, 2002). Angiotensin 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 number 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 *et al.*, 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 from 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 an
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 the 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 a 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 insulin receptor substrate, 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 the 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 center 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-trisphosphate 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, 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 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., 1999).
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 Akt 2 (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-Cbl/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-permeant 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 tyrosine 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, CIP4.2 (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 pathogenetic role for abnormal regulation of PTPases 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 hyperinsulinemic 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 635% 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 preceptors 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 1 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 11 gives details about the different GLUTs found in mammalian tissues.
<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 testis, intermediate in brain</td>
<td>Not known</td>
<td>Ducluzeau et al., 2002</td>
</tr>
<tr>
<td>GLUT 9</td>
<td>Brain/leukocytes</td>
<td>Not known</td>
<td>Dodge et al., 2000</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></td>
<td></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^r) mice</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Viable yellow (A^w/r) mice</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Brown-fat–ablated mice</td>
<td>++</td>
<td>+</td>
<td>0</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 streptozotocin–induced diabetes</td>
<td>-</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td>Spontaneously hypertensive rats</td>
<td>+</td>
<td>0</td>
<td>0</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</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 humans
(Shepherd et al., 2000)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>GLUT 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Muscle</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>0</td>
</tr>
<tr>
<td>Pancreatic transplantation in subjects with type 1 diabetes</td>
<td>-</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>0</td>
</tr>
<tr>
<td>Insulin resistance in relatives of subjects with type 1 diabetes</td>
<td>0</td>
</tr>
<tr>
<td>Obesity</td>
<td>0</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>0</td>
</tr>
<tr>
<td>Aging</td>
<td>-</td>
</tr>
<tr>
<td>Uremia</td>
<td>0</td>
</tr>
<tr>
<td>Polycystic ovary syndrome</td>
<td>ND</td>
</tr>
<tr>
<td>Pseudoacromegaly</td>
<td>0</td>
</tr>
<tr>
<td>Exercise</td>
<td>+</td>
</tr>
<tr>
<td>Sulfonylurea therapy</td>
<td>0</td>
</tr>
<tr>
<td>Weight loss</td>
<td>0</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 per deciliter (2 to 10 mmol per liter), 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 percent 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 tyrosine 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 movement 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 B is normal (Krook et al., 1998).

Several proteins have been identified in GLUT 4 containing vesicles, most of which are also present in other exocytotic 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 of mechanisms used by synaptic vesicles. This model proposes that proteins similar to those involved in synaptoosome 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 the GLUT 4 containing vesicle.

Theoretically, there are at least three ways in which insulin might modulate GLUT 4 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 the 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).
The insulin dependant glucose transporter GLUT 4 is translocated by a PI3-K dependant pathway including PKB/Akt and PKC stimulation downstream of PI3K.

PI3-K = Phosphatidylinositol 3-kinase; PI3,4,5 P = phosphatidylinositol 3,4,5-trisphosphate; PKB = Protein Kinase B; PKC = Protein Kinase C; PDK = Phosphate dependant kinase; IRS = Insulin Receptor Kinase

A general hypothesis called the SNARE (soluble NSF attachment protein receptors; NSF, Nethylnmaleimide-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 fusion 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, (Bjornholm 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 acyltransferase, 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 synthesise 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 levels 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 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 (Hartman et al., 2002; Haugen et al., 2001) and serum levels (Steppan et al., 2001). Li and Lazar (2002) created a transcription factor that activated transcription of PPARg-responsive genes in the absence of ligand by fusion of the potent viral transcriptional activator VP16 to PPARg2 (VP16-PPARg). Resistin gene expression was reduced in VP16-PPARg 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 antidiabetic and antiobesity 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 the 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 β cell (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 (Ørskov, 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 GLP-1Receptor-/- 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; Byrne et al., 1998; Rachman et al., 1997). GLP-1 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-1, 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
The suppressor of cytokine signaling (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 1 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 suppressor of cytokine signaling (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 play a role in the negative regulation of the signaling of insulin and IGF-I (Senn et al., 2003). Senn et al. (2003) reported that SOCS-3 is a potential mechanism in resistance in hepatocytes. SOCS-1 and SOCS-3 cause inhibition of tyrosine phosphorylation of insulin receptor substrates (Ueki et al., 2004).

Diabetes mellitus

Administration of STZ (100 mg/kg) on the day of birth of diabetes mellitus can be developed (Okamoto, 1981). Injecting or 5th day (Portha et al., 1979a) of the birth can also be in rats. Day of birth exhibit insulin deficient acute diabetes mellitus showed high plasma glucose and about 93% decrease in plasma insulin content. The hyperglycaemia observed in the neonates transient. It is reported that neonatal rats are resistant to STZ (Connier-Weir et al., 1981). It was found that only by 8 weeks of age and showed mild hyperglycaemia (Portha et al., 1979a).

When Sprague-Dawley pups injected intraperitoneally on the 2nd 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 characterised by a low insulin release in vivo in response to glucose or amino acids (Okamoto, 1981). In insulin secretion studies of the
10-16 week old 0 day neonatal STZ-diabetic rats, there was a complete loss of β-cell sensitivity to glucose (Portha et al., 1979a). The impairment of glucose-induced insulin release in this rat model is clearly related to a defect in oxidative glycolysis. This leads to a severe 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 ionic 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 ratio. 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 was incomplete (Bonnier-Weir et al., 1981; Portha et al., 1979a).

Streptozotocin and Nicotinamide induced diabetes model

Pellegrino et al. (1998) reported about the use of nicotinamide with streptozotocin to induce diabetes, which simulates type 2 diabetes in certain aspects. Administration of STZ 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 beta-cytotoxicity 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 a direct 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 relative dosages 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, was 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 a marked glucose intolerance, as expected. With regard to postload 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 nonesterified 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 reportedly 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 antidiabetic 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-fed 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
antiobesity and antidiabetic 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 antidiabetic thiazolidinediones (Khoursheed 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 animals 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. 
Pharmaceutical 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 mutations result in reduced energy expenditure and lead to diabetes and 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 impaired glucose tolerance (IGT), mild hyperglycemia, pronounced hyperinsulinemia, and marked reduction in insulin sensitivity (Terrettaz, and Jeanrenaud, 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 antidiabetic compounds.

**KK and KKA$^Y$ 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 KKA$^Y$ mice, a congenic strain harboring the $A^Y$ allele at the agouti locus (Suto et al., 1999). The $A^Y$ 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 uptake 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).
2.4. *Punica granatum* Linn.

**Family:** Punicaceae

**Common names**

English-pomegranate tree; Hindi- Anar, dhalim; Malayalam- Dadiman; Tamil- Madulam, Madulangam; Sanskrit- Dadima; Urdu- Anarmitha

**Description**

*Punica granatum* (Punicaceae) is a large deciduous shrub or small tree up to 10 m in height with smooth dark grey bark. Flowers are mostly solitary.

**Distribution**

It is distributed from Balkans to the Himalayas and cultivated throughout India.

**Uses in Traditional medicine**

In Ayurveda, the root is used as external vermicide. The bark and seeds are reputed to cure bronchitis. The unripe fruit is a good appetizer and tonic. The ripe fruit is esteemed as a tonic, astringent to the bowels. It is used to treat biliousness, tridosha, thirst, burning sensation of the body, fever, heart disease, sore throat and stomatitis. The rind of fruits is credited as an anthelmintic, and to relieve diarrhoea and dysentery.

In Unani, the bark is valued as astringent and to strengthen gums and used in the treatment of piles and colic. The unripe fruit is consumed as tonic, laxative and diuretic. It is prescribed to treat sore throat, sore eyes, brain diseases, spleen complaints, chest troubles, scabies, bronchitis and ear ache. The seeds are recommended as astringent and used to treat vomiting, sore eyes, biliousness, scabies, liver and kidney disorders.

The flowers of *P. granatum* (Gulnar in Unani) are efficacious to treat diabetes, either as a single drug or in polyherbal formulations in Unani medicine (Hakim, 1997; Jurjani 1878, Majoosi, 1889). The flowers are also reputed as styptic to the gums. It is used to treat biliousness, sore eyes, ulcers and sore throat. A paste of the flowers is applied to hydrocele.

**Previously reported studies**

**Phytochemistry**

Betainic alkaloid and hydroquinone pyridinium alkaloid have been isolated from the leaves of pomegranates *P. granatum* (Schmidt et al., 2005). A radical scavenging antioxidant - punicalagin was isolated and identified from pith and carpellary membrane
of pomegranate fruit. Activity-guided repeated fractionation of the methanol extract on a silica gel column, followed by preparative HPLC, yielded a compound which exhibited strong radical scavenging activity against DPPH free radical (Kulkarni et al., 2004). From *P. granatum* heartwood two ellagic acid rhamnosides, 3-O-methylellagic acid 4-O-alpha-L-rhamnopyranoside and 3,4'-O-dimethylellagic acid 4-O-alpha-L-rhamnopyranoside were isolated together with brevifolincarboxylic acid, 3-O-methylellagic acid and 4,4'-O-dimethylellagic acid. 

*P. granatum* heartwood contained two ellagitannins, diellagic acid rhamnosyl glucopyranoside and 5-O-galloylpunicacortein D were isolated and characterized together with four known tannin metabolites, punicacortein D, punicalin, punicalagin and 2-O-galloylpunicalin (Toumy and Rauwald, 2003). Phenolic compounds identified in freshly prepared pomegranate juices were gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, o- and p-coumaric acids, catechin, phloridzin and quercetin (Poyrazoglu et al., 2002).

Antioxidant activities of freeze-dried preparations of a 70% acetone extract of pomegranate (*P. granatum* L.) and its three major anthocyanidins (delphinidin, cyanidin, and pelargonidin) were evaluated. Anthocyanidins inhibited a Fenton reagent OH generating system possibly by chelating with ferrous ion. Anthocyanidins scavenged superoxide radical in a dose-dependent manner. The ID$_{50}$ values of delphinidin, cyanidin, and pelargonidin were 2.4, 22, and 456 µM, respectively. In contrast, anthocyanidins did not effectively scavenge nitric oxide. Anthocyanidins inhibited hydrogen peroxide-induced lipid peroxidation in the rat brain homogenates (Noda et al., 2002).

Phytochemical investigation of the bark of *P. granatum* led to the isolation and characterization of a new flavonoid diglucoside, quercetin-3,4' -dimethyl ether-7-O-alpha-L-arabinofuranosyl (1→6)-β-D-glucopyranoside, along with the known compounds quercetin, pelargonidine-3,5-diglucoside and ellagic acid (Chauhan and Chauhan, 2001). The leaves of *P. granatum* contained the gallotannins, 1,2,4-tri-O-galloyl-beta-glucopyranose and 1,3,4-tri-O-galloyl-beta-glucopyranose together with the ellagitannins, 1,4-di-O-galloyl-3,6-(R)-hexahydroxydiphenyl-beta-glucopyranose and brevifolin carboxylic acid 10-monopotassium sulphate (Hussein et al., 1997). A triglyceride, di-O-
pimicyl-O-octadeca-8Z,11Z, 13E-trienylglycerol, has been isolated and characterized from the seeds of *P. granatum* from India and Iran (Yusuph and Mann, 1997).

From the leaves of *P. granatum* the unique phenolic, N-(2',5'-dihydroxyphenyl)pyridinium chloride, as well as the known flavone glycosides, apigenin 4'-O-beta-glucopyranoside, luteolin 4'-O-beta-glucopyranoside, luteolin 3'-O-beta-gluсopyranoside and luteolin 3'-O-beta-xylopyranoside, were isolated and identified (Nawwar et al., 1994). Brevifolin carboxylic acid, brevifolin, corilagin, 3,6-(R)-hexahydroxydiphenol-(alpha/beta)-1C-4-glucopyranose, 1,2,6-tri-O-galloyl-beta-4C-1-glucopyranose, 1,4,6-tri-O-galloyl-beta-4C-1-glucopyranose, ellagic acid, 3,4,8,9,10-pentahydroxydibenz(o,d)pyran-6-one, granatin-B and punicafolin were isolated from the leaves of *P. granatum* (Nawwar et al., 1994a).

The structures of punicalin and punicalagin, isolated from the bark of *P. granatum* (pomegranate), were identified as 4,6-(S,S)-gallagyl-D-glucose and 2,3-(S)-hexahydroxydiphenol-(alpha/beta)-1C-4-glucopyranose, respectively, on the basis of chemical and spectroscopic evidence. A new hydrolyzable tannin, 2-O-galloyl-4,6-(S,S)-gallagyl-D-glucose was also isolated (Tanaka et al., 1986). A chemical examination of the bark of *P. granatum* has led to the isolation of five new ellagittannins, punicacortins A, B, C and D, and punigluconin, together with the known ellagittannins, casuariin and casuarinin (Tanaka et al., 1986a).

A new ellagittannin, punicafolin has been isolated from the leaves of *P. granatum* and characterized by physiochemical data and spectral evidence as 1,2,4-tri-O-galloyl-3,6-(R)-hexahydroxydiphenol-.beta.-D-glucose. The occurrence of the known tannins, granatins A and B, corilagin, strictinin, 1,2,4,6-tetra-O-galloyl-.beta.-D-glucose and 1,2,3,4,6-penta-O-galloyl-.beta.-D-glucose in the leaves has been demonstrated (Tanaka et al., 1985). Two crystalline tannins, named punicalagin and punicalin were isolated from a mixture of tannins, obtained from the peels of pomegranates (Mayer et al., 1977).

**Bioactivities**

The antioxidant activities of three parts (peel, juice, and seed) and extracts of three pomegranate varieties in China were investigated by using a chemiluminescence method in vitro. The peel extract of white pomegranate had the best scavenging ability on hydrogen peroxide, which had the lowest IC$_{50}$ value (0.032 ± 0.003 µg/mL) in the nine
extracts. The seed extract of white pomegranate (the IC\textsubscript{50} value was 3.67 ± 0.03 μg/mL) was the most powerful on the DNA damage-preventing effect in all of the extracts (Guo et al., 2007).

The antioxidant capacity in cell free-systems of preparations from various parts of pomegranate has been compared with their cytoprotective - bona fide antioxidant - activity in cultured human cells (U937 promonocytes and HUVEC endothelial cells) exposed to an array of oxidizing agents. Pomegranate juice, arils only juice and aqueous rinds extract were investigated. In cell-free assays - 1,1-diphenyl-2-picrylhydrazyl (DPPH), chemiluminescence luminol/xanthine/xanthine oxidase and lipoxygenase assays - all the preparations displayed good antioxidant capacity. Rind extract was capable of preventing the deleterious cytotoxicity (Sestili et al., 2007).

The effect of minimum inhibitory concentrations of the \textit{P. granatum} phytotherapeutic gel and miconazole oral gel on the adherence of microorganisms (three standard streptococci strains, mutans ATCC 25175, sanguis ATCC 10577 and mitis ATCC 9811) to glass was assessed in the presence of 5% sucrose. The \textit{Punica granatum} L. gel had greater efficiency in inhibiting microbial adherence than the miconazole (Vasconcelos et al., 2006).

In a double-blind, placebo-controlled trial to clinically evaluate the protective and ameliorative effects of ellagic acid-rich pomegranate extract, thirteen healthy volunteers per group were randomly assigned to three groups; namely, high dose (200 mg/d ellagic acid), low dose (100 mg/d ellagic acid) and control (0 mg/d ellagic acid: placebo). Treatments were continued for four weeks. Each subject received a 1.5 minimum erythema dose (MED) of UV irradiation on an inside region of the right upper arm, based on the MED value measured on the previous day. Luminance (L), melanin and erythema values were measured before the start of the test food intake, and after 1, 2, 3 and 4 wk following the start of the test food intake. As a result, decreasing rates of L values from the baseline in the low- and high-dose groups were inhibited by 1.35% and 1.73% respectively, as compared to the control group. Further, a stratified analysis using subjects with a slight sunburn revealed an inhibited decrease of L values compared with the control group at 1, 2 (p<0.01, respectively) and 4 wk (p<0.05) after the start of the test food intake in the low-dose group, and at 2 and 3 wk (p<0.05) in the high-dose
Ellagic acid-rich pomegranate extract, ingested orally, has an inhibitory effect on a slight pigmentation in the human skin caused by UV irradiation (Kasai et al., 2006).

The effect of concentrated pomegranate juice consumption on lipid profiles of type II diabetic patients with hyperlipidemia (total cholesterol or triglycerides $\geq 200$ mg/dL) was assessed. After consumption of concentrated pomegranate juice significant reductions were seen in total cholesterol ($p < 0.006$), low-density lipoprotein-cholesterol (LDL-c) ($p < 0.006$), LDL-c/high-density lipoprotein-cholesterol (HDL-c) ($p < 0.001$), and total cholesterol/HDL-c ($p < 0.001$). However, there were no significant changes in serum triacylglycerol and HDL-c concentrations (Esmailzadeh et al., 2006).

In an experiment, transgenic mice (APP(sw)/Tg2576) with Alzheimer's disease pathology received either pomegranate juice or sugar water control from 6 to 12.5 months of age. Pomegranate treated mice learned water maze tasks more quickly and swam faster than controls. Mice treated with the juice had significantly less (approximately 50%) accumulation of soluble Aβ42 and amyloid deposition in the hippocampus as compared to control mice (Hartman et al., 2006).

In normal human bronchial epithelial cells (NHBE) and human lung carcinoma A549 cells, the growth inhibitory effects of pomegranate fruit extract was compared. Treatment of pomegranate fruit extract (50-150 µg/ml) for 72 h was found to result in a decrease in the viability of A549 cells but had only minimal effects on NHBE cells as assessed by the MTT and Trypan blue assays. Extract treatment of A549 cells also resulted in dose-dependent arrest of cells in G0-G1 phase of the cell cycle (as assessed by DNA cell cycle analysis) (Khan et al., 2007).

A new polyphenol compound named pomegranate, together with, ellagic acid, 3,3',4'-tri-O-methylellagic acid, ethyl brevifolin-carboxylate, urolic and maslinic acids, and daucosterol were isolated from the ethanolic extract of the flowers of *P. granatum*. Maslinic acid exhibited antioxidant activity, evaluated by measurement of LDL susceptibility to oxidation (Wang et al., 2006).

The anti-inflammatory effect of methanol extract of *P. granatum* was investigated. The extract was pretreated in BV2 microglial cells and cells were stimulated to induce inflammation by lipopolysaccharide (LPS). The results suggested that *P. granatum*...
The oral administration of a human acceptable dose of pomegranate fruit extract to athymic nude mice implanted with CWR22Rnu1 cells resulted in significant inhibition of tumor growth with concomitant reduction in secretion of prostate-specific antigen (PSA) in the serum (Malik and Mukhtar, 2006).

The effects of pomegranate juice on inflammatory cell signaling proteins in the HT-29 human colon cancer cell line. At a concentration of 50 mg/L, pomegranate juice significantly suppressed TNF-α induced COX-2 protein expression by 79% (Adams et al., 2006).

Pomegranate flower extract was found to exhibit a potent protective activity in acute oxidative tissue injury animal model: ferric nitritotriacetate (Fe-NTA) induced hepatotoxicity in mice. Intraperitoneal administration of 9 mg/kg body wt. Fe-NTA to mice induced oxidative stress and liver injury. Pretreatment with pomegranate flower extract at a dose regimen of 50-150 mg/kg body wt. for a week significantly and dose dependently protected against Fe-NTA induced oxidative stress as well as hepatic injury. The extract afforded up to 60% protection against hepatic lipid peroxidation and preserved glutathione levels and activities of antioxidant enzymes viz., catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase (Kaur et al., 2006).

In the course of screening for anti-dementia agents from natural products, two beta-secretase (BACE1) inhibitors were isolated from the husk of pomegranate by activity-guided purification. They were identified as ellagic acid and punicalagin with IC50 values of 3.9 x 10(-6) and 4.1 x 10(-7) M and Ki values of 2.4 x 10(-5) and 5.9 x 10(-7) M, respectively (Kwak et al., 2005).

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Pomegranate juice and wine have become increasingly popular because of the important biological activities, including antioxidant activity (Schubert et al., 1999) and cardiovascular protection (Aviram et al., 2002) attributed to the plant. The aqueous-ethanolic extract of P. granatum flowers decreased blood glucose in normal and alloxan induced diabetic rats (Jafri et al., 2000). 6 week oral administration of methanol extract from P. granatum flowers (500 mg/kg, daily) inhibited glucose loading-induced increase
of plasma glucose levels in Zucker diabetic fatty rats. Gallic acid was identified as the main constituent for activity and it acts through activation of PPAR-γ receptors (Huang et al., 2005). *P. granatum* flower extract markedly inhibited the increase of plasma glucose levels after sucrose loading. In vitro *P. granatum* extract demonstrated a potent inhibitory effect on α-glucosidase activity (Li et al., 2005).

*P. granatum* flower extract improved abnormal cardiac lipid metabolism in Zucker diabetic fatty rats by activating PPAR-α and thereby lowering circulating lipid and inhibiting its cardiac uptake (Huang et al., 2005a). *P. granatum* flower extract reduced the up-regulated cardiac mRNA expression of ET-1, ETA, inhibitor KBα and c-jun and normalised the down-regulated mRNA expression of inhibitor KBα in Zucker diabetic fatty rats. It diminishes cardiac fibrosis in Zucker diabetic fatty rats by modulating cardiac ET-1 and NF-κB signalling (Huang et al., 2005b).

The extracts from fruits of pomegranate have been reported to have antimicrobial (Anesini and Perez, 1993), antiviral (Zhang et al., 1995) and anticancer (Kim et al., 2002) properties. Pomegranate peel extract had markedly higher antioxidant capacity than the pulp extract in scavenging or preventive capacity against superoxide anion, hydroxyl and peroxyl radicals as well as inhibiting copper sulphate-induced LDL oxidation (Li et al., 2006).

Oral administration of pomegranate fruit extract (0.1% and 0.2%, wt/vol) to athymic nude mice implanted with androgen-sensitive CWR22Rv1 cells resulted in a significant inhibition in tumor growth concomitant with a significant decrease in serum prostate-specific antigen levels (Malik et al., 2005). The effects of pomegranate juice on patients who have ischemic coronary heart disease was investigated and found to improve stress-induced myocardial ischemia in patients who have coronary heart disease (Sumner et al., 2005).

When osteoarthritis chondrocytes or cartilage explants pretreated with pomegranate fruit extract and then stimulated with IL-1β at different time points in vitro, degradation of cartilage was inhibited (Ahmad et al., 2005). Pomegranate juice reduced cholesterol accumulation in macrophages. Macrophage cholesterol biosynthesis was inhibited by 50% (P<.01) after cell incubation with pomegranate juice (Fuhrman et al., 2005). Pomegranate juice showed antiproliferative activity against all cell lines by inhibiting
proliferation from 30% to 100%. At 100 μg/ml, pomegranate juice, ellagic acid, punicalagin and standardized total pomegranate tannin (TPT) extract induced apoptosis in HT-29 colon cells. However, in the HCT116 colon cells, ellagic acid, punicalagin and TPT but not pomegranate juice induced apoptosis. The trend in antioxidant activity was pomegranate juice > TPT > punicalagin > ellagic acid (Seeram et al., 2005).

Long-term pomegranate juice intake increased intracavernous blood flow, improved erectile response and smooth muscle relaxation in erectile dysfunction and control groups while having no significant effect on NOS expression. PJ intake prevented erectile tissue fibrosis in the erectile dysfunction group (Azadzoi et al., 2005). Maternal dietary supplementation with pomegranate juice was found to be neuroprotective for the neonatal brain. Pomegranate juice also diminished caspase-3 activation by 84% in the hippocampus and 64% in the cortex (Loren et al., 2005).

Fractions rich in flavonoids obtained from the fruits of *P. granatum* extract, orally administered to rats at dose of 10 mg/kg/day, showed potential antiperoxidative effect. Concentrations of malondialdehyde, hydroperoxides and conjugated dienes were significantly decreased in liver while the activities of the enzymes catalase, superoxide dismutase (SOD), glutathione peroxidase and glutathione reductase showed significant elevation. Concentrations of glutathione in the tissues were also increased (Sudheesh and Vijayalakshmi, 2005).

Four pure chemicals, ellagic acid, caffeic acid, luteolin and punicic acid, all important components of the aqueous compartments or oily compartment of pomegranate fruit (*P. granatum*), showing known anticancer activities, were tested as potential inhibitors of in vitro invasion of human PC-3 prostate cancer cells. All compounds significantly inhibited invasion when employed individually (Lansky et al., 2005). Pomegranate fruit extract protects against the adverse effects of UV-B radiation by inhibiting UV-B-induced modulations of NF-kappaB and MAPK pathways (Afaq et al., 2005). Pomegranate fruit extract possesses antiskin-tumor-promoting effects in CD-1 mouse. Pomegranate fruit extract is capable of inhibiting conventional as well as novel biomarkers of 12-O-tetradecanoylphorbol-13-acetate -induced tumor promotion (Afaq et al. 2005a). Administration of 70% methanolic extract of *Punica granatum* fruit rind (250 mg/kg and
500 mg/kg) showed a percentage of inhibition in 22.37, 74.21 and 21.95, 63.41 in aspirin- and ethanol-induced gastric ulceration, respectively (Ajaikumar et al., 2005). Pomegranate cold-pressed (Oil) or supercritical carbon dioxide extracted seed oil, fermented juice polyphenols, and pericarp polyphenols on human prostate cancer cell xenograft growth in vivo, and/or proliferation, cell cycle distribution, apoptosis, gene expression, and invasion across Matrigel, in vitro. All pomegranate derived materials potently suppressed proliferation, xenograft growth, and invasion of human prostate cancer cells (Albrecht et al., 2004). Flavonoid-rich fractions from fresh and fermented pomegranate juice and from an aqueous extraction of pomegranate pericarps were investigated as potential differentiation-promoting agents of human HL-60 promyelocytic leukemia cells. Fermented pomegranate juice and aqueous extraction of pomegranate pericarps were strong promoters of differentiation. The extracts had proportional inhibitory effects on HL-60 cell proliferation (Kawai and Lansky, 2004). Aqueous extract of P. granatum were highly effective against Escherichia coli O157:H7 with the best MIC and MBC values of 0.19 and 0.39 mg/ml respectively (Voravuthikunchai et al., 2004). The methanolic extract of dried pomegranate (P. granatum) peels extract was formulated as a 10% (wt/wt) water-soluble gel and was studied for its wound healing property against an excision wound on the skin of Wistar rats. Animals treated with 2.5% gel showed moderate healing, whereas the group treated with 5.0% gel showed good healing (Murthy et al., 2004). P. granatum seed oil rich in cis (c), trans (t) 11, c13-18:3 as conjugated linolenic acids suppressed azoxymethane -induced colon carcinogenesis, and the inhibition is associated in part with the increased content of conjugated linolenic acids in the colon and liver and/or increased expression of PPAR-γ protein in the colon mucosa (Kohno et al., 2004). P. granatum fruit rind powder was able to restore the immune system suppressed by dexamethasone in rabbits (Ross et al., 2004a). When a gel containing the extract of P. granatum as an antifungal agent against candidosis associated with denture stomatitis was evaluated in group of patients satisfactory and regular response was obtained, indicating that the extract of P. granatum may be used as a topical antifungal agent for the treatment of candidosis associated with denture stomatitis (de Souza et al., 2003).
Methanolic extract of pomegranate peels showed marked reducing power in potassium ferricyanide reduction method. Acetone extract showed the highest antibacterial activity, followed by methanol and water extract (Negi and Jayaprakasha, 2003). Ethyl acetate acetone, methanol and water extracts of dried and powdered pomegranate peels exhibited marked antioxidant capacity, but the water extract was the lowest. All the extracts decreased sodium azide mutagenicity in *Salmonella typhimurium* strains (TA100 and TA1535), either weakly or strongly. At 2500 μg/plate all the extracts showed strong antimutagenicity. The antimutagenicity of the water extract was followed by acetone, ethyl acetate and methanol extracts (Negi et al., 2003). Pretreatment of the rats with a methanolic extract of pomegranate peel at 50 mg/kg (in terms of catechin equivalents) followed by CCl₄ treatment caused preservation of catalase, peroxidase, and SOD to values comparable with control values, whereas lipid peroxidation was brought back by 54% as compared to control (Murthy et al., 2002).

Fermented juice, aqueous pericarp extract and cold-pressed or supercritical carbon dioxide extracted seed oil of organically grown pomegranates were assessed in vitro for possible chemopreventive or adjuvant therapeutic potential in human breast cancer. In both MCF-7 and MB-MDA-231 cells, fermented pomegranate juice polyphenols consistently showed about twice the anti-proliferative effect as fresh pomegranate juice polyphenols. Pomegranate seed oil effected 90% inhibition of proliferation of MCF-7 at 100 μg/ml medium, 75% inhibition of invasion of MCF-7 across a Matrigel membrane at 10 μg/ml, and 54% apoptosis in MDA-MB-435 estrogen receptor negative metastatic human breast cancer cells at 50 μg/ml. In a murine mammary gland organ culture, fermented juice polyphenols effected 47% inhibition of cancerous lesion formation induced by the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) (Kim et al., 2002).

*P. granatum* fruit rind powder (PGFRP) at the dose of 100 mg/kg orally as aqueous suspension was found to stimulate the cell-mediated and humoral components of the immune system in rabbits. PGFRP elicited an increase in antibody titer to typhoid-H antigen. It also enhanced the inhibition of leucocyte migration in Leucocyte Migration Inhibition test (Ross et al., 2001).

Soxhlet and cold water extracts of dried flowers of *P. granatum* reduced the heart rate and force of contraction of the isolated frog heart. The effect produced by both the
extracts was blocked by atropine which indicated the presence of cholinergic component in *P. granatum* flowers (Jolly et al., 2000). The pomegranate juice or its methanol eluate from C18 cartridge competed with 17beta-estradiol for estrogen receptor (ER) binding and also stimulated proliferation of human ER-positive cell (MCF-7) in vitro. Furthermore, they effectively increased uterine weight in the ovariectomized rat (Maru et al., 2001).

Ethanolic extracts of *P. granatum* was found to exhibit interesting alpha-amylase inhibitory activity (Prasanth et al., 2001). The pomegranate fermented juice and cold pressed seed oil showed strong antioxidant activity close to that of butylated hydroxyanisole (BHA) and green tea (*Thea sinensis*), and significantly greater than that of red wine (*Vitis vinifera*). Flavonoids extracted from cold pressed seed oil showed 31-44% inhibition of sheep cyclooxygenase and 69-81% inhibition of soybean lipoxygenase (Schubert et al., 1999).

Tannin from the pericarp of *P. granatum* was found to be an effective component against genital herpes virus (HSV-2). The tannin not only inhibited HSV-2 replication, but also shown stronger effects of killing virus and blocking its absorption to cells (Zhang et al., 1995). Seven highly active inhibitors against carbonic anhydrase (CA, EC 4.2.1.1), punicalin, punicalagin, granatin B, gallagyldilactone, casuarinin, pedunculagin and tellimagrandin I, and four weakly active inhibitors, gallic acid, granatin A, corilagin and ellagic acid, were isolated from the pericarps of *P. granatum* (Satomi et al., 1993).

The aqueous extract of fruit rind of *P. granatum* was found to be more active on tape worms than on earth worms and round worms. The effect was 5-7 times lower than that of reference substance piperazine citrate (Hukkeri et al., 1993). Aqueous and alcohol extracts of the fruit rind of *P. granatum* exhibited significant activity in rats, when compared to loperamide hydrochloride, a standard anti-diarrhoeal drug (Pillai, 1992). Aqueous and methanolic extracts of *P. granatum* L. prevented implantation in 50% of rats (Prakash, 1986).
2.5. *Nelumbo nucifera* Gaertn.

**Family:** Nymphaeaceae

**Common names:** English-Chinese water lily, Indian lotus; Hindi-Ambuj, kamal, lalkamal, padam; Malayalam-Tamara; Konkani- Salloc; Sanskrit- Abja, kamala; Tamil- Ambal, Sivapputamarai, tamarai; Urdu-Nilufer.

**Description**

A large aquatic herb with slender, elongate, branched, creeping stems sending out roots at the nodes. Leaves membranous glabrous; petioles very long, rough with small distant prickles. Flowers solitary, 10-25 cm diameter, white or rosy, peduncles coming off from the node of the stem, sheathing at the base. Sepals small. Petals long elliptic, obtuse and finely veined.

**Distribution**

It is distributed throughout the warmer parts of India and from eastwards of Persia (Iran) to Australia.

**Uses in traditional medicine**

**Ayurveda:** the whole plant is sweet, cool; slightly bitter, gives tone to the breast, removes worms, allays thirst and used to treat fever, biliousness and vomiting. The root is bitter, it cures cough and biliousness and allays thirst and is cooling to the body. The stem is used to treat blood complaints, vomiting and leprosy. The tender leaves are bitter, cooling and useful in burning sensation of the body and used to treat piles and leprosy. The flower is sweet and cooling; it allays cough, thirst, blood defects, skin eruptions and symptoms of poisoning and used to treat fever and biliousness. The anthers are cooling, aphrodisiac, astringent and used to relieve diarrhoea, bleeding piles, inflammations and poisoning. It is useful to cure ulcers and sores of mouth. The fruit is bitter and astringent, sweet and cooling and prescribed to remove blood impurities and foul breath. The seeds are sweet and flavoury, astringent, slightly bitter and aphrodisiac. They are efficacious to overcome diarrhoea and dysentery, burning sensation of the body, vomiting and leprosy. The honey is an excellent tonic and useful in diseases of eye. The plant with other drugs is considered as antidote to snake and scorpion venoms (Kirtikar & Basu, 2000).
Unani: The root is used as diuretic; it is good in throat troubles, chest pain, spermatorrhoea, leucoderma and small pox. The white flower is a good tonic for the heart and brain, allays thirst; improves watery eyes; good in bronchitis and for internal injuries. The seeds are cool, diuretic, tonic to uterus and use to treat menorrhagia and leucorrhoea and used to treat fever and chest complaints.

The flowers of *N. nucifera* are used to treat diabetes, either as a single drug or in polyherbal formulations in Unani medicine (Hakim, 1997; Israel, 1977).

**Phytoconstituents and bioactivities**

(S)-arnnepavine (C19H23O3N; MW313) from *N. nucifera* inhibited PHA-induced cell proliferation and cytokine production in a major way by blocking membrane-proximal effectors such as Itk and PLC-γ in a PI-3K-dependent manner (Liu et al., 2007).

Kaempferol isolated from extracts of *N. nucifera* reduced reactive oxygen species levels and augmented GSH levels in a dose-dependent manner in the aged rat gingival tissues. Kaempferol was shown to effect a significant reduction in iNOS and TNF-alpha protein levels, as compared to control gingival tissue samples (Kim et al., 2007).

A methanol extract of the stamens of *N. nucifera* was shown to exert an inhibitory effect on rat lens aldose reductase. Molecules such as kaempferol 3-O-alpha-L-rhamnopyranosyl-(1-->6)-beta-d-glucopyranoside (5) and isorhamnetin 3-O-alpha-L-rhamnopyranosyl-(1-->6)-beta-d-glucopyranoside isolated from *N. nucifera* exhibited the highest degree of rat lens aldose reductase inhibitory activity in vitro (Lim et al., 2006).

The inhibitory effects of isoliensinine, a bisbenzylisoquinoline alkaloid extracted from the seed embryo of the traditional Chinese medicinal herb *N. nucifera* on the proliferation of porcine coronary arterial smooth muscle cells induced by angiotensin II (Xiao et al., 2006).

In this study, we isolated two new isorhamnetin glycosides, designated as nelumboroside A and nelumboroside B, as well as the previously-characterized isorhamnetin glucoside and isorhamnetin rutinoside, from the n-BuOH fraction of Nelumbo nucifera stamens showed marked antioxidant activities in the DPHH, and ONOO- assays (Hyun et al., 2006).

*N. nucifera* leaves extract impaired digestion, inhibited absorption of lipids and
carbohydrates, accelerated lipid metabolism and up-regulated energy expenditure. It prevented the increase in body weight, parametrial adipose tissue weight and liver triacylglycerol levels in mice with obesity induced by a high-fat diet (Ono et al., 2006). The effects of (S)-armepavine isolated from *N. nucifera* on MRL/MpJ-lpr/lpr mice, which have similar disease features to human systemic lupus erythematosus (SLE). MRL/MpJ-lpr/lpr mice were treated orally with (S)-armepavine for 6 weeks and their SLE characteristics were evaluated. The results revealed that (S)-armepavine prevented lymphadenopathy and elongated life span of MRL/MpJ-lpr/lpr mice. It seemed to be mediated by inhibition of splenocytes proliferation, suppression of interleukin-2 (IL-2), interleukin-4, interleukin-10, and interferon-gamma (IFN-gamma) gene expressions, reduction of glomerular hypercellularity (Liu et al., 2006).

Antioxidant activity of hydro alcoholic extract of *N. nucifera* seeds was studied using in vitro and in vivo models. The HANN exhibited strong free radical scavenging activity as evidenced by the low IC$_{50}$ values in both DPPH (1,1-diphenyl-2-picryl hydrazyl) (6.12 ± 0.41 µg/ml) and nitric oxide (84.86 ± 3.56 µg/ml) methods. Administration of HANN to Wistar rats at 100 and 200 mg/kg body weight for 4 days prior to carbon tetrachloride treatment caused a significant dose dependent increase (p < 0.05 to p < 0.001) in the level of superoxide dismutase and catalase and a significant decrease (p < 0.05 to p < 0.001) in the level of thiobarbituric acid reactive substances, when compared to CCl$_4$ treated control in both liver and kidney (Rai et al., 2006).

Inhibitory effects of ethanolic extract of *N. nucifera* on herpes simplex virus type 1 (HSV-1) replication were investigated. By a bioassay-guided fractionation procedure, NN-B-5 was identified from seeds of *N. nucifera*. NN-B-5 significantly blocked HSV-1 multiplication in HeLa cells without apparent cytotoxicity (Kuo et al., 2005).

The procyanidins of nonedible parts of lotus (*N. nucifera*) were determined for the first time. The extract contained monomers, dimers, and tetramers of procyanidins, in which the amounts of dimers are greatest, and catechin and epicatechin are the base units. The effects of the procyanidins on lipid autoxidation, lipoxygenase activities, and free radical scavenging were also studied. The results showed that 0.1% procyanidins have a strong antioxidant activity in a soybean oil system, better than BHT at the same concentration; inhibited lipoxygenase activity by > 90% at a concentration of 62.5 µg/mL, with an IC$_{50}$
value of 21.6 μg/mL; and had IC$_{50}$ inhibitory values rate to hydroxyl radical of 10.5 mg/L and a scavenging effect on superoxide of 17.6 mg/L (Ling et al., 2005).

Antioxidant activities of the Korean traditional lotus liquor made from lotus blossom and leaves were investigated. The antioxidant activities are dose-dependent and reached a plateau (about 80% inhibition) when the concentration of lotus liquor exceeded 25 μg in a modified linoleic acid peroxidation induced by haemoglobin. The scavenging activities of DPPH exerted by lotus liquor as well as alpha-tocopherol were tested. Linear response curves were also obtained and the IC$_{50}$ were estimated as 5.6 μg for alpha-tocopherol, 17.9 μg for lotus liquor. Lotus liquor also has a potent superoxide radical scavenging activity, with value of 0.93 unit/mg as superoxide dismutase equivalents. The IC$_{50}$ was estimated as 1.07 ± 0.04 mg for lotus liquor (Lee et al., 2005).

Isoliensinine exerted antiproliferative effect on porcine coronary arterial smooth muscle cells induced by phenylephrine, and its mechanisms were related to decrease the overexpression of growth factors (PDGF-beta, bFGF), protooncogene (c-fos, c-myc) and hsp70 (Xiao et al., 2005). The effects of isoliensinine, a bisbenzylisoquinoline alkaloid extracted from the Chinese traditional medicine seed embryo of N. nucifera on bleomycin-induced pulmonary fibrosis in mice were investigated. Administration of isoliensinine remarkably suppressed the increase in hydroxyproline content and abated the lung histological injury induced by bleomycin. Isoliensinine could obviously enhance the SOD activity and decrease the MDA level in a concentration-dependent manner. Moreover, isoliensinine also significantly inhibited the over-expression of TNF-α and TGF-β1 induced by bleomycin (Xiao et al., 2005a).

(±)-1(R)-Coclaurine (1) and (−)-1(S)-norcoclaurine (2), together with quercetin 3-O-beta-D-glucuronide (3), were isolated from the leaves of N. nucifera (Nymphaccae), and identified as anti-HIV principles. Compounds 1 and 2 demonstrated potent anti-HIV activity with EC$_{50}$ values of 0.8 and <0.8 μg/mL, respectively, and therapeutic index (TI) values of >125 and >25, respectively. benzylisoquinoline, aporphine, and bisbenzylisoquinoline alkaloids, including liensinine (4), negferine (5), and isoliensinine (6), which were previously isolated from the leaves and embryo of N. nucifera, were evaluated for anti-HIV activity. Compounds 4-6 showed potent anti-HIV activities with EC$_{50}$ values of <0.8 μg/mL and TI values of >9.9, >8.6, and >6.5, respectively.
Nuciferine, an aporphine alkaloid, had an EC\textsubscript{50} value of 0.8 \(\mu\)g/mL and TI of 36 (Kashiwada et al., 2005).

The extracts from \textit{N. nucifera} used in treatment of tissue inflammation in traditional Chinese medicine, inhibited human peripheral blood mononuclear cells proliferation activated with phytohemagglutinin. By a bioassay-guided fractionation procedure, NN-B-4 identified from \textit{N. nucifera} ethanolic extracts significantly suppressed activated human peripheral blood mononuclear cells proliferation (Liu et al., 2004).

A dose-dependent protective effect against reactive oxygen species induced cytotoxicity was observed when Caco-2 cells were treated with 10 mM H\textsubscript{2}O\textsubscript{2} in combination with the methanol extract of the lotus leaf (0.1-0.3 mg/ml). In vitro assay revealed that the extract exhibited scavenging activities on free radicals and hydroxyl radicals, and metal binding ability as well as reducing power, which may explain in part the mechanism behind the extract's ability to protect cells from oxidative damage (Wu et al., 2003).

Lotus plumule and blossom possessed strong reducing powers and free radical scavenging abilities. Methanol extract of lotus plumule exhibited ferrous ion chelating capabilities, which might contribute to the difference in antioxidant activities between lotus plumule and lotus blossom when analyzing the preventive effects on fatty acid peroxidation (Wang et al., 2003).

Ethanol extracts from \textit{N. nucifera} showed potent free radical scavenging effects with a median inhibition concentration of 6.49 \(\mu\)g/ml. Treatment of hepatocytes with extracts of \textit{N. nucifera} inhibited both the production of serum enzymes and cytotoxicity by CCl\textsubscript{4}. The genotoxic and cytotoxic effects of aflatoxin B1 were also inhibited by extract in dose-dependent manner (Sohn et al., 2003).

A methanol extract of the stamens of \textit{N. nucifera} showed strong antioxidant activity in the peroxynitrites system, and marginal activity in the DPPH and total reactive oxygen species systems. Kaempferol showed good activity in all models, compounds kaempferol 3-O-beta-D-glucuronopyranosyl methylester and kaempferol 3-O-beta-D-glucuronopyranoside exhibited activity against DPPH and peroxynitrites tests. Compounds kaempferol 3-O-beta-D-glucopyranoside and kaempferol 3-O-beta-D-galactopyranoside showed activity against only peroxynitrites test (Jung et al., 2003).
The antiarrhythmic effect of daurisoline and neferine which is an alkaloid isolated from *N. nucifera* and their mechanisms of actions have been studied. The antiarrhythmic effect of daurisoline is more potent than that of dauricine (Qian, 2002). Lotusine concentration-dependently increased the cyclic AMP level of the rat myocardium. The effect of lotusine was stronger than that of amrinone (Zhao et al., 2002). *N. nucifera* showed some thyroregulatory activity. It lowered the serum concentrations of thyroid hormones and glucose (Tahiliani and Kar, 2000).

Oral administration of the ethanolic extract of rhizomes of *N. nucifera* markedly reduced the blood sugar level of normal, glucose-fed hyperglycemic and streptozotocin-induced diabetic rats. The extract improved glucose tolerance and potentiated the action of exogenously injected insulin in normal rats (Mukherjee et al., 1997). The anti-inflammatory activity of the methanol extract of *N. nucifera* rhizome as well as of betulinic acid, a steroidal triterpenoid isolated from it, were evaluated on carrageenin and serotonin induced rat paw edema. Methanol extract at doses of 200 and 400 mg/kg and betulinic acid at doses of 50 mg/kg and 100 mg/kg p.o., showed significant antiinflammatory activity in both the models of inflammation in rats (Mukherjee et al., 1997a).

The petroleum ether extract of the seeds of *N. nucifera* and its fractions were administered orally to sexually immature female rats and mature male rats on alternate day for 15 days. The treatment caused a remarkable delay in sexual maturation in prepubertal female rats as evidenced from age of vaginal opening and first estrus (cornified smear) and a significant reduction in the sperm count and motility in mature male rats. In both the cases treatment resulted in accumulation of cholesterol and ascorbic acid and reduction in DELTA-5-3-beta-hydroxysteroid dehydrogenase and glucose-phosphate dehydrogenase activity in the ovary and testis of female and male rat respectively (Gupta et al., 1996).

The crude protein of the *N. nucifera* seeds was found to be 14.81%. Moreover the crude protein caused a significant decrease (44.80%) in the blood glucose level of diabetic albino rats after two weeks of treatment (Ibrahim and El-Eraqy, 1996). Neferine, a hypotensive agent with antiarrhythmic action, is a dibenzyl isoquinoline alkaloid isolated from Chinese medicinal herb *N. nucifera*. Neferine was shown to significantly inhibit
rabbit platelet aggregation induced by ADP, collagen, arachidonic acid and platelet-activating factor (PAF) with IC-50 of 16, 22, 193 and 103 μmol/L. Neferine was found to increase vascular 6-keto-PGF-1α and platelet cAMP levels in dose-dependent manner, but inhibit AA stimulated TXA-2 release from platelets (Yu and Hu, 1997).

The ethanol extract of *N. nucifera* was intraperitoneally administered and was significantly and dose dependently effective in eliminating superoxides in the plasma (Yoshizaki *et al.*, 1996). Methanolic extract of rhizomes of *N. nucifera* was investigated for different psychopharmacological actions in rats and mice. The extract was found to cause reduction in spontaneous activity, decrease in exploratory behaviour pattern by the head dip and Y-maze test, reduction in muscle relaxant activity by rotarod, 30 degree inclined screen and traction test and potentiated the pentobarbitone induced sleeping time in mice significantly (Mukherjee *et al.*, 1996).

The aerial parts of the *N. nucifera* were found to contain quercetin, myricetin, kaempferol 3-0 glucoside, quercetin 3-0-glucoside and luteolin 7-0-glucoside. The glycosidal flavonoids kaempferol 3-0 glucoside and luteolin 7-0-glucoside showed significant antimicrobial, anti-inflammatory and analgesic activities with no ulcerations (Wassel *et al.*, 1996).

Antipyretic activity of methanolic extract of rhizome of *N. nucifera* was studied on normal body temperature and yeast induced pyrexia in rats. Yeast suspension (10 ml/kg, sc) increased rectal temperature after 19 hr of administration. The extract, in doses of 200, 300 or 400 mg/kg (po) produced significant dose dependent lowering of normal body temperature and yeast provoked elevation of body temperature in rats (Mukherjee *et al.*, 1996a). Methanolic extract of rhizomes of *N. nucifera* treated animals showed significant inhibitory activity against castor oil induced diarrhoea and inhibited significantly PGE-2 induced enteropooling in rats. It also showed significant reduction in gastrointestinal motility following charcoal meal in rats (Mukherjee *et al.*, 1995).

A reduction of triglyceride and cholesterol was seen after treatment with a decoction of *N. nucifera* (La *et al.*, 1995). Linaelinesine an alkaloid extracted from the green seed embryo of *N. nucifera* 100 μmol/L was shown to concentration-dependently decrease the action potential amplitude, the maximal velocity of phase 0 depolarization and prolong the sinus
cycle length of slow action potential in isolated sinoatrial node pacemaker cells of rabbits (Wang et al., 1993).

Liensinine, an alkaloid extracted from the green seed embryo of *N. nucifera* has been shown to have anti-arrhythmic action; its mechanism may be related to blockade of Ca\(^{2+}\) Na\(^{+}\) influx. Liensinine 3 mg/kg i.v. may temporarily inhibit all parameters of haemodynamics in anesthetized or pithed rats. The degrees of inhibitory effect of Liensinine 12 mg/kg on all haemodynamic parameters nearly corresponded to these of verapamil 1 mg/kg. Liensinine 100 μmol/L reduced the contractile force of isolated left atria and the spontaneously beating rate of isolated right atria of rabbits in concentration-dependent manner (Wang et al., 1992).

Rhizomes of lotus had potent antifungal activity against *Aspergillus niger*, *Trichoderma viride*, and *Penicillium* spp. (Matthews and Hass, 1993). Petroleum ether extract of seeds of *N. nucifera*, administered (ip) to sexually mature female albino swiss mice at a dose of 3 mg/kg body weight on alternate days for 15 days after 18 hr of fasting, exhibited significant contraceptive, antiestrogenic and antiprogestational activities (Mazumder et al., 1992). Neferine, an alkaloid extracted from the green seed embryo of *N. nucifera* Gaertn, has been shown to have anti-arrhythmic action. Neferine 1-10 mg/kg IV dose-dependently decreased the monophasic action potential amplitude, prolonged the monophasic action potential duration. It also decreased LVP, dP/dt, prolonged SCL, and reduced arterial blood pressure in a dose-dependent manner. These effects were similar to those of quinidine, and different from tetrandrine (Li et al., 1990).

Effects of neferine on transmembrane potentials of guinea-pig myocardium (Li et al., 1989) and on transmembrane potential in rabbit sinoatrial nodes and clusters of cultured myocardial cells from neonatal rats were studied. Neferine has an inhibitory effect on the slow trans-membrane Na\(^{+}\) and/or Ca\(^{2+}\) current of myocardium (Li et al., 1989a).

Neferine possesses a hypotensive effect in experimental animals. Nepferine (2 mg/kg) given intravenously to rats decreased only blood pressure markedly. At the dose of 0.6 mg/kg neferine produced an obvious reduction of the peripheral resistance in the perfused hind legs of cats. In addition, the extent of decreasing diastolic blood pressure was greater than that of systolic blood pressure (Hu et al., 1989).
Neferine 100 μmol/L, in a concentration-dependent manner, inhibited the force of contraction and excitability, prolonged the functional refractory period on the left atrium, and reduced the automaticity and contractility of the right atrium. Neferine 30 μmol/L suppressed and shifted the isoprenaline dose-effect curve to the right non-parallelly, an action different from the action of propranolol. Neferine, as verapamil, showed dualistic action in Ca²⁺ antagonism (Li et al., 1988).

The antihemorrhagic principle in Nelumbins Receptaculum, dried receptacle of N. nucifera was isolated by a combination of partition, gel filtration through Sephadex LH-20 and column chromatography over silica gel, and identified as quercetin [2-(3,4-dihydroxy)-3,5,7-trihydroxy-4H-1-benzopyran-4-one] (Ishida et al., 1988).

Effects of neferine on isolated rabbit myocardium were investigated. 30 min after adding neferine 30 μmol/L, the contractility of the papillary muscle decreased from 100% of control to 60 ± SD 10% and the duration-intensity curve was shifted to the right (indicating the decrease of excitability, P < 0.01) while the concentration of epinephrine inducing automaticity was increased 12 ± 1 times (p < 0.01) and the functional refractory period was prolonged from 196 ± 8 ms to 238 ± 4 ms (p < 0.01). 10 min after neferine 0.3 mmol/L was administrated the positive staircase of the left atrium was markedly inhibited, and the post-rest potentiation was decreased to 27 ± 16% 20 min later (Li et al., 1988a).

Rats fed on with a high fat diet containing 1.5% cholesterol and 1.0% cholic acid was treated with N. nucifera aqueous extract for 5 days. Significant decreases in serum total cholesterol, free cholesterol and phospholipids were observed in the high fat-loaded groups given the extract. Total cholesterol in the liver did not show any reduction in the groups received the extract (Onishi et al., 1984). The alkaloid L-5,6-dimethoxyaporphine (L-nuciferine) [obtained from N. nucifera] was studied with iontophoretic techniques, and its interaction with excitant amino acids on neurons of the pigeon [Columba livia] optic tectum was tested. This compound antagonizes the action of L-glutamate and, to a weaker extent, that of L-aspartate and D,L-homocysteate. In comparison, L5,6-dimethoxyaporphine did not reduce the acetylcholine evoked excitation (Felix and Frangi et al., 1977).
A poly herbal formulation containing *N. nucifera* improved the fluid deformation, expand blood vessel, improve blood circulation, and reduce blood fat and viscosity, and can be used to treat cerebral thrombosis and cerebral infarction (Gao, 2005, Patent no. CN 1565606). A poly herbal formulation containing *N. nucifera* leaves lowered the blood sugar (Li, 2003, patent no. CN 1439383). The stamen extract of *N. nucifera* suppressed UV-B induced formation of interleukin-1α in mouse keratinocytes. The embryo extracts stimulated lipolysis activity in rat adipocytes (Ito, 2005). Lotus plant extract showed inhibition of α-glucosidase enzyme in vitro. And also prevented the rise of blood glucose in mice loaded with sugar (Tsuboi, 2005, Patent no. WO 2005041995).