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Le complicanze cardiovascolari nel diabete mellito: studio delle basi molecolari

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To my Family

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Abbreviation List

DCCT	Diabetes Control and Complications Trial
DFO	Deferoxamine
DM	Diabetes Mellitus
EDIC	Epidemiology of Diabetes Interventions and Complications study
GSK-3β	Glycogen Synthase Kinase 3 β
HAEC	Human Aortic Endothelial Cells
HbA1C	hemoglobin A1c
HK	Hexokinase
MnSOD	Manganese Superoxide Dismutase
PP2A	Protein Phosphatase 2A
ROS	Reactive Oxygen Species
VDAC	Voltage dependent Anions Channel

ABSTRACT

Diabetes is characterized by development of specific microvascular complications and by a high incidence of accelerated atherosclerosis.

The assumption underlying current clinical treatment is that lowering the level of time-averaged glucose concentration, measured as hemoglobin A1c (HbA1c), prevents the development and progression of microvascular complications. This current treatment recommendation, adopted by diabetes professional societies around the world, is based on data from the 1993 Diabetes Control and Complications Trial (DCCT).

Recent Diabetes Control and Complications Trial data analyses show that 89% of the variation in microvascular complications risk in type 1 diabetes is not captured by HbA1c values (time-averaged glucose concentration). Recent experimental evidence from Dr Brownlee's lab, shows that transient exposure to threshold levels of high glucose reprograms human endothelial cells to continue overproducing reactive oxygen species in the presence of physiologic glucose concentrations. This persistent ROS overproduction causes an equally persistent overexpression of pro-inflammatory genes in normal glucose due to hystone modifications in the proximal promoter of the NF-κB subunit p65.

Since in normal cells the epigenetic changes are rapidly reversed by histone demethylases and histone methyltransferases my thesis work aimed to understand how transient exposure to high glucose reprograms endothelial cells, and characterize the critical regulatory networks that shift vascular endothelial cells to a state of persistent excess ROS production after transient exposure to high glucose.

We found that transient spikes of hyperglycemia cause persistent mitochondrial overproduction of ROS during subsequent periods of prolonged normal glucose, causing persistent activation of the epigenetic changes and resultant vascular inflammatory gene expression. We identified a multi-component positive feedback loop induced by transient exposure to high glucose in human vascular endothelial cells which maintains persistently increased ROS production in normal glucose, thus we verified that transient disruption of any of the elements in the feed back loop rapidly restores the system to its normal state, including reversing persistent increased ROS production, persistent hyperglycemia-induced epigenetic changes and persistent increased NF-κB-dependent pro-inflammatory gene expression.

Our results highlight the dramatic and long-lasting effects that short-term hyperglycemic spikes can have on vascular cells and suggest that transient spikes of hyperglycemia may be an HbA1c–independent risk factor for diabetic complications. Moreover we understood the mechanism underlying the continue overproducing of reactive oxygen species in the presence of physiologic glucose concentrations in human endothelial cells. This knowledge will provide the basis for developing new type 1 diabetes treatment paradigms that more effectively prevent the development and progression of microvascular complications.

INTRODUCTION

Diabetes mellitus (DM) refers to a group of common metabolic disorders characterized by hyperglycemia. The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems, leading to end-stage renal disease (ESRD), non-traumatic lower extremity amputations, and adult blindness. It also predisposes to cardiovascular diseases.
The assumption underlying current clinical treatment is that lowering the level of time-averaged glucose concentration, measured as hemoglobin A1c (HbA1c), prevents the development and progression of microvascular complications. This current treatment recommendation, adopted by diabetes professional societies around the world, is based on data from the 1993 Diabetes Control and Complications Trial (DCCT), where intensive therapy reduced the risk of sustained retinopathy progression by 73% compared with standard treatment. After the announcement of the DCCT results, many patients who had been in standard therapy group adopted more intensive therapeutic regiments, and their level of glycemic control improved. The post-DCCT HbA1c values for both groups have become nearly identical during the approximate 10 years of follow-up in the Epidemiology of Diabetes Interventions and Complications Study (EDIC). Surprisingly, the effects of 6.5-years difference in HbA1c, during the DCCT on the incidence if retinopathy and nephropathy, have persisted. People in standard treatment group continued to have a higher incidence of microvascular complications even with an improvement in glycemic control during the EDIC. HbA1c and duration of diabetes (glycemic exposure) explained only about 11% of the variation in retinopathy risk for the entire study population, suggesting that the remaining 89% of the variation in risk is presumably explained by other factors not captured by measurement of HbA1c.
What factors not captured by HbA1c measurements might explain the remaining 89% of microvascular complications risk for people with type 1 diabetes? Since HbA1c represents the time-averaged mean level of glycemia, it provides no information about how closely the fluctuations of blood glucose levels around that mean mimic the normal narrow range of blood glucose excursion, and patients with identical HbA1c values differ significantly in amplitude and duration of glycemic spikes.

Oxidative stress plays a pivotal role in the development of diabetes vascular and neurologic complications. High intracellular concentrations of glucose cause increased superoxide production from the mitochondria. This increased superoxide production causes the activation of the five major pathways implicated in the pathogenesis of complications over the past 40 years: polyol pathway flux, increased formation of AGEs (advanced glycation end products), increased expression of the receptor for AGEs, (RAGE) and its activating ligands, activation of protein kinase C isoforms, and overactivity of the hexosamine pathway. Through these pathways, increased intracellular reactive oxygen species (ROS) cause defective angiogenesis in response to ischemia and activate a number of proinflammatory pathways as demonstrated by overexpression of superoxide dismutase in transgenic diabetic mice that prevents diabetic retinopathy, nephropathy, and cardiomyopathy.

Experimental evidence both in cultured primary human endothelial cells and in non-diabetic mice shows that short exposure to hyperglycemia (6-16 hr) triggers persistent increases in proinflammatory gene expression during long subsequent periods of normal glycemia (6 days) by inducing persistent epigenetic changes in the proximal promoter of NF-κB p65,

increasing p65 expression. This in turn causes persistent increased expression of the proinflammatory proteins monocyte chemotactic protein-1, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, interleukin-6, and inducible nitric oxide synthase.
These persistent changes in gene expression are induced by spikes of hyperglycemia that have durations too short (6-16 hours followed by 6 days of normal glycemia) to influence HbA1C values. Since regulation of gene expression by methylation of histone lysines is rapidly reversible by the action of demethlases and histone methyltransferases, however, the mechanism responsible for the persistence of these epigenetic changes has remained puzzling.
The ability of hyperglycemia to elicit epigenetic changes may be associated with diabetes complications. Further exploration into epigenetic mechanisms can yield new insights into the pathogenesis of diabetes and its complications and uncover potential therapeutic targets and treatment options to prevent the continued development of diabetic complications even after glucose control has been achieved.

AIM OF THE STUDY

The existing paradigm for treatment of type 1 diabetes is to lower hemoglobin A1c values, based on the assumption that hemoglobin A1c explains a large proportion of microvascular complication risk. However, recent analysis of the Diabetes Control and Complications Trial data shows that 89% of the variation in retinopathy risk in type 1 diabetes is not captured by HbA1c values (time-averaged glucose concentration).

Transient exposure to threshold levels of high glucose reprograms human endothelial cells to continue overproducing reactive oxygen species in the presence of physiologic glucose concentrations for many days. This persistent ROS overproduction causes an equally persistent overexpression of pro-inflammatory genes in normal glucose. The specific aims of this thesis are to identity and characterize critical regulatory networks that shift vascular endothelial cells to a state of persistent excess ROS production after transient exposure to high glucose.

CHAPTER 1

Diabetes Mellitus

Diabetes mellitus (DM) refers to a group of common metabolic disorders characterized by hyperglycemia. Depending on the etiology of the DM, factors contributing to hyperglycemia include reduced insulin secretion, decreased glucose utilization, and increased glucose production. The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems, leading to end-stage renal disease (ESRD), nontraumatic lower extremity amputations, and adult blindness. It also predisposes to cardiovascular diseases.

1.1 Classification

DM is classified on the basis of the pathogenic process that leads to hyperglycemia, as opposed to earlier criteria such as age of onset or type of therapy. The two broad categories of DM are designated type 1 and type 2. Type 1 DM is the result of complete or near-total insulin deficiency. Type 2 DM is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production and is preceded by a period of abnormal glucose homeostasis classified as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT).

Table 1 Etiologic Classification of Diabetes Mellitus

I. Type 1 diabetes (beta cell destruction, usually leading to absolute insulin deficiency)
- A. Immune-mediated
- B. Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly insulin secretory defect with insulin resistance)

III. Other specific types of diabetes

A. Genetic defects of beta cell function characterized by mutations in:
1. Hepatocyte nuclear transcription factor (HNF) 4α (MODY 1)
2. Glucokinase (MODY 2)
3. HNF-1α (MODY 3)
4. Insulin promoter factor-1 (IPF-1; MODY 4)
5. HNF-1β (MODY 5)
6. NeuroD1 (MODY 6)
7. Mitochondrial DNA
8. Subunits of ATP-sensitive potassium channel
9. Proinsulin or insulin

B. Genetic defects in insulin action
1. Type A insulin resistance
2. Leprechaunism
3. Rabson-Mendenhall syndrome
4. Lipodystrophy syndromes

C. Diseases of the exocrine pancreas—pancreatitis, pancreatectomy, neoplasia, cystic fibrosis, hemochromatosis, fibrocalculous pancreatopathy, mutations in carboxyl ester lipase

D. Endocrinopathies—acromegaly, Cushing's syndrome, glucagonoma, pheochromocytoma, hyperthyroidism, somatostatinoma, aldosteronoma

E. Drug- or chemical-induced—glucocorticoids, vacor (a rodenticide), pentamidine, nicotinic acid, diazoxide, β-adrenergic agonists, thiazides, hydantoins, asparaginase, α-interferon, protease inhibitors, antipsychotics (atypicals and others), epinephrine

F. Infections—congenital rubella, cytomegalovirus, coxsackievirus

G. Uncommon forms of immune-mediated diabetes— "stiff-person" syndrome, anti-insulin receptor antibodies

H. Other genetic syndromes sometimes associated with diabetes— Wolfram's syndrome, Down's syndrome, Klinefelter's syndrome, Turner's syndrome, Friedreich's ataxia, Huntington's chorea, Laurence-Moon-Biedl syndrome, myotonic dystrophy, porphyria, Prader-Willi syndrome

IV. Gestational diabetes mellitus (GDM)

Two features of the current classification of DM diverge from previous classifications. First, the terms *insulin-dependent diabetes mellitus* (IDDM) and *non-insulin-dependent diabetes mellitus* (NIDDM) are obsolete. Since many individuals with type 2 DM eventually require insulin treatment for control of glycemia, the use of the term NIDDM generated considerable confusion. A second difference is that age is not a criterion in the classification system. Although type 1 DM most commonly develops before the age of 30, an autoimmune beta cell destructive process can develop at any age. It is estimated that between 5 and 10% of individuals who develop DM after age 30 years have type 1 DM. Although type 2 DM more typically develops with increasing age, it is now being diagnosed more frequently in children and young adults, particularly in obese adolescents (Harrison's Principles of Internal Medicine textbook, 18e 2011).

1.2 Epidemiology

The worldwide prevalence of DM increased dramatically over the past 20 years, from ~ 30 million cases in 1985 to about 285 million in 2010. Approximately 1.6 million individuals (>20 years) were newly diagnosed with diabetes in 2010. Based on current trends, the International Diabetes Federation expects that 438 million individuals will have diabetes by the year 2030. Among type 1 and type 2 DM, the prevalence of type 2 DM is rising much more rapidly, presumably because of increasing obesity and the aging of the population. DM increases with aging. In 2010, the prevalence of DM in the United Sates was estimated to be 0.2% in individuals aged <20 years and 11.3% in individuals aged >20 years. In individuals aged >65 years, the prevalence of DM was 26.9%. The prevalence is similar in men and women throughout most age ranges (11.8% and 10.8%, respectively, in individuals aged >20 years). Worldwide estimates project that in 2030 the greatest number of individuals with diabetes will be aged 45–64 years (Harrison's Principles of Internal Medicine textbook, 18e 2011).

1.3 Insulin biosynthesis, secretion and action

1.3.1 Biosynthesis

Insulin is produced in the beta cells of the pancreatic islets. It is initially synthesized as a single-chain 86-aminoacid precursor polypeptide, known as preproinsulin. Subsequent proteolytic processing removes the N-terminal signal peptide, generating proinsulin. Proinsulin is structurally related to insulin-like growth factors I and II, which bind weakly to the insulin receptor. Cleavage of an internal 31-residue fragment from proinsulin generates the C peptide and the A (21 amino acids) and B (30 amino acids) chains of insulin, which are connected by disulfide bonds. The mature insulin molecule and C peptide are stored together and co-secreted from secretory granules in the beta cells. Because C peptide is cleared more slowly than insulin, it is a useful marker of insulin secretion and allows discrimination of endogenous and exogenous sources of insulin in the evaluation of hypoglycemia. Pancreatic beta cells co-secrete islet amyloid polypeptide (IAPP) or amylin, a 37-amino-acid peptide, along with insulin. The role of IAPP in normal physiology is not fully defined, but it is the major component of the amyloid fibrils found in the islets of patients with 2 DM, and an analogue is sometimes used in treating type 1 and type 2 DM. Human insulin is produced by recombinant DNA technology; structural alterations at one or more amino acid residues modify its physical and pharmacologic characteristics.

1.3.2 Secretion

Glucose is the key regulator of insulin secretion by the pancreatic beta cell, although several other molecules (e.g. amino acids, ketones, various nutrients, gastrointestinal peptides, and neurotransmitters) also influence insulin secretion. Glucose levels >3.9 mmol/L (70 mg/dL) stimulate insulin synthesis, primarily by enhancing protein translation and processing. Glucose stimulation of insulin secretion begins with its transport into the beta cell by a facilitative glucose transporter (Fig.1.1). Glucose phosphorylation by glucokinase is the rate-limiting step that controls glucose-regulated insulin secretion. Further metabolism of glucose-6-phosphate via glycolysis generates ATP, which inhibits the activity of an ATP-sensitive K^+ channel. This channel consists of two separate proteins: one is the binding site for certain oral hypoglycemics (e.g., sulfonyl-ureas, meglitinides); the other is an inwardly rectifying K^+ channel protein. Inhibition of this K^+ channel induces beta cell membrane depolarization, which opens voltage-dependent calcium channels (leading to an influx of calcium), and stimulates insulin secretion. Insulin secretory profiles reveal a pulsatile pattern of hormone release, with small secretory bursts occurring about every 10 min, superimposed upon greater amplitude oscillations of about 80–150 min. Incretins are released from neuroendocrine cells of the gastrointestinal tract following food ingestion and amplify glucose-stimulated insulin secretion and suppress glucagon secretion. Glucagon-like peptide 1 (GLP-1), the most potent incretin, is released from L cells in the small intestine and stimulates insulin secretion only when the blood glucose is above the fasting level. Incretin analogues, are used to enhance endogenous insulin secretion.

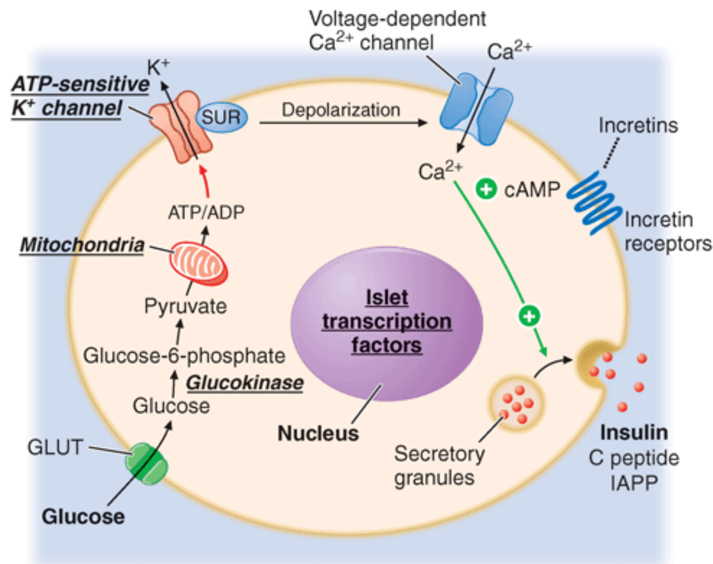


Figure 1.1 Mechanisms of glucose-stimulated insulin secretion and abnormalities in diabetes. Glucose and other nutrients regulate insulin secretion by the pancreatic beta cell. Glucose is transported by a glucose transporter (GLUT1 in humans, GLUT2 in rodents); subsequent glucose metabolism by the beta cell alters ion channel activity, leading to insulin secretion. The SUR receptor is the binding site for some drugs that act as insulin secretagogues. SUR, sulfonylurea receptor; ATP, adenosine triphosphate; ADP, adenosine diphosphate, cAMP, cyclic adenosine monophosphate. IAPP, islet amyloid polypeptide or amylin.

1.3.3 Action

Once insulin is secreted into the portal venous system, ~50% is removed and degraded by the liver. Unextracted insulin enters the systemic circulation where it binds to receptors in target sites. Insulin binding to its receptor stimulates intrinsic tyrosine kinase activity, leading to receptor autophosphorylation and the recruitment of intracellular signaling molecules, such as insulin receptor substrates (IRS) (Fig.1.2). IRS and other adaptor proteins initiate a complex cascade of phosphorylation and dephosphorylation reactions, resulting in the widespread metabolic and mitogenic effects of insulin. As an example, activation of the phosphatidylinositol-3′-kinase (PI-3-kinase) pathway stimulates translocation of a facilitative glucose transporter (e.g., GLUT4) to the cell surface, an event that is crucial for glucose uptake by skeletal muscle and fat. Activation of other insulin receptor signaling pathways induces glycogen synthesis, protein synthesis, lipogenesis, and regulation of various genes in insulin-responsive cells.

Glucose homeostasis reflects a balance between hepatic glucose production and peripheral glucose uptake and utilization. Insulin is the most important regulator of this metabolic equilibrium, but neural input, metabolic signals, and other hormones (e.g.,glucagon) result in integrated control of glucose supply and utilization. In the fasting state, low insulin levels increase glucose production by promoting hepatic gluconeogenesis and glycogenolysis and reduce glucose uptake in insulin-sensitive tissues (skeletal muscle and fat), thereby promoting mobilization

of stored precursors such as amino acids and free fatty acids (lipolysis). Glucagon, secreted by pancreatic alpha cells when blood glucose or insulin levels are low, stimulates glycogenolysis and gluconeogenesis by the liver and renal medulla. Postprandially, the glucose load elicits a rise in insulin and fall in glucagon, leading to a reversal of these processes. Insulin, an anabolic hormone, promotes the storage of carbohydrate and fat and protein synthesis. The major portion of postprandial glucose is utilized by skeletal muscle, an effect of insulin-stimulated glucose uptake. Other tissues, most notably the brain, utilize glucose in an insulin-independent fashion (Harrison's Principles of Internal Medicine textbook, 18e 2011).

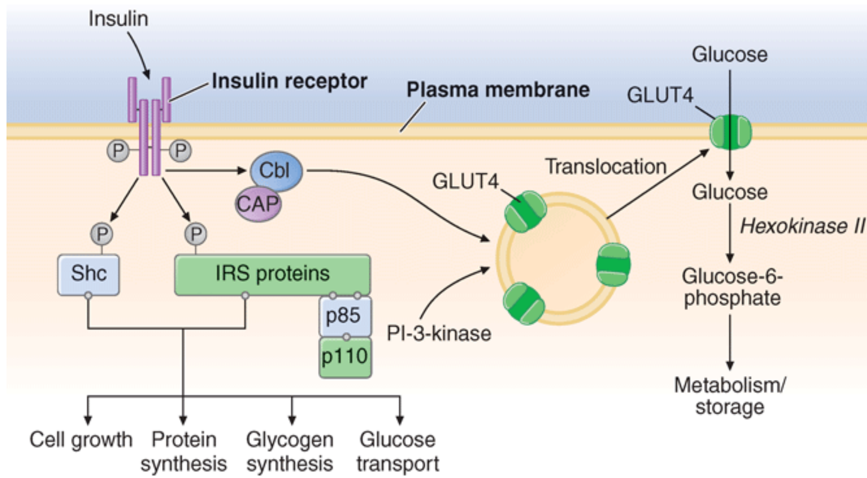


Figure 1.2 Insulin signal transduction pathway in skeletal muscle. The insulin receptor has intrinsic tyrosine kinase activity and interacts with insulin receptor substrates (IRS and Shc) proteins. A number of "docking" proteins bind to these cellular proteins and initiate the metabolic actions of insulin [GrB-2, SOS, SHP-2, p110, and phosphatidylinositol-3′-kinase (PI-3-kinase)]. Insulin increases glucose transport through PI-3-kinase and the Cbl pathway, which promotes the translocation of intracellular vesicles containing GLUT4 glucose transporter to the plasma membrane

1.4. Type 1 DM

Type 1 DM is the result of interactions of genetic, environmental, and immunologic factors that ultimately lead to the destruction of the pancreatic beta cells and insulin deficiency. Type 1 DM results from autoimmune beta cell destruction, and most, but not all, individuals have evidence of islet-directed autoimmunity. This autoimmune process is thought to be triggered by an infectious or environmental stimulus and to be sustained by a beta cell–specific molecule. In the majority, immunologic markers appear after the triggering event but before diabetes becomes clinically overt. Beta cell mass then begins to decrease, and insulin secretion progressively declines, although normal glucose tolerance is maintained. The rate of decline in beta cell mass varies widely among individuals, with some patients progressing rapidly to clinical diabetes and others evolving more slowly. Features of diabetes do not become evident until a majority of beta cells are destroyed (70–80%). At this point, residual functional beta cells exist but are insufficient in number to maintain glucose tolerance. The events that trigger the transition from glucose intolerance to frank diabetes are often associated with increased insulin requirements, as might occur during infections or puberty. After the initial clinical presentation of type 1 DM, a "honeymoon" phase may ensue during which time glycemic control is achieved with modest doses of insulin or, rarely, insulin is not needed. However, this fleeting phase of endogenous insulin production from residual beta cells disappears as the autoimmune process destroys remaining beta cells, and the individual becomes insulin deficient. Some individuals with long-standing type 1 diabetes produce a small amount of insulin (as reflected by C-peptide production) and some individuals have insulin-positive cells in the pancreas at autopsy.

1.4.1 Genetic Considerations

Susceptibility to type 1 DM involves multiple genes. The concordance of type 1 DM in identical twins ranges between 40 and 60%, indicating that additional modifying factors are likely involved in determining whether diabetes develops. The major susceptibility gene for type 1 DM is located in the HLA region on chromosome 6. Polymorphisms in the HLA complex account for 40–50% of the genetic risk of developing type 1 DM. This region contains genes that encode the class II major histocompatibility complex (MHC) molecules, which present antigen to helper T cells and thus are involved in initiating the immune response. The ability of class II MHC molecules to present antigen is dependent on the amino acid composition of their antigen-binding sites. Amino acid substitutions may influence the specificity of the immune response by altering the binding affinity of different antigens for class II molecules.

Most individuals with type 1 DM have the HLA DR3 and/or DR4 haplotype. Refinements in genotyping of HLA loci have shown that the haplotypes DQA1*0301, DQB1*0302, and DQB1*0201 are most strongly associated with type 1 DM. These haplotypes are present in 40% of children with type 1 DM as compared to 2% of the normal U.S. population. However, most individuals with predisposing haplotypes do not develop diabetes.

In addition to MHC class II associations, genome association studies have identified at least 20 different genetic loci that contribute susceptibility to type 1 DM

(polymorphisms in the promoter region of the insulin gene, the CTLA-4 gene, interleukin-2 receptor, *CTLA4*, and PTPN22, etc.). Genes that confer protection against the development of the disease also exist. The haplotype DQA1*0102, DQB1*0602 is extremely rare in individuals with type 1 DM (<1%) and appears to provide protection from type 1 DM.

Although the risk of developing type 1 DM is increased ten fold in relatives of individuals with the disease, the risk is relatively low: 3–4% if the parent has type 1 diabetes and 5–15% in a sibling (depending on which HLA haplotypes are shared). Hence, most individuals with type 1 DM do not have a first-degree relative with this disorder.

1.4.2 Pathophysiology

Although other islet cell types [alpha cells (glucagon-producing), delta cells (somatostatin-producing), or PP cells (pancreatic polypeptide-producing)] are functionally and embryologically similar to beta cells and express most of the same proteins as beta cells, they are spared from the autoimmune destruction. Pathologically, the pancreatic islets are infiltrated with lymphocytes (a process termed *insulitis*). After all beta cells are destroyed, the inflammatory process abates, the islets become atrophic, and most immunologic markers disappear. Studies of the autoimmune process in humans and in animal models of type 1 DM (NOD mouse and BB rat) have identified the following abnormalities in the humoral and cellular arms of the immune system: (1) islet cell autoantibodies; (2) activated lymphocytes in the islets, peripancreatic lymph nodes, and systemic circulation; (3) T lymphocytes that proliferate when stimulated with islet proteins; and (4) release of cytokines within the insulitis. Beta cells seem to be particularly susceptible to the toxic effect of some cytokines [tumor necrosis factor α (TNF-α), interferon γ, and interleukin 1 (IL-1)]. The precise mechanisms of beta cell death are not known but may involve formation of nitric oxide metabolites, apoptosis, and direct CD8+ T cell cytotoxicity. The islet destruction is mediated by T lymphocytes rather than islet autoantibodies, as these antibodies do not generally react with the cell surface of islet cells and are not capable of transferring DM to animals. Suppression of the autoimmune process at the time of diagnosis of diabetes slows the decline in beta cell destruction, but the safety of such interventions is unknown.

Pancreatic islet molecules targeted by the autoimmune process include insulin, glutamic acid decarboxylase (GAD, the biosynthetic enzyme for the neurotransmitter GABA), ICA-512/IA-2 (homology with tyrosine phosphatases), and a beta cell–specific zinc transporter (ZnT-8). Most of the auto-antigens are not beta cell–specific, which raises the question of how the beta cells are selectively destroyed. Current theories favor initiation of an autoimmune process directed at one beta cell molecule, which then spreads to other islet molecules as the immune process destroys beta cells and creates a series of secondary auto-antigens. The beta cells of individuals who develop type 1 DM do not differ from beta cells of normal individuals, since islets transplanted from a genetically identical twin are destroyed by a recurrence of the autoimmune process of type 1 DM.

1.4.3 Immunologic markers

Islet cell autoantibodies (ICAs) are a composite of several different antibodies directed at pancreatic islet molecules such as GAD, insulin, IA-2/ICA-512, and ZnT-8, and serve as a marker of the autoimmune process of type 1 DM. Assays for autoantibodies to GAD-65 are commercially available. Testing for ICAs can be useful in classifying the type of DM as type 1 and in identifying non-diabetic individuals at risk for developing type 1 DM. ICAs are present in the majority of individuals (>85%) diagnosed with new-onset type 1 DM, in a significant minority of individuals with newly diagnosed type 2 DM (5–10%), and occasionally in individuals with GDM (<5%). At present, the measurement of ICAs in non-diabetic individuals is a research tool because no treatments have been approved to prevent the occurrence or progression to type 1 DM. Clinical trials are testing interventions to slow the autoimmune beta cell destruction.

1.4.4 Environmental factors

Numerous environmental events have been proposed to trigger the autoimmune process in genetically susceptible individuals; however, none have been conclusively linked to diabetes. Identification of an environmental trigger has been difficult because the event may precede the onset of DM by several years. Putative environmental triggers include viruses (coxsackie, rubella, enteroviruses most prominently), bovine milk proteins and nitrosourea compounds.
(Harrison's Principles of Internal Medicine textbook, 18e 2011)

1.5 Type 2 DM

Insulin resistance and abnormal insulin secretion are central to the development of type 2 DM. Although the primary defect is controversial, most studies support the view that insulin resistance precedes an insulin secretory defect but that diabetes develops only when insulin secretion becomes inadequate. Type 2 DM likely encompasses a range of disorders with common phenotype of hyperglycemia. Most of our current understanding of the pathophysiology and genetics is based on studies of individuals of European descent. It is becoming increasing apparent that DM in other ethnic groups (Asian, African, and Latin American) has a different, but yet undefined, pathophysiology. In these groups, DM that is ketosisprone (often obese) or ketosis-resistant (often lean) is commonly seen.

1.5.1 Genetic Considerations

Type 2 DM has a strong genetic component. The concordance of type 2 DM in identical twins is between 70 and 90%. Individuals with a parent with type 2 DM have an increased risk of diabetes; if both parents have type 2 DM, the risk approaches 40%. Insulin resistance, as demonstrated by reduced glucose utilization in skeletal muscle, is present in many non-diabetic, first-degree relatives of individuals with type 2 DM. The disease is polygenic and multifactorial, since in addition to genetic susceptibility, environmental factors (such as obesity, nutrition, and physical activity) modulate the phenotype. The genes that predispose to type 2 DM are incompletely identified, but recent genome-wide association studies have identified a large number of genes that convey a relatively small risk for type 2 DM (>20 genes, each with a relative risk of 1.06–1.5). Most prominent is a variant of the transcription factor 7–like 2 gene that has been associated with type 2 diabetes in several populations and with impaired glucose tolerance in one population at high risk for diabetes. Genetic polymorphisms associated with type 2 diabetes have also been found in the genes encoding the peroxisome proliferators–activated receptor-γ, inward rectifying potassium channel, zinc transporter, IRS, and calpain 10. The mechanisms by which these genetic loci increase the susceptibility to type 2 diabetes are not clear, but most are predicted to alter islet function or development or insulin secretion. While the genetic susceptibility to type 2 diabetes is under active investigation (estimation that <10% of genetic risk is determined by loci identified thus far), it is currently not possible to use a combination of known genetic loci to predict type 2 diabetes.

1.5.2 Pathophysiology

Type 2 DM is characterized by impaired insulin secretion, insulin resistance, excessive hepatic glucose production, and abnormal fat metabolism. Obesity, particularly visceral or central (as evidenced by the hip-waist ratio), is very common in type 2 DM (80% or more are obese). In the early stages of the disorder, glucose tolerance remains near-normal, despite insulin resistance, because the pancreatic beta cells compensate by increasing insulin output. As insulin resistance and compensatory hyperinsulinemia progress, the pancreatic islets in certain individuals are unable to sustain the hyperinsulinemic state. A further decline in insulin

secretion and an increase in hepatic glucose production lead to overt diabetes with fasting hyperglycemia. Ultimately, beta cell failure ensues.

1.5.3 Abnormal Muscle and Fat Metabolism

Insulin resistance, the decreased ability of insulin to act effectively on target tissues (especially muscle, liver, and fat), is a prominent feature of type 2 DM and results from a combination of genetic susceptibility and obesity. Insulin resistance impairs glucose utilization by insulin-sensitive tissues and increases hepatic glucose output; both effects contribute to the hyperglycemia. Increased hepatic glucose output predominantly accounts for increased FPG levels, whereas decreased peripheral glucose usage results in postprandial hyperglycemia. In skeletal muscle, there is a greater impairment in non-oxidative glucose usage (glycogen formation) than in oxidative glucose metabolism through glycolysis. Glucose metabolism in insulin-independent tissues is not altered in type 2 DM.

The precise molecular mechanism leading to insulin resistance in type 2 DM has not been elucidated. Insulin receptor levels and tyrosine kinase activity in skeletal muscle are reduced, but these alterations are most likely secondary to hyperinsulinemia and are not a primary defect. Therefore, "post-receptor" defects in insulin-regulated phosphorylation/dephosphorylation appear to play the predominant role in insulin resistance. For example, a PI-3-kinase signaling defect might reduce translocation of GLUT4 to the plasma membrane. Other abnormalities include the accumulation of lipid within skeletal myocytes, which may impair mitochondrial oxidative phosphorylation and reduce insulin-stimulated mitochondrial ATP production. Impaired fatty acid oxidation and lipid accumulation within skeletal myocytes also may generate reactive oxygen species such as lipid peroxides. Of note, not all insulin signal transduction pathways are resistant to the effects of insulin (e.g., those controlling cell growth and differentiation using the mitogenic-activated protein kinase pathway). Consequently, hyperinsulinemia may increase the insulin action through these pathways, potentially accelerating diabetes-related conditions such as atherosclerosis.

The obesity accompanying type 2 DM, particularly in a central or visceral location, is thought to be part of the pathogenic process. The increased adipocyte mass leads to increased levels of circulating free fatty acids and other fat cell products. For example, adipocytes secrete a number of biologic products (non-esterified free fatty acids, retinol-binding protein 4, leptin, TNF-α, resistin, and adiponectin). In addition to regulating body weight, appetite, and energy expenditure, adipokines also modulate insulin sensitivity. The increased production of free fatty acids and some adipokines may cause insulin resistance in skeletal muscle and liver. For example, free fatty acids impair glucose utilization in skeletal muscle, promote glucose production by the liver, and impair beta cell function. In contrast, the production by adipocytes of adiponectin, an insulin-sensitizing peptide, is reduced in obesity, and this may contribute to hepatic insulin resistance. Adipocyte products and adipokines also produce an inflammatory state and may explain why markers of inflammation such as IL-6 and C-reactive protein are often elevated in type 2 DM. In addition, inflammatory cells have been found infiltrating adipose tissue. Inhibition of inflammatory signaling pathways such as the nuclear factor κB (NF-κB) pathway appears to reduce insulin resistance and improve hyper-glycemia in animal models.

1.5.4 Impaired Insulin Secretion

Insulin secretion and sensitivity are interrelated. In type 2 DM, insulin secretion initially increases in response to insulin resistance to maintain normal glucose tolerance. Initially, the insulin secretory defect is mild and selectively involves glucose-stimulated insulin secretion. The response to other non-glucose secretagogues, such as arginine, is preserved. Abnormalities in proinsulin processing is reflected by increased secretion of proinsulin in type 2 diabetes. Eventually, the insulin secretory defect progresses to a state of inadequate insulin secretion. The reason(s) for the decline in insulin secretory capacity in type 2 DM is unclear. The assumption is that a second genetic defect—superimposed upon insulin resistance—leads to beta cell failure. Beta cell mass is decreased by approximately 50% in individuals with long-standing type 2 diabetes. Islet amyloid polypeptide or amylin is co-secreted by the beta cell and forms the amyloid fibrillar deposit found in the islets of individuals with long-standing type 2 DM. Whether such islet amyloid deposits are a primary or secondary event is not known. The metabolic environment of diabetes may also negatively impact islet function. For example, chronic hyperglycemia paradoxically impairs islet function ("glucose toxicity") and leads to a worsening of hyperglycemia. Improvement in glycemic control is often associated with improved islet function. In addition, elevation of free fatty acid levels ("lipotoxicity") and dietary fat may also worsen islet function.

1.5.5 Increased Hepatic Glucose and Lipid Production

In type 2 DM, insulin resistance in the liver reflects the failure of hyperinsulinemia to suppress gluconeogenesis, which results in fasting hyperglycemia and decreased glycogen storage by the liver in the postprandial state. Increased hepatic glucose production occurs early in the course of diabetes, though likely after the onset of insulin secretory abnormalities and insulin resistance in skeletal muscle. As a result of insulin resistance in adipose tissue, lipolysis and free fatty acid flux from adipocytes are increased, leading to increased lipid [very low density lipoprotein (VLDL) and triglyceride] synthesis in hepatocytes. This lipid storage or steatosis in the liver may lead to non-alcoholic fatty liver disease and abnormal liver function tests. This is also responsible for the dyslipidemia found in type 2 DM [elevated triglycerides, reduced high-density lipoprotein (HDL), and increased small dense low-density lipoprotein (LDL) particles] (Harrison's Principles of Internal Medicine textbook, 18e 2011).

1.6 INSULIN RESISTANCE SYNDROMES

The insulin resistance condition comprises a spectrum of disorders, with hyperglycemia representing one of the most readily diagnosed features. The *metabolic syndrome*, the *insulin resistance syndrome*, or *syndrome X* are terms used to describe a constellation of metabolic derangements that includes insulin resistance, hypertension, dyslipidemia (decreased HDL and elevated triglycerides), central or visceral obesity, type 2 diabetes or IGT/IFG, and accelerated cardiovascular disease.

A number of relatively rare forms of severe insulin resistance include features of type 2 DM or IGT. Mutations in the insulin receptor that interfere with binding or signal transduction are a rare cause of insulin resistance. *Acanthosis nigricans* and signs of hyperandrogenism (hirsutism, acne, and oligomenorrhea in women) are also common physical features. Two distinct syndromes of severe insulin resistance have been described in adults: (1) type A, which affects young women and is characterized by severe hyperinsulinemia, obesity, and features of hyperandrogenism; and (2) type B, which affects middle-aged women and is characterized by severe hyperinsulinemia, features of hyperandrogenism, and autoimmune disorders. Individuals with the type A insulin resistance syndrome have an undefined defect in the insulin-signaling pathway; individuals with the type B insulin resistance syndrome have autoantibodies directed at the insulin receptor. These receptor autoantibodies may block insulin binding or may stimulate the insulin receptor, leading to intermittent hypoglycemia (Harrison's Principles of Internal Medicine textbook, 18e 2011.

CHAPTER 2

Complications of Diabetes Mellitus

2.1 Biochemistry and molecular cell biology

All forms of diabetes, both inherited and acquired, are characterized by hyperglycemia, a relative or absolute lack of insulin, and the development of diabetes-specific microvascular pathology in the retina, renal glomerulus, and peripheral nerve. Diabetes is also associated with accelerated atherosclerotic macrovascular disease affecting arteries that supply the heart, brain, and lower extremities. The risk of cardiovascular complications is increased by two-fold to six-fold in subjects with diabetes. Overall, life expectancy is about 7 to 10 years shorter than for people without diabetes mellitus because of increased mortality from diabetic complications (Skyler, 1996).

2.1.2 Shared Pathophysiologic Features of Microvascular Complications

In the retina, glomerulus, and vasa nervorum, diabetes-specific microvascular disease is characterized by similar pathophysiologic features. Although all diabetic cells are exposed to elevated levels of plasma glucose, hyperglycemic damage is limited to those cell types (e.g., endothelial cells) that develop intracellular hyperglycemia. Endothelial cells develop intracellular hyperglycemia because, unlike many other cells, they cannot downregulate glucose transport when exposed to extracellular hyperglycemia. As illustrated in Figure 2.1, vascular smooth muscle cells (VSMC), which are not damaged by hyperglycemia, show an inverse relationship between extracellular glucose concentration and subsequent rate of glucose transport measured as 2-deoxyglucose uptake (Fig. 2.1, *upper part).* In contrast, vascular endothelial cells show no significant change in subsequent rate of glucose transport after exposure to elevated glucose concentrations (see Fig. 2.1, *lower part)(Kaiser et al., 1993).*

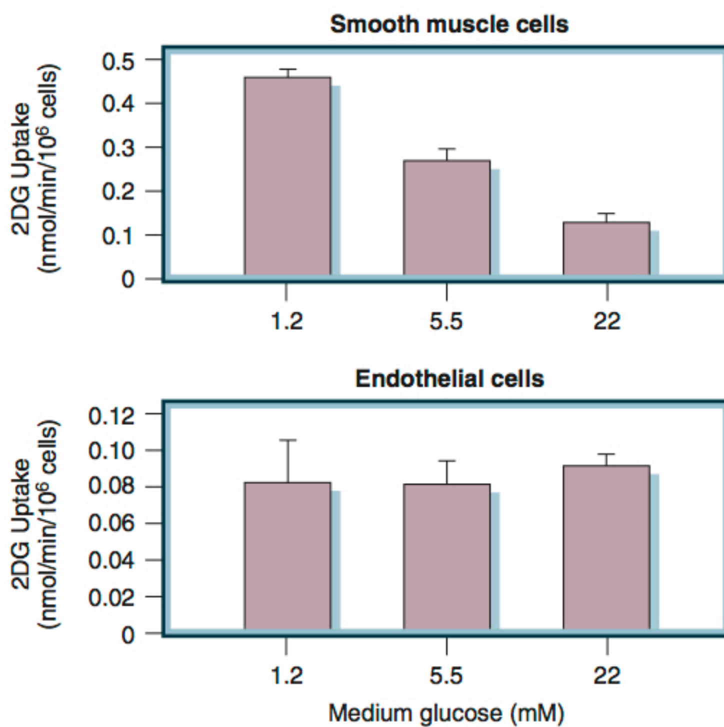


Figure 2.1 Lack of downregulation of glucose transport in cells affected by diabetic complications. *Upper panel,* 2-deoxyglucose (2DG) uptake in vascular smooth muscle cells pre-exposed to 1.2, 5.5, or 22 mmol/l glucose. *Lower panel,* 2DG uptake in bovine endothelial cells pre-exposed to 1.2, 5.5, or 22 mmol/l glucose. (Kaiser et al., 1993)

2.1.3 Abnormal Endothelial Cell Function

Early in the course of diabetes mellitus, before structural changes are evident, hyperglycemia causes abnormalities in blood flow and vascular permeability in the retina, glomerulus, and peripheral nerve vasa nervorum (Tooke et al., 1996; Kihara et al., 1991). The increase in blood flow and intracapillary pressure is thought to reflect a hyperglycemia-induced decrease in nitric oxide (NO) production on the efferent side of capillary beds and possibly an increased sensitivity to angiotensin II. Morover, the common pathophysiologic feature of diabetic microvascular disease is progressive narrowing and eventual occlusion of vascular lumina, which results in inadequate perfusion and function of the affected tissues.

Early hyperglycemia-induced microvascular hypertension and increased vascular permeability contribute to irreversible microvessel occlusion by three processes

- The first process is an abnormal leakage of periodic acid–Schiff (PAS)-positive, carbohydrate-containing plasma proteins, which are deposited in the capillary wall and can stimulate perivascular cells such as pericytes and mesangial cells to elaborate growth factors and extracellular matrix.

- The second process is extravasation of growth factors, such as transforming growth factor-β1 (TGF-β1), which directly stimulates overproduction of extracellular matrix components (Kopp et al., 1996) and can induce apoptosis in certain complication-relevant cell types.

- The third process is hypertension-induced stimulation of pathologic gene expression by endothelial cells and supporting cells, which include GLUT1, growth factors, growth factor receptors, extracellular matrix components, and adhesion molecules that can activate circulating leukocytes (Chien et al., 1998).

2.1.4 Microvascular Cell Loss and Vessel Occlusion

The progressive narrowing and occlusion of diabetic microvascular lumina are also accompanied by microvascular cell loss. In the retina, diabetes mellitus induces programmed cell death of Müller cells and ganglion cells (Hammes et al., 1995), pericytes, and endothelial cells (Mizutani et al., 1996). In the glomerulus, declining renal function is associated with widespread capillary occlusion and podocyte loss, but the mechanisms underlying glomerular cell loss are not yet known. In the vasa nervorum, degeneration of endothelial cells and pericytes occur (Giannini and Dyck, 1993), and these microvascular changes appear to precede the development of diabetic peripheral neuropathy (Giannini and Dyck, 1995).

2.1.5 Genetic Determinants of Susceptibility to Microvascular Complications

Clinicians have long observed that different patients with similar duration and degree of hyperglycemia differ markedly in their susceptibility to microvascular complications. Such observations suggested that genetic differences exist that affected the pathways by which hyperglycemia damages microvascular cells.

A role for a genetic determinant of susceptibility to diabetic nephropathy is most strongly supported by familial clustering, with an estimated heritability of at least 40% (Wagenknecht et al., 2001a). In two studies of families in which two or more siblings had T1DM, the risk of nephropathy in a diabetic sibling was 83% or 72% if the probrand diabetic sibling had advanced diabetic nephropathy, but only 17% or 22% if the index patient did not have diabetic nephropathy (Fig. 2.2) (Seaquist et al., 1989; Quinn et al., 1996) or retinopathy.

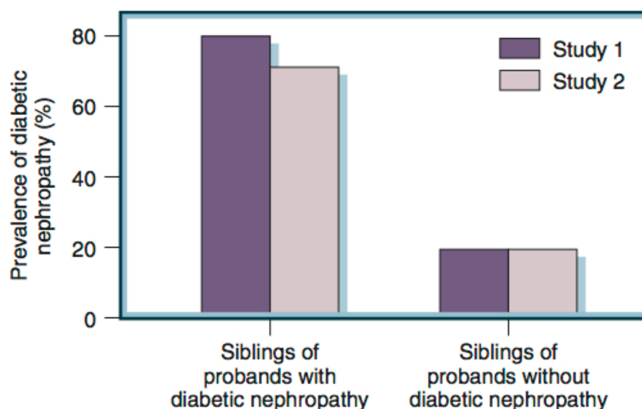


Figure 2.2 Familial clustering of diabetic nephropathy. Prevalence of diabetic nephropathy in two studies of diabetic siblings of probands with or without diabetic nephropathy. Adapted from (Seaquist et al., 1989; Quinn et al., 1996)

Numerous associations have been made between various genetic polymorphisms and the risk of diabetic complications. Examples include the 5′ insulin gene polymorphism (Raffel et al., 1991), the G2m^{23+} immunoglobulin allotype (Stewart et al., 1993), angiotensin-converting enzyme (ACE) insertion/deletion polymorphisms (Marre et al., 1994; Marre et al., 1997), HLA-DQB10201/0302 alleles (Agardh et al., 1996), polymorphisms of the aldose reductase gene (Oates and Mylari, 1999), and a polymorphic CCTTT(n) repeat of nitric oxide synthase 2A (NOS2A) (Warpeha et al., 1999). An individual-based genetic association study of subjects from the DCCT/EDIC found that multiple variations in superoxide dismutase 1 were significantly associated with persistent microalbuminuria and severe nephropathy (Al Kateb et al., 2008). In all of these studies, there was no indication that the polymorphic gene actually plays a functional role rather than simply being in linkage disequilibrium with the locus encoding the unidentified relevant genes.

As genes are identified that affect susceptibility to diabetic complications, a new area of research has emerged that will make it possible to identify genetic modifiers of the clinical manifestations of complications.

2.2 Pathophysiologic Features of Macrovascular Complications

Unlike microvascular disease, which occurs only in patients with diabetes mellitus, macrovascular disease resembles that in subjects without diabetes. However, subjects with diabetes have more rapidly progressive and extensive CVD than non-diabetic persons (Granger et al., 1993).

The importance of hyperglycemia in the pathogenesis of diabetic macrovascular disease is suggested by studies that show how hyperglycemia is a continuous risk factor for macrovascular disease (Gerstein, 1999; Gall et al., 1995; Kuusisto et al., 1994; Salomaa et al., 1995; Laakso and Kuusisto, 1996).

However, data from the United Kingdom Prospective Diabetes Study (UKPDS) show for microvascular disease end points, that there is an almost 10-fold

increase in risk as HbA1c increases from 5.5% to 9.5%, whereas over the same HbA1c range, macrovascular risk increases only about two-fold (1998). Hyperglycemia is not nearly as central a determinant of diabetic macrovascular disease as it is in microvascular disease like insulin resistance. Insulin resistance is commonly associated with a pro-atherogenic dyslipidemia with a characteristic lipoprotein profile that includes a high level of very-low-density lipoprotein (VLDL) and low levels of high-density lipoprotein (HDL) and small, dense low-density lipoprotein (LDL). Both low HDL and small, dense LDL are independent risk factors for macrovascular disease. This profile arises as a direct result of increased net free fatty acid (FFA) release by insulin-resistant adipocytes (Fig. 2.3) (Ginsberg, 2000).

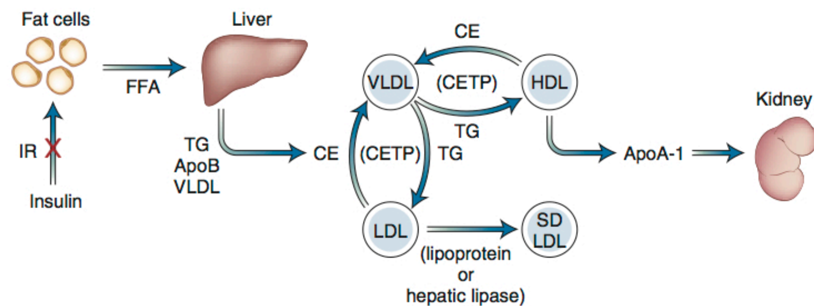


Figure 2.3 Schematic summary relating insulin resistance (IR) to the characteristic dyslipidemia of type 2 diabetes mellitus. IR at the adipocyte results in increased free fatty acid (FFA) release. Increased FFA flux stimulates secretion of very-low-density lipoprotein (VLDL), causing hypertriglyceridemia (TG). VLDL stimulates a reciprocal exchange of TG to cholesteryl ester (CE) from both high-density lipoprotein (HDL) and low-density lipoprotein (LDL), catalyzed by CE transfer protein (CETP). TG-enriched HDL dissociates from apolipoprotein (Apo) A-1, leaving less HDL for reverse cholesterol transport TG-enriched. LDL serves as a substrate for lipases that convert it to atherogenic small, dense LDL particles (SD LDL). (Ginsberg, 2000)

2.2.1 Impaired Collateral Blood Vessel Formation from Bone Marrow Progenitor Cells

It has become apparent that diabetic complications result not only from damage to vascular cells but also from a defective repair process. Normally, in response to acute ischemia, new blood vessel growth rescues stunned areas of the heart or central nervous system, reducing morbidity and mortality. In response to chronic ischemia, collateral vessel development reduces the size and severity of subsequent infarction. In response to ischemia, circulating endothelial progenitor cells from the bone marrow promote the regeneration of blood vessels, acting in concert with cells and extracellular matrix at the site of injury. In experimental diabetes, however, these circulating endothelial progenitor cells are depleted and dysfunctional. As a result, diabetic animals have decreased vascular density after hind limb ischemia. Similarly, in human diabetes, endothelial progenitor cells are also depleted and dysfunctional (Nathan et al., 2005).

Diabetes is associated with poor outcomes after acute vascular occlusive events. This results in part from a failure to form adequate compensatory microvasculature in response to ischemia.

High glucose induces decreased HIF-1α functional activity by impaired

formation of the HIF-1α heterodimer with arylhydrocarbon receptor nuclear translocator (ARNT) and by impaired binding of the coactivator p300 to the HIF-1α–ARNT heterodimer. Hyperglycemia-induced covalent modification of p300 by the dicarbonyl metabolite methylglyoxal is responsible for this decreased association. In diabetic mouse models of impaired angiogenesis and wound healing, decreasing mitrochondrial formation of reactive oxygen species (ROS) normalizes both ischemia-induced new vessel formation and wound healing (Krolewski et al., 1996; Wagenknecht et al., 2001b).

2.3 Mechanisms of Hyperglycemia-Induced Damage

2.3.1 Increased Polyol Pathway Flux

Aldose reductase is a NADPH-dependent oxidoreductase that catalyzed the reducion of a wide variety of carbonyl compounds, including glucose. Aldose reductase has a low-affinity (high Michaelis constant [K_m]) for glucose and at the normal glucose concentrations, found in nondiabetic patients, the metabolism of glucose by this pathway constitutes a small percentage of total glucose utilization. In a hyperglycemic environment, however, increased intracellular glucose results in increased enzymatic conversion to the polyalcohol sorbitol, with concomitant decreases in NADPH. In the polyol pathway, sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase, with nicotinamide adenine dinucleotide (NAD^+) reduced to NADH. A number of mechanisms have been proposed to explain the potential detrimental effects of hyperglycemia-induced increases in polyol pathway flux. These include sorbitol-induced osmotic stress, decreased activity of the sodium-potassium adenosine triphosphatase (Na^+,K^+-ATPase) pump, increased cytosolic NADH/NAD^+, and decreased cytosolic NADPH. More recently, it has been proposed that oxidation of sorbitol by NAD^+ increases the cytosolic NADH/ NAD^+ ratio, thereby inhibiting activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH) and increasing the concentrations of triose phosphate (Williamson et al., 1993). Elevated triose phosphate concentrations could increase the formation of both methylglyoxal, a precursor of AGEs, and (via α-glycerol-3-phosphate) diacylglycerol (DAG) thus activating PKC. It has also been proposed that reduction of glucose to sorbitol by NADPH consumes the cofactor NADPH. Because NADPH is required for regeneration of reduced glutathione, this could induce or exacerbate intracellular oxidative stress. Indeed, overexpression of human aldose reductase increased atherosclerosis in diabetic mice and reduces the expression of genes that regulate regeneration of glutathione (Vikramadithyan et al., 2005). *In vivo* studies of polyol pathway inhibition have yielded inconsistent results. In a 5-year study in dogs, aldose reductase inhibition prevented diabetic neuropathy but failed to prevent retinopathy or capillary basement membrane thickening in the retina, kidney, or muscle (Engerman et al., 1994). Several negative clinical trials have questioned the relevance of this mechanism in humans (Trial of sorbinil 1990). However, the positive effect of aldose reductase inhibition on diabetic neuropathy has been confirmed in humans in a rigorous multidose, placebo-controlled trial with the potent aldose reductase inhibitor, zenarestat (Greene et al., 1999).

2.3.2 Increased Intracellular Formation of Advanced Glycation End Products

AGEs were originally thought to arise from non-enzymatic reactions between extracellular proteins and glucose. However, the rate of AGE formation from glucose is orders of magnitude slower than the rate of AGE formation from glucose-derived dicarbonyl precursors generated intracellularly, and it now seems likely that intracellular hyperglycemia is the primary initiating event in the formation of both intracellular and extracellular AGEs (Degenhardt et al., 1998). AGEs can arise from

intracellular auto-oxidation of glucose to glyoxal (Wells-Knecht et al., 1995), decomposition of the Amadori product to 3-deoxyglucosone, and fragmentation of glyceraldehyde-3-phosphate to methylglyoxal (Fig. 2.4) (Takahashi et al., 1993).
Intracellular production of AGE precursors damages target cells by three general mechanisms:

- Intracellular proteins modified by AGEs have altered function.
- Extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with matrix receptors (integrins) on cells.
- Plasma proteins modified by AGE precursors bind to AGE receptors on cells such as macrophages, inducing receptor-mediated ROS production.

This AGE-receptor ligation activates the pleiotropic transcription factor nuclear factor-κB (NF-κB), causing pathologic changes in gene expression (Oldfield et al., 2001)

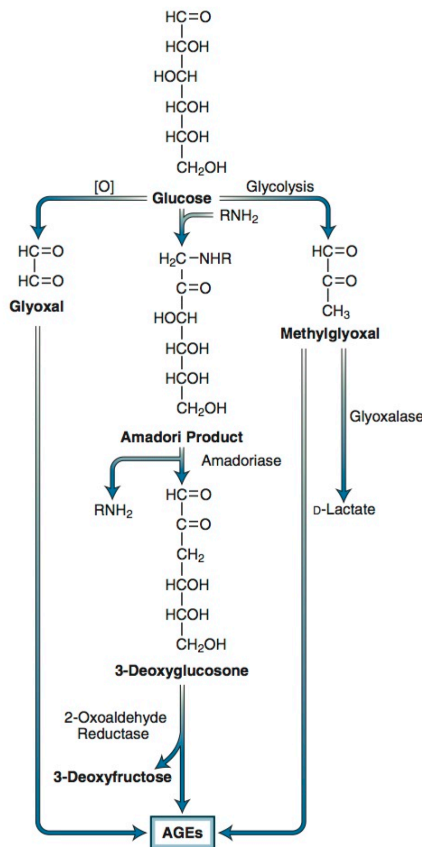


Figure 2.4 Potential pathways leading to the formation of advanced glycation end products (AGEs) from intracellular dicarbonyl precursors. Glyoxal arises from the auto-oxidation of glucose, 3-deoxyglucosone arises from decomposition of the Amadori product, and methylglyoxal arises from fragmentation of glyceraldehyde-3-phosphate. These reactive dicarbonyls react with amino groups of proteins to form AGEs. Methylglyoxal and glyoxal are detoxified by the glyoxalase system. (Adapted from Shinohara M,Thornalley PJ, Giardino I, et al. Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation end-product formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. J Clin Invest. 1998;101:1142-1147.)

2.3.3 Hyperglycemia-Induced Protein Kinase C Activation.

The PKC family comprises at least 11 isoforms, 9 of which are activated by the lipid second messenger, DAG. Intracellular hyperglycemia increases DAG content in cultured microvascular cells and in the retina and renal glomeruli of diabetic animals (Inoguchi et al., 1992; Craven et al., 1990; Shiba et al., 1993).

In early experimental diabetes, activation of PKC-β isoforms has been shown to mediate retinal and renal blood flow abnormalities (Ishii et al., 1996), perhaps by depressing NO production and increasing endothelin-1 activity.

In addition to affecting hyperglycemia-induced abnormalities of blood flow and permeability, activation of PKC contributes to increased microvascular matrix protein accumulation by inducing the expression of TGF-β1, fibronectin, and α1 type IV collagen in cultured mesangial cells (Pugliese et al., 1994; Studer et al., 1993) and in glomeruli of diabetic rats (Craven et al., 1994).

2.3.4 Increased Hexosamine Pathway Flux

A fourth hypothesis about how hyperglycemia causes diabetic complications (Sayeski and Kudlow, 1996a; Kolm-Litty et al., 1998; Daniels et al., 1993; McClain et al., 1992) states that glucose is shunted into the hexosamine pathway. In this pathway, fructose-6-phosphate is diverted from glycolysis to provide substrate for the rate-limiting enzyme of this pathway, glutamine:fructose 6-phosphate amidotransferase (GFAT). GFAT converts fructose 6-phosphate to glucosamine 6-phosphate, which is then converted to UDP-N-Acetylglucosamine. Specific O-GlcNAc transferases use this for post-translational modification of specific serine and threonine residues on cytoplasmic and nuclear proteins by O-GlcNAc. Inhibition of GFAT blocks hyperglycemia-induced increases in the transcription of both TGF-α and TGF-β_1 (Sayeski and Kudlow, 1996b).

Although it is not entirely clear how increased flux through the hexosamine pathway mediates hyperglycemia-induced increases in the gene transcription of key genes such as TGF-α, TGF-β_1, and PAI-1, it has been shown that hyperglycemia causes a 4-fold increase in O-GlcNAcylation of the transcription factor Sp1, which mediates hyperglycemia-induced activation of the PAI-1 promoter in vascular smooth muscle cells (Chen et al., 1998) and of TGF-β1 and PAI-1 in arterial endothelial cells (Du et al., 2000a).

Of particular relevance to diabetic vascular complications is the inhibition of eNOS activity in arterial endothelial cells by O-GlcNAcylation at the Akt activation site of eNOS protein (Yamagishi et al., 2001; Hart, 1997; Musicki et al., 2005). Hyperglycemia also increases GFAT activity in aortic smooth muscle cells, which increases O-GlcNAc-modification of several proteins in these cells (Akimoto et al., 2001).

Finally, diabetic hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear O-GlcNAcylation, which reduced sarcoplasmic reticulum Ca(2+)-ATPase 2a (SERCA2a) mRNA and protein expression, and decreased SERCA2a promoter activity (Clark et al., 2003). In isolated perfused rat hearts, increased GlcNAcylation inhibited phenylephrine-induced inotropy by impairing capacitative Ca^{2+} entry (CCE), the influx of Ca^{2+} through plasma membrane channels activated in response to depletion of endoplasmic or sarcoplasmic reticulum Ca^{2+} stores (Pang et al., 2004).

2.4 Different Hyperglycemia-Induced Pathogenic Mechanisms Reflect a Single Process

Although specific inhibitors of aldose reductase activity, AGE formation, and PKC activation ameliorate various diabetes-induced abnormalities in animal models, there has been no apparent common element linking the four mechanisms of hyperglycemia-induced damage.

Hyperglycemia increases ROS production inside cultured bovine aortic endothelial cells. To understand how this occurs, a brief overview of glucose metabolism is helpful (Fig. 2.5).

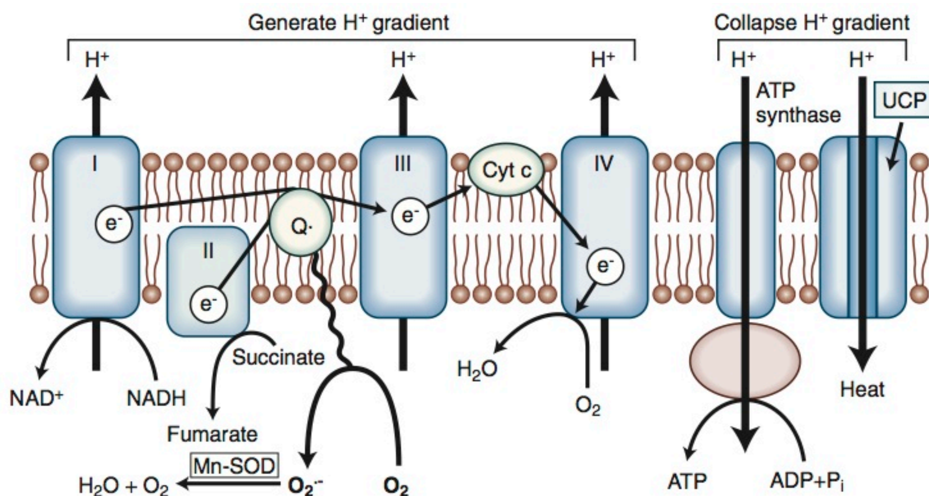


Figure 2.5 Production of superoxide by the mitochondrial electron transport chain. Increased hyperglycemia-derived electron donors from the tricarboxylic acid cycle (NADH and $FADH_2$) generate a high mitochondrial membrane potential ($\Delta\mu\ H^+$) by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III and increases the half-life of free radical intermediates of coenzyme Q, which reduce O_2 to superoxide. ADP, adenosine diphosphate;ATP, adenosine triphosphate; Mn-SOD, manganese superoxide dismutase; NAD+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; Pi, inorganic phosphate; UCP, uncoupling protein. (From Boss O, Hagen T, Lowell BB. Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. Diabetes. 2000;49:143-156.)

Intracellular glucose oxidation begins with glycolysis in the cytoplasm, which generates NADH and pyruvate. Cytoplasmic NADH can donate reducing equivalents to the mitochondrial electron transport chain via two shuttle systems, or it can reduce pyruvate to lactate, which exits the cell to provide substrate for hepatic gluconeogen- esis. Pyruvate can also be transported into the mitochondria, where it is oxidized by the tricarboxylic acid (TCA) cycle to produce CO_2, H_2O, four molecules of NADH, and one molecule of reduced flavin adenine dinucleotide ($FADH_2$). Mitochondrial NADH and $FADH_2$ provide energy for ATP production via oxidative phosphorylation by the electron transport chain.

Electron flow through the mitochondrial electron transport chain is carried out by four inner membrane–associated enzyme complexes, plus cytochrome *c* and the mobile carrier, ubiquinone (Wallace, 1992). NADH derived from cytosolic

glucose oxidation and mitochondrial TCA cycle activity donates electrons to NADH:ubiquinone oxidoreductase *(complex I).* Complex I ultimately transfers its electrons to ubiquinone. Ubiquinone can also be reduced by electrons donated from several $FADH_2$-containing dehydrogenases, including succinate:ubiquinone oxidoreductase *(complex II)* and glycerol-3-phosphate dehydrogenase. Electrons from reduced ubiquinone are then transferred to ubiquinol:cytochrome-c oxidoreductase *(complex III)* by the ubisemiquinone radical-generating Q cycle (Trumpower, 1990). Electron transport then proceeds through cytochrome *c,* cytochrome-c oxidase *(complex IV),* and, finally, molecular oxygen.

Electron transfer through complexes I, III, and IV generates a proton gradient that drives ATP synthase *(complex V).* When the electrochemical potential difference generated by this proton gradient is high, the life of superoxide-generating electron transport intermediates such as ubisemiquinone is prolonged. There appears to be a threshold value above which superoxide production is markedly increased (Fig. 2.5) (Korshunov et al., 1997).

Investigators using inhibitors of both the shuttle that transfers cytosolic NADH into mitochondria and the transporter that transfers cytosolic pyruvate into the mitochondria showed that the TCA cycle is the source of hyperglycemia-induced ROS in endothelial cells. Overexpression of uncoupling protein 1 (UCP-1), a specific protein uncoupler of oxidative phosphorylation capable of collapsing the proton electrochemical gradient (Casteilla et al., 1990), also prevented the effect of hyperglycemia. These results demonstrated that hyperglycemia-induced intracellular ROS are produced by the proton electrochemical gradient generated by the mitochondrial electron transport chain. Overexpression of manganese superoxide dismutase (Mn-SOD), the mitochondrial form of this antioxidant enzyme (Manna et al., 1998), prevents the effect of hyperglycemia. Prevention of mitochondrial superoxide production also completely prevents activation of the polyol pathway, AGE formation, PKC, and the hexosamine pathway.

Therefore, hyperglycemia-induced mitochondrial production of ROS is both necessary and sufficient for activation of each of these pathways.
How does hyperglycemia-induced ROS activate AGE formation, PKC, the hexosamine pathway, and the polyol pathway?

Diabetes in animals and patients, and hyperglycemia in cells, all decrease the activity of the key glycolytic enzyme GAPDH in cell types that develop intracellular hyperglycemia. Inhibition of GAPDH activity by hyperglycemia does not occur when mitochondrial overproduction of superoxide is prevented by either UCP-1 or MnSOD (Nascimento et al., 2006). When GAPDH activity is inhibited, the levels of all the glycolytic intermediates that are upstream of GAPDH increase. This then increases the flux into the 5 pathways described earlier. Increased levels of the upstream glycolytic metabolite glyceraldehyde 3-phosphate activates 2 major pathways. It activates the AGE pathway because the major intracellular AGE precursor methylglyoxal is formed non-enzymatically from glyceraldehyde 3-phosphate. Hyperglycemia-induced methylglyoxal formation has recently been shown to cause both increased expression of the RAGE and its activating ligands S100 calgranulins and HMGB1 (Yao and Brownlee, 2010).

Increased glyceraldehyde 3-phosphate also activates the classic PKC pathway, because the physiological activator of PKC, DAG, is also formed from glyceraldehyde 3-phosphate. Further upstream, levels of the glycolytic metabolite fructose 6-phosphate increase, which then increases flux through the hexosamine pathway, where fructose 6-phosphate is converted by the enzyme GFAT to UDP–

N-acetylglucosamine (UDP-GlcNAc). Finally, inhibition of GAPDH increases intracellular levels of the first glycolytic metabolite, glucose. This increases flux through the polyol pathway, where the enzyme aldose reductase reduces it (or glyceraldehyde 3-phosphate), consuming NADPH in the process. Inhibition of GAPDH activity in 5 mmol/L glucose using antisense DNA elevates the activity of each of the major pathways of hyperglycemic damage to the same extent as that induced by hyperglycemia (Fig. 2.6).

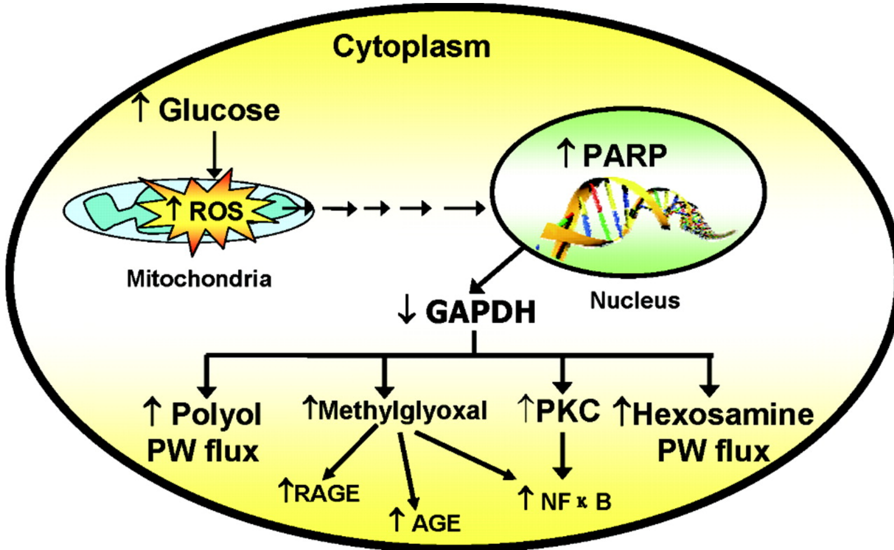


Figure 2.6 Schematic showing elements of the unifying mechanism of hyperglycemia-induced cellular damage. (Adpted from Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005; 54: 1615–1625).

Hyperglycemia-induced superoxide inhibits GAPDH activity in vivo by modifying the enzyme with polymers of ADP-ribose (Du et al., 2003). By inhibiting mitochondrial superoxide production with either UCP-1 or MnSOD, both modification of GAPDH by poly(ADP-ribose) and reduction of its activity by hyperglycemia were prevented. Most importantly, both modification of GAPDH by poly(ADP-ribose) and reduction of its activity by hyperglycemia were also prevented by a specific inhibitor of poly(ADP-ribose) polymerase (PARP), the enzyme that makes these polymers of ADP-ribose. Normally, PARP resides in the nucleus in an inactive form, waiting for DNA damage to activate it. When increased intracellular glucose generates increased ROS in the mitochondria, free radicals induce DNA strand breaks, thereby activating PARP. Both hyperglycemia-induced processes are prevented by either UCP-1 or MnSOD (Du et al., 2000b).Once activated, PARP splits the NAD^+ molecule into its 2 component parts: nicotinic acid and ADP-ribose. PARP then proceeds to make polymers of ADP-ribose, which accumulate on GAPDH and other nuclear proteins. GAPDH is commonly thought to reside exclusively in the cytosol. However, it normally shuttles in and out of the nucleus, where it plays a critical role in DNA repair (Fig. 2.6) (Du et al., 2003).

CHAPTER 3

Metabolic Memory

The first investigation of a metabolic memory was reported over 20 years ago (Engerman and Kern, 1987) in the retina of diabetic dogs who were switched to good glucose control after either 2 months or 2.5 years of poor glucose control and then analyzed at 5 years after beginning the study. The animals switched to good glucose control at 2 months had little evidence of retinopathy and identical HbA1c, as their control counterparts receiving good glucose control throughout the study. In contrast, the animals switched to good control at 2.5 years had a similar incidence of retinopathy as their control counterparts who received poor glucose control throughout the 5-years study. Just after this first study was published, a study (Roy et al., 1990) showed that there was a memory of basement membrane collagen IV, fibronectin mRNA induction in isolated endothelial cells and in the kidneys of streptozotocin (STZ)-induced diabetic rats 1 week after glucose normalization after 2 weeks of high glucose.

This metabolic memory phenomenon emerged, clinically, when the results of a large type 1 diabetes clinical trial, the Diabetes Complications and Control Trial (DCCT) and its follow-up Epidemiology of Diabetes Interventions and Complications (EDIC) trial, came to light. In the DCCT, type 1 diabetic patients were placed on either standard or intensive treatment regimens to normalize their glucose levels. Because the progression of microvascular complications was so profoundly reduced in patients with intensive glucose control, the DCCT ended early after a mean time of 6.5 years and all patients were placed onto intensive therapy (DCCT study 1993). Then in the EDIC follow-up trial with the same patient population, it was found that patients on the standard treatment regimen during the DCCT still had a higher incidence of microvascular diabetic complications such as nephropathy and retinopathy compared with their counterparts receiving intensive therapy throughout the trial several years after switching to intensive therapy, despite the fact that the mean HbA1c levels of the groups were nearly equivalent (EDIC study 2002; 2003). HbA1c and duration of diabetes (glycemic exposure) explained only about 11% of the variation in retinopathy risk for the entire study population, suggesting that the remaining 89% of the variation in risk is presumably explained by other factors independent of HbA1C (Lachin et al., 2008).

The metabolic memory has also been shown to be present in type 2 diabetes. Data from the follow-up of the United Kingdom Prospective Diabetes Study (UKPDS) have shown that type 2 diabetic patients, like type 1 diabetic patients in the DCCT-EDIC, who were on the standard treatment regimen during the study still have a higher incidence of microvascular and cardiovascular complications compared with their counterparts receiving intensive therapy throughout the trial and the follow-up period (Holman et al., 2008). This suggests that early metabolic control has enduring beneficial effects also in type 2 diabetes.

3.1 Generation of irreversible biochemical response from a transient stimulus

Sustained cellular responses to a transient stimulus are features of many biologic systems. An instructive example relevant to the major problem being addressed by this proposal is the differentiation of xenopus ooctyes after a brief exposure to progesterone (Schmitt and Nebreda, 2002). An important conceptual breakthrough was based on the observation that two progesterone-activated kinases - Cdc2 and MAPK - activate themselves, but also activate each other. However, since the phosphorylation events involved are rapid and reversible, simply knowing that positive feedback of kinase activity was involved in progesterone-induced oocyte differentiation did not did by itself explain how oocytes stably maintain the differentiated phenotype - with persistently activated kinases Cdc2 and MAPK - in the absence of progesterone.

Theoretical modeling studies by Ferrell and Xiong predicted that the positive feedback features involving the two kinases could maintain a stable biochemical phenotype in differentiated oocytes (Xiong and Ferrell, Jr., 2003). This model predicted that inhibiting the positive feedback loops would cause a mature egg to dedifferentiate, adopting the phenotype of the immature oocyte. Using three different experimental methods to inhibit the positive feedback between MAPK and a protein upstream of MAPK – Mos - changed the activation of the two kinases after brief progesterone exposure from sustained to transient. The oocyte bistable switch-like response is showed schematically in figure 3.1.

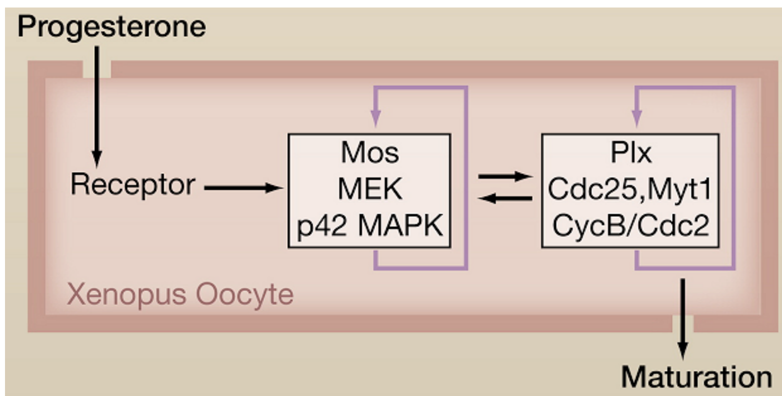


Figure 3.1 Bistable switch-like response based on positive feedback regulates xenopus oocyte differentiation in response to transient progesterone exposure. From (Xiong and Ferrell, Jr., 2003).

A similar role for bistable switch-like responses based on positive feedback has been found for the differentiation and dedifferentiation of human embryonic stem (ES) cells. The transcription factors OCT4, SOX2, and NANOG were identified as critical determinants of ES cell differentiation by genome-wide transcriptional studies (Xiong and Ferrell, Jr., 2003). In the same way that critical kinases regulate the switch to differentiated xenopus oocytes, these transcription factors switch ES cells from undifferentiated to differentiated by means of positive feedback loops, and transient delivery of four such reprogramming transcription factors can also dedifferentiate human differentiated cells to cells similar to human

embryonic stem cells (Boyer et al., 2005). In general, bistable switch-like responses occur when there is a functional element in the regulatory network that exhibits a threshold-like response to a stimulus (Burrill and Silver, 2010). This characteristic can be quantitatively described by a Hill function in which the Hill coefficient is high. As illustrated in Figure 3.2, a smaller Hill coefficient (the exponent *n)* gives a graded response to increasing concentrations of X (n=1 in the figure). In contrast, a larger Hill coefficient results in a bistable, switch-like response (n=4 in the figure).

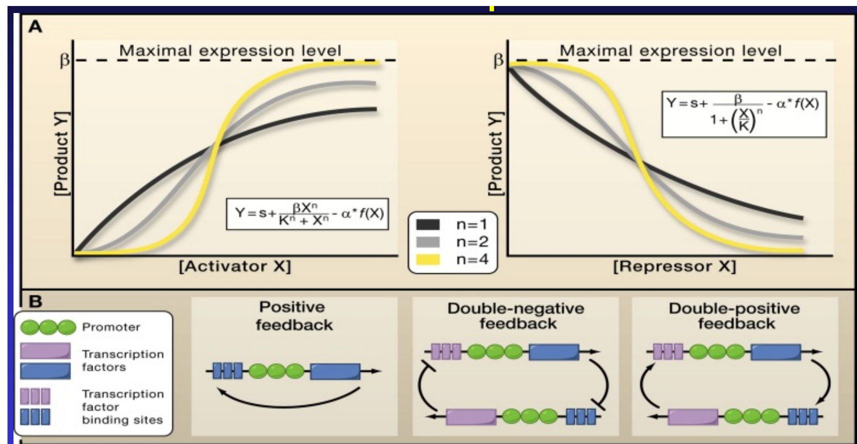


Figure 3.2 Gene Circuits for Cellular Memory (A) Dynamics of regulated gene circuits described by Hill functions. (Left) The concentration of product Y is plotted as a function of the concentration of activator X, as described by Hill functions with n = 1, 2, and 4. β is the maximal expression level from Y's promoter when X is bound, and K defines the concentration of X needed to reach the threshold activation of Y. (Right) The concentration of product Y is plotted as a function of the concentration of repressor X, as described by Hill functions with n = 1, 2, and 4. β is the maximal expression level from Y's promoter when X is unbound, and K defines the concentration of X needed to reach the threshold for repression of Y.(B) Network motifs that achieve biological memory (Burrill and Silver, 2010).

This concept is a critical feature underlying the switch of cell phenotype from one stable state to another. The underlying molecular components of these regulatory systems may be signaling molecules, silencing methylation modifications of histones, transcription factors, and metabolic reactions (Loh et al., 2006). With transcription factors, a stimulus can cause a single transcription factor to self-activate, or cause two transcription factors to regulate each other, producing either two negative or two positive feedback interactions. Each can trigger a switch between two steady states when a threshold concentration of the activator or repressor protein is reached.

CHAPTER 4

Material and Methods

4.1 Materials

Human aortic endothelial cells (HAEC) were obtained from Invitorgen (Grand Island NY). EGM2 Media plus growth factor additives were obtained from Lonza, (Walkerville, Md).
PP2A inhibitor, okadiac acid, deferoxamine mesylate, and GSK-3β activity assay kit were from Sigma-Aldrich (St Louis ,Mo). [γ^{32}P] ATP was from GE Health Care Life Sciences (Piscataway, N.J.). CM-H2DCFDA and JC-1 were from Invitrogen (Grand Island NY). Protein A agarose was from Roche (Nutley, NJ). AKT activity assay kit was obtained from Cell Signaling Technology (Danvers, MA). GSK3-β inhibitory peptide Myr-NGKEAPPAPPQSPP was obtained from EMD (San Diago CA). Antibodies to GSK3 β, p-GSK3-β (S9) were obtained from Cell Signaling Technology (Danvers, MA) and BD Bioscience (San Diego,Calif). Antibodies to Hexokinase II and to VDAC were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). Secondary antibodies for western blot and immunoprecipitation-WB were obtained from Invitrogen (Grand Island NY) and Sigma-Aldrich (St Louis, MO). PP2A Activity assay kit was bought from R&D Systems (Minneapolis MN). EPR tube for free iron measurement was bought from wilmad-lab glass (Vineland NJ). Mn-SOD, empty vector and Catalase adenovirus were obtained as previously described (D'Apolito et al., 2010). Constitutively active GSK-3β adenovirus and plasmids with the cDNA of constitutively activated Akt1 (Akt-1CA), and dominant negative Akt (Akt-DN) were a kind gift of Dr Birnbaum. Akt-1CA, Akt-DN cDNAs were subcloned in a shuttle vector for adenoviral particle packaging.

4.2 Cell Culture conditions

Confluent HAECs (passage 1-4) were maintained in EGM2 media containing 0.4% FBS plus growth factor additives. Cells were incubated for varying times with either 5 mM glucose, 25 mM glucose, with or without various inhibitors and target genes over expression: GSK-3β inhibitory peptide (10mM), PP2A inhibitors (okadiac acid and microcystin-LR, 100 nM and 500 mM respectively), deferoxamine (100mM). Mn-SOD, constitutively activated Akt, Akt-DN and GSK-3β were used at an MOI of 100. The cells were infected with viruses with the target genes 24 hours prior to high glucose treatment.

4.3 Reactive oxygen species quantization

Cells were plated in 96-well cell culture plates. Intracellular reactive oxygen species were detected using the fluorescent probe CM-H2DCFDA. Cells were loaded with 10 µM CM-H2DCFDA after 2 times wash with non-phenol red MEM medium, incubated for 45 min at 37 °C, and analyzed with an HTS 7000 Bio Assay Fluorescent Plate Reader (Perkin Elmer) using the HTSoft program.

4.4 Mitochondrial membrane potential

Mitochondrial membrane potential was assessed using the dual emission potentiometric dye, JC-1 from Life Technologies. JC-1 aggregates fluoresce red (~597 nm), while monomers fluoresce green (~539 nm). Briefly, after indicated treatment cells were stained with 1μM JC-1 for 20' at 37 °C. Pictures were taken using an IX51 inverted microscope (Olympus America Inc.).

Image merge was performed with the public domain software ImageJ (NIH; http://rsb.info.nih.gov/ij).

4.5 Immunoprecipitation and Western blotting

Immunoprecipitated proteins electrophoresed on 10% PAGE gels were transferred onto nitrocellulose membranes. The immunoblots were developed with 1:1000 dilutions of primary antibody and anti-RABBIT IRDye™ 800CW (green) and anti-MOUSE (or goat) ALEXA680 (red). Membranes were scanned and quantitated by the ODYSSEY Infrared Imaging System (LICOR, NE).

4.6 Determination of GSK-3β activity

GSK-3β activity was measured using a kit from Sigma-Aldrich following the instruction of manufacture. In brief, cells were washed in cold PBS twice and lysed in 500 μl of lysis buffer and then scraped off and centrifuged (13000 g, 5min, 4°C). Protein was quantitated by the Bradford method. 200mg of cell lysate was added to the EZview Red Protein G Affinity Gel Equilibration of beads, which should be well equilibrated before use according the manufacture's instruction, and rotated overnight at 4°C. Two negative control samples (Negative Control 1-no substrate and Negative Control 2-no antibody) should be prepared at the same time. Manufacturer's assay procedure was followed accordingly.

4.7 Determination of Akt1 activity

Cells were washed in cold PBS 1X and lysed in 300 μl of lysis buffer (137 mM NaCl, 20 mM Tris-HCL pH7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP40, protease inhibitors tablet (Roche), phosphatase inhibitors cocktail 1+2, 100mM NaF, 10mM Na-Pyrophosphate, 1 mM microcystein). Cells were rocked at 4°C for 20 min, and then scraped off and centrifuged (13000 g, 5mn, 4°C). Proteins were quantitated by the Bradford method. Akt activity was measured following the instruction of the ELISA kit from Cell Signaling Technology.

4.8 Determine of PP2A Activity

PP2A activity was analyzed using a kit form R&D System. The HAECs were cultured until confluent in 10mm dish. After washed with ice-cold PBS twice, the cells were lysed with lysis buffer. Enzyme activity measurement was then performed following the manufacturer's instructions.

4.9 Determination of intracellular free iron

EPR Sample Preparation
The HAECs (10^6/sample) were scraped in PBS and spun down at 1,000 x g for 10 min. The cell pellet was resuspended in 1ml of PBS to wash. The cell pellet was resuspended in 200 µl of assay buffer, 20 mm Tris-Cl, pH 7.4, 10% glycerol and 2mM deferoxamine mesylate. Exactly 200µl of this sample was transferred into an EPR tube, and the sample was frozen on dry ice or liquid nitrogen immediately and stored at –70°C until EPR measurements were performed.

High spin mononuclear ferric iron (electron spin S = 5/2) chelated in a rhombic environment gives rise to a characteristic EPR spectrum dominated by a peak at apparent g-value (g’) equal to approximately 4.3. This signal has been referred to as “free” or “garbage” iron because it often originates from non-specifically bound iron or iron released upon the denaturization of protein active sites. The free iron EPR spectrum is distinct from that obtained from iron bound to heme or present in iron-sulfur clusters. In the this study, the g’=4.3 signal is used to monitor the concentration of free iron present in primary human aortic endothelial cells upon treatment with high glucose and Mn-SOD over expression. In the cell, free iron can be in both the high spin ferrous (Fe^{2+}) and ferric (Fe^{3+}) states. High spin Fe^{2+} is an S=2 paramagnetic species that is typically not detectable at the temperatures and EPR frequencies employed in this study. In order to make it EPR-detectable, the ferrous species is converted to high spin ferric by treatment with deferoxamine mesylate (DF) which binds to Fe^{2+}, lowers its redox potential and allows molecular oxygen to oxidize it to Fe^{3+}. The relative amount of the DF:Fe^{3+} complex can then be determined by measuring the amplitude of the g’=4.3 EPR peak. More quantitative determination of iron concentration can be done using procedures employing S = 5/2 Fe or S = ½ Cu^{2+} or Vo^{2+} standards.

Experimental section

EPR spectra were registered on a Varian E-line 112 X-band spectrometer equipped with a rectangular TE_{102} resonator. A liquid nitrogen finger dewar was inserted into the resonator to keep the sample at 77K during the measurement. Typical spectral acquisitions parameters were as follows. EPR frequency: 9.11 GHz; power: 20 mW; modulation amplitude: 20 G; time constant: 0.5 seconds; scan time: 2 minutes; number of scans averaged: 4 – 9. Spin concentrations were estimated by comparing the amplitude of the g’=4.3 signal with spectra obtained from standards of known concentrations of DF:Fe^{3+} in buffer solution (Woodmansee and Imlay, 2002).

4.10 Chromatin Immunoprecipitation Experiments

ChIP experiments were performed as previous described (Milne et al., 2009). Antibodies to unmodified H3 (catalog# ab1791) and to Histone H3 mono methyl K4 (catalog# ab8895) are from Abcam. Results were quantified comparing input vs. IP signal directly as described (Milne et al., 2009).

4.11 Statistics

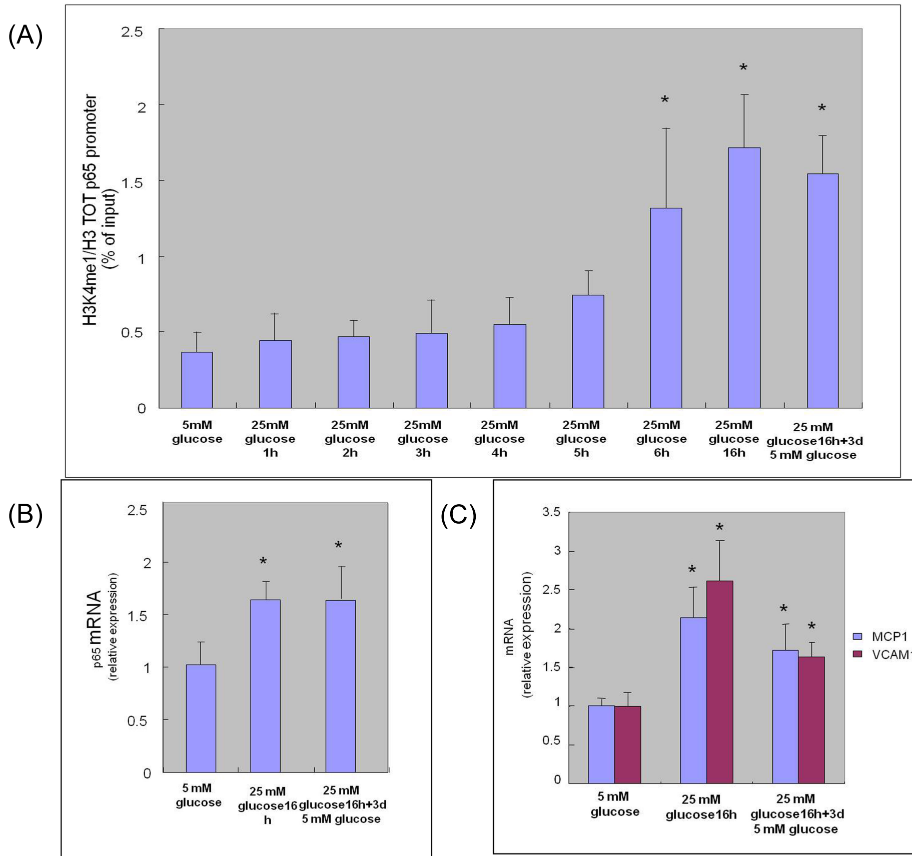
Data were analyzed using one-factor ANOVA to compare the means of all the groups. The Tukey–Kramer multiple comparisons procedure was used to determine which pairs of means were different.

CHAPTER 5

Results

5.1 Transient High Glucose causes sustained ROS production

A previous study by Brownlee et al. shows that transient hyperglycemia induced persistent epigenetic changes in the promoter of the NF-κB p65 subunit in both Human Aortic Endothelial Cells (HAECs) and in non-diabetic mice. In the proximal promoter region of p65, the increased monomethylation of histone 3 lysine 4 (H3K4me1) by the histone methyltransferase Set7 caused a sustained increase in p65 mRNA, leading to a sustained increase in expression of the proatherogenic NF-kB–responsive genes MCP-1 and VCAM-1. Both the persistent epigenetic changes and the increased inflammatory gene expression, induced by transient hyperglycemia, were prevented by reducing mitochondrial superoxide production (El Osta et al., 2008). Since in normal cells these epigenetic changes were rapidly reversed by histone demethylases and histone methyltransferases (Wysocka et al., 2005) we hypothesized that epigenetic changes remain after transient hyperglycemia because of persistent ROS generation during subsequent period of normoglycemia.



<u>Figure 5.1</u> Impact of transient high glucose on Histone methylation and p65 expression. (A) Time course of ChIP H3K4me1 after transient hyperglycemia in HAECs. (B) NF-kB p65 subunit mRNA levels after transient hyperglycemia. (C) MCP-1 VCAM-1 mRNA expression after transient hyperglycemia. Control cells were maintained at 5 mM glucose. Data are the mean ± S.E.M from 5 independent experiments. * $p<0.05$

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We performed a chromatin immunoprecipitation (ChIP) time course to define when the monomethylation in histone 3 lysine 4 occurred in HAECs. A significant increase occurred after 6 and 16 hours of high glucose and remained at comparable levels after 3 days of low glucose (Fig. 5.1.A). As demonstrated in the precedent paper (El Osta et al., 2008) the increased H3K4me1 caused an increase in p65 mRNA and a subsequent augmentation of MCP-1 and VDAC-1 mRNA levels (Fig. 5.1.B-C).
6 hours Glucose exposure induce a X fold increase in ROS production in HAEC. This increase was still present after 2 days of normal glucose. This increase was prevented by overexpression of manganese superoxide dismutase (MnSOD) (Brownlee, 2001) demonstrating that transient high glucose induces an increase in mitochondrial ROS production (Fig. 5.2).

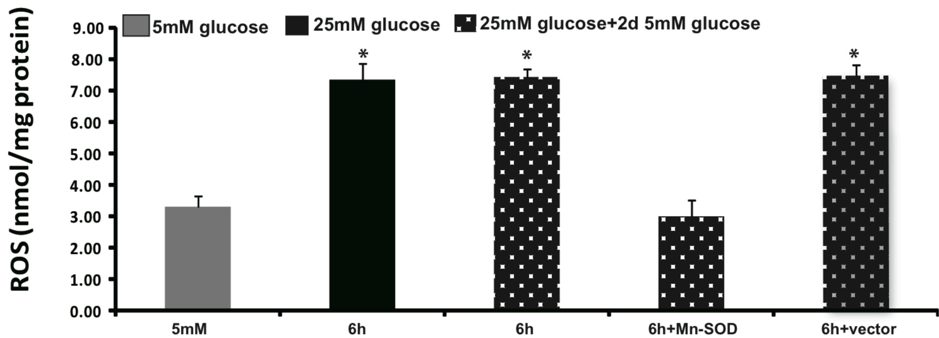
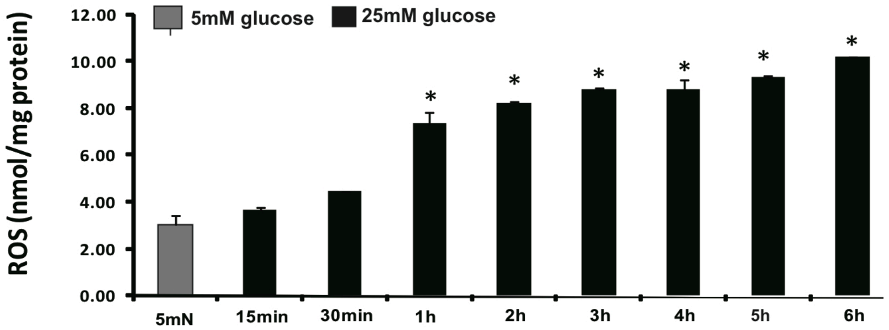


Figure 5.2 Impact of glucose levels on ROS levels. ROS levels measured by 2',7'-dichlorfluorescein-diacetate (DCFH-DA) in HAECs exposed to 25 mM glucose at the indicated time. Cells at 5 mM glucose were used as control. Data are the mean ± S.E.M from n independent experiments. * $p<0.01$.

High glucose exposure induce a rapid increase in ROS production, reaching a maximum after 1 hour and staying at the same levels up to 6h (Fig. 5.3.A). To better understand the time of high glucose exposure able to cause the augmentation of ROS levels in cells subsequently exposed to normal glucose condition, ROS levels were measured in cells transiently exposed to high glucose (25 mM) for different intervals of time and then returned for 2 days in physiological glucose concentration (5 mM). Figure 5.3.B shows that there was no significant increase of ROS levels compared with the control (HAECs in 5 mM glucose for 3 days) when the high-glucose treatment did not exceed the 4h. Conversely, the levels of ROS were still significantly higher after two days in 5 mM glucose, when high glucose exposure was longer than 4 hours.

(A)



(B)

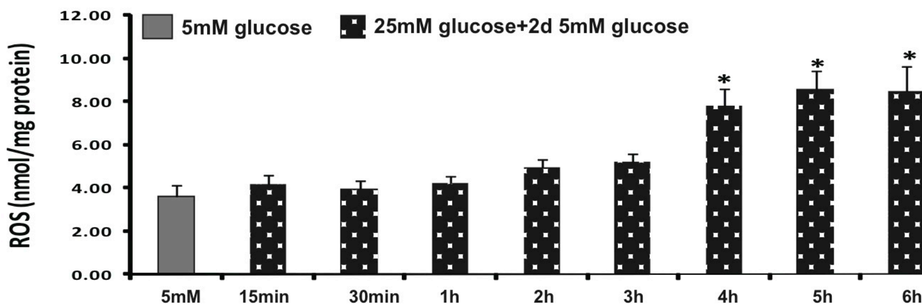
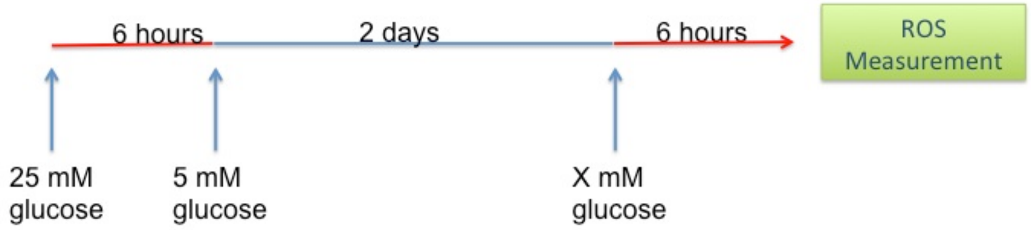


Figure 5.3 Time course of ROS production measured by DCFH-DA. (A) HAECs were exposed to 25mM glucose at the indicated intervals. Cells exposed at 5mM glucose were considered as control. (B) HAECs were exposed to 25 mM glucose at the indicated interval and then returned to 5mM glucose for 2 days. Cells maintained at 5mM glucose were considered as control. Data are the mean ± S.E.M from 5 independent experiments. * $p<0.01$.

Our data showed post-hyperglycemia persistent high levels of ROS. The continuous ROS production, in normal glucose level, highlights a modification in mitochondria physiology as demonstrated by the experiment in Figure 5.4. HAECs were pretreated for 6h in high glucose (25 mM) plus two days in low glucose (5 mM) and then treated for 6h with different glucose concentration from 0 to 25 mM (Fig. 5.4, diagram-A). Cells maintained in 5 mM glucose and then treated with different concentration of glucose (Fig. 5.4, diagram–B) were used as control. Figure 5.4 shows the ROS levels as function of the glucose concentration of the second treatment (represented by the X treatment in the upper diagram). The control cells (B condition-blu line) required 10 mM glucose at least to increase the ROS levels, whereas the cells pre-treated with high glucose (A condition-red line) had higher levels of ROS even in glucose concentration below 5 mM. This resulted in a shift to the left of the glucose concentration ROS curve.

(A)



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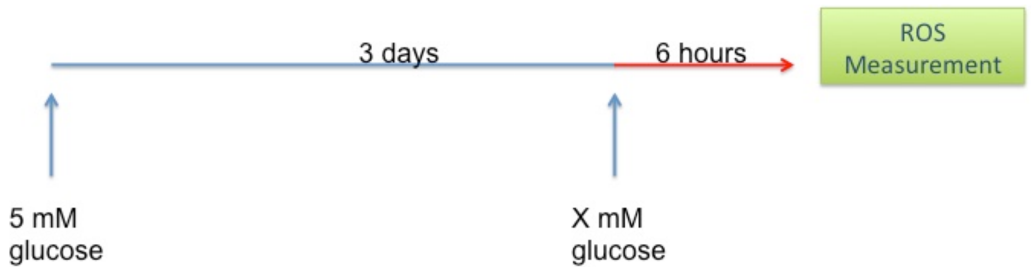


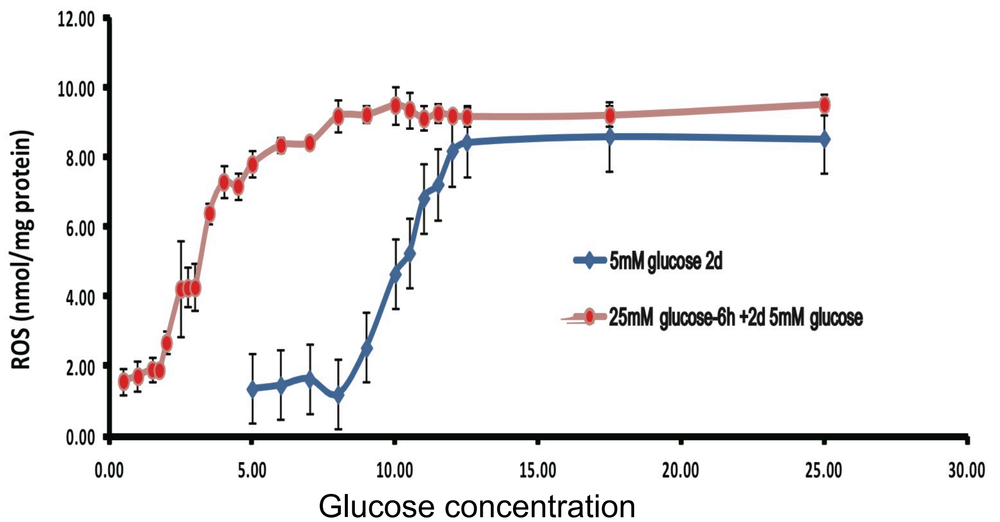


Figure 5.4 ROS levels in HAECs with different glucose pretreatment. HAECs pretreated for 6h in 25 mM glucose followed 2 days in 5 mM glucose (red line) and HAECs treated as control with 5 mM glucose (blue line). Then the cells were treated for 6h at the indicated glucose concentration (from 0 to 25mM with a 0.5 mM increase per experimental point). Data are the mean ± S.E.M from 5 independent experiments. * $p<0.01$.

5.2 Sustained increase in mitochondrial ROS production is maintained by a multi-component positive feedback loop

Based on the previous observations we hypothesized that the sustained increase of mitochondrial ROS by transient hyperglycemia is based on a feedback loop that changes the mitochondria proprieties. Our aim was to identify the critical regulatory network that shifts vascular endothelial cells to a state of persistent ROS production after transient exposure to high glucose.
The physiological rate of mitochondrial ROS production is inversely proportional to the availability of cytosolic ADP (Cadenas and Davies, 2000). Thus, low ADP levels induce an increase in the magnitude of the mitochondrial membrane potential ($\Delta\Psi$), which, in turn, decreases the respiratory rate, leading to the stimulation of ROS generation. In fact, mitochondrial ROS generation is strongly dependent on $\Delta\Psi$ levels, because high H^+ gradients increase O_2^- and H_2O_2 formation (Korshunov et al., 1997). Hexokinase II (HKII) activity is critical for sustaining a constant ADP steady-state cycling which sets $\Delta\Psi$ down to lower levels and, consequently, decreases the mitochondrial ROS formation in rat brain cells (da Silva et al., 2004). HKII binds the outer mitochondrial membrane (OMM) interacting with the voltage-dependent anion channel (VDAC) and regulating the exchange of metabolites into and out the mitochondria (Pastorino et al., 2002).
In our system we hypothesized that there was a dissociation of HKII from VDAC-1 with a consequent impaired ATP/ADP ratio. In order to prove this correlation we induced HKII detachment from VDAC-1 by using a peptide formed by the N-terminal 15 amino acids of HKII linked to a HIV-1 TAT sequence to allow its entry into the cell (Chiara et al., 2008).

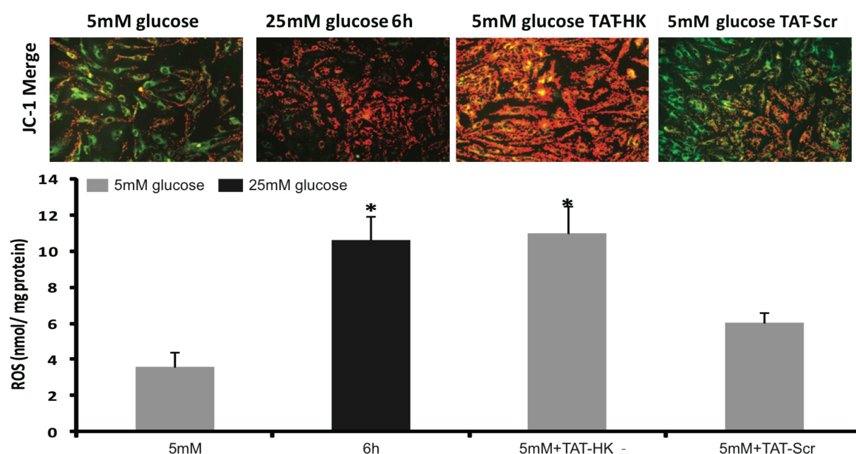
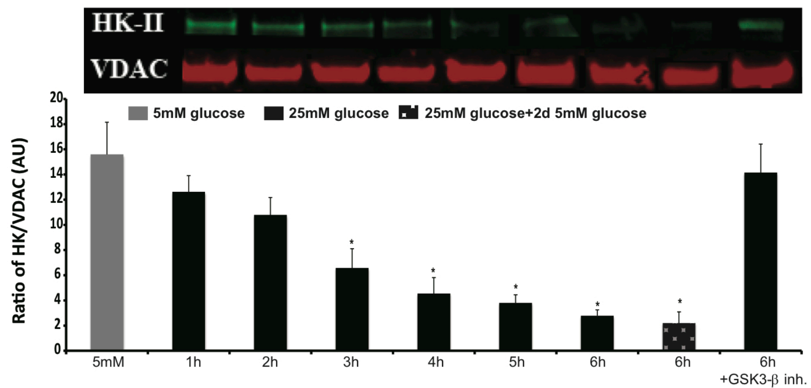


Figure 5.5 Impact of TAT-HK on $\Delta\Psi$ and ROS production. HAECs were treated with TAT-HK 1 μM for 1 hour in 5 mM glucose medium. The TAT-Scr was used as control. In the upper panel: Mitochondrial membrane potential measured by JC-1 probe. In the lower panel ROS production measured by DCFH-DC. Data are the mean ± S.E.M from 5 independent experiments. * $p<0.01$.

Treatment with 1μM of TAT-HK for 1 h induced an increase in mitochondrial membrane potential as showed in the upper level of Figure 5.5. The cells were

stained with JC-1 and the high potential promoted the formation of dye aggregates (red color). TAT-Scr was used as negative control. HKII detachment was also able to induce high levels of ROS in cells grown in 5 mM glucose (Fig. 5.5 lower panel).
Thus we evaluated, by immunoprecipitation, the VDAC-HKII association as function of the time of high-glucose exposure. Figure 5.6.A shows that the dissociation of HKII from VDAC-1 occurred after 3h of high glucose treatment. Moreover, the dissociation persisted even when glucose levels returned to 5 mM concentration. GSK3β mediates VDAC-HKII dissociation through threonine 51 phosphorylation (Pastorino et al., 2005a). Figure 5.6.B shows that GSK-3β inhibitor prevented the phosphorylation threonine 51 and, subsequently, the dissociation of HKII from VDAC-1. A time course of VDAC-1 phosphorylation proved that phosphorylation started at 3 hours of high glucose treatment and was persistent after 6 hours of transient hyperglycemia (Fig. 5.6.B).

(A)



(B)

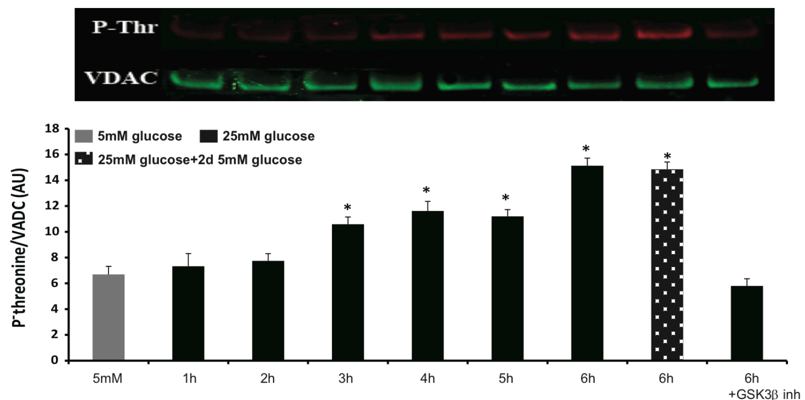
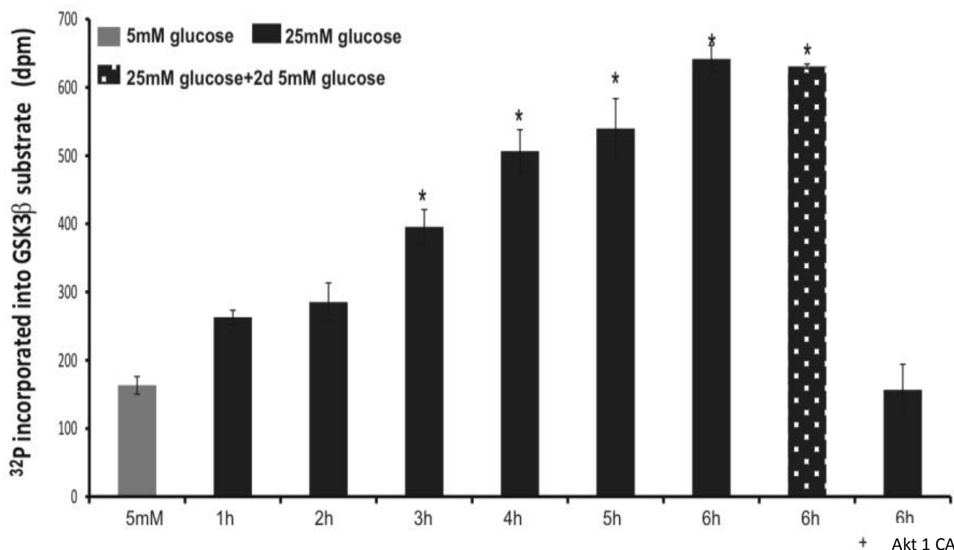


Figure 5.6 High glucose effect on VDAC-HKII association. Immunoprecipitation blotting of HAECs treated with 25 mM glucose at different time. (A) VDAC-HKII association. (B) VDAC threonine phosphorylation. Pre-treatment with GSK-3β inhibitor (10μM) for 3 hours prevents VDAC phosphorylation and as a consequence VDAC-HKII dissociation. Data are the mean ± S.E.M from 5 independent experiments. * p<0.01.

Activation of GSK-3β is associated with mitochondrial dysfunction and cellular injury (Murphy, 2004; Morrison et al., 2002); we found an up-regulation of GSK-3β activity after 3 hours of high glucose treatment. This upregulation reached a stable maximum level at 6 hours (Figure 5.7). Moreover, when HAECs were returned to 5 mM glucose medium after 6 hours exposure to high glucose, the increased GSK-3β activity persisted unchanged for at least 2 days of normal glucose levels. It is well known that GSK-3β can be inactivated by Akt-1, through the phosphorylation of the single serine residue serine 9, located in the regulatory N-terminal domain (Frame and Cohen, 2001). Indeed, prior overexpression of Akt-1, by adenoviral infection, prevented GSK-3β hyper-activity (Fig. 5.7 – last bar).



<u>Figure 5.7</u> Time course of GSK-3β activity measured as 32P incorporated in a substrate. Akt-1 adenoviral infection (100 MOI) prevented hyper-activation of GSK-3β in high glucose condition. No effect with empty vector used as a control (data not showed). Data are the mean ± S.E.M from 5 independent experiments. * $p<0.01$.

Having demonstrated that the overexpression of Akt-1 by adenoviral infection prevented GSK-3β induction, Akt-1 function has been evaluated in our condition. As showed in Figure 5.8 the activity started to decrease significantly after 3 h of high glucose. The decreased activity was persistent even after 2 days of low glucose levels after transient exposure for 6 hours to high glucose medium. Because Akt-1 activity is known to be negatively regulated by the protein phosphatase 2A (PP2A) (Millward et al., 1999), HAECs were pretreated with the PP2A inhibitors, Okadaic Acid (OKA) and Microcystin-LR (Mic). Figure 5.8 shows that the inhibition of PP2A prevented the decrease of Akt-1 activity.

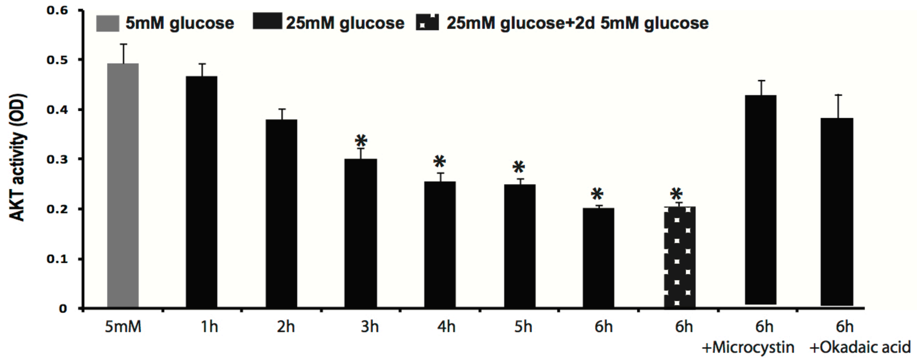


Figure 5.8 Time course of Akt-1 activity. Akt-1 activity measured by ELISA assay. PP2A inhibitor Microcystin-LR (500μm) and Okadaic Acid (100nM) prevented Akt-1 decrease activity in High Glucose condition. Data are the mean ± S.E.M from 5 independent experiments. * $p<0.01$.

Decreased Akt-1 activity is caused by dephosphorylation of Thr 308 by PP2A (Millward et al., 1999). A time course proved a time-depend increase in PP2A activity since 2 h of high glucose treatment reaching a maximum at 6 hours. Remarkably, this increase in PP2A activity persisted during subsequent 2 days in normal glycemia. PP2A hyperactivity was completely prevented inhibiting hyperglycemia-induced ROS by overexpressing MnSOD and Catalase and by using desferoxamine, an iron chelator (Fig. 5.9).

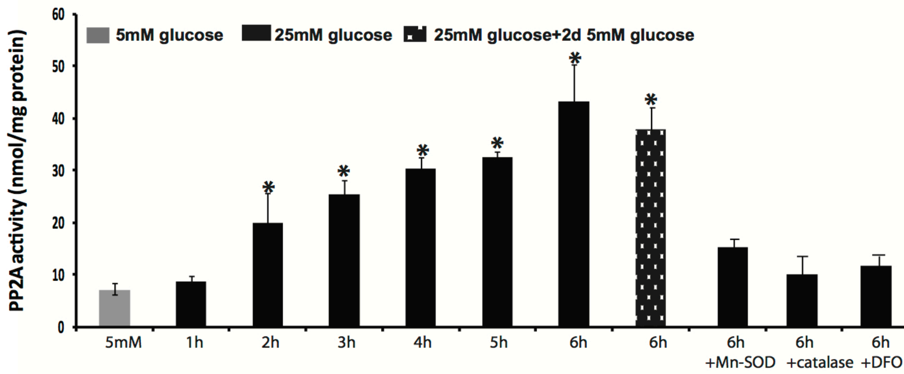


Figure 5.9 Time course of PP2A activity measured by immunoprecipitation assay kit. Adenoviral overexpression of MnSOD, catalase (100 MOI) and desferoxamine (DFO 100 mM) prevent PP2A hyper-activity. Data are the mean ± S.E.M from 5 independent experiments. * $p<0.01$.

Iron–sulphur (FeS) clusters are ROS-sensitive components of many (>120) cellular proteins, like proteins involved in electron transfer. Oxidation of FeS causes loss of protein function with releases of free Fe, which may participate in Fenton reaction (Kuo et al., 2008). The Fenton reaction forms hydroxyl radical from hydrogen peroxide in the presence of ferrous ions (Raha and Robinson, 2000):

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\cdot} + Fe^{3+}$$

PP2A has been showed to be ROS sensitive with controversial effects on its activity (Cicchillitti et al., 2003; Chen et al., 2009). In our system hydroxyl radicals were able to incresase PP2A (Fig. 5.9).
Since we found a decrease in PP2A activity after treatment with Desferoxamine, we performed an experiment to quantify free iron by electron paramagnetic spectroscopy (EPR).
In our system after 2 hours of high glucose there was an increase in cellular free iron. This was prevented by the overexpression of MnSOD demonstrating that mitochondrial ROS are responsible of free iron release (Fig. 5.10).

Figure 5.10 Time course of Free Iron. Intracellular Free measured by Electron Paramagnetic Spectroscopy (EPR). Adenoviral overexpression of MnSOD, (100 MOI) prevents Iron release. Data are the mean ± S.E.M from 5 independent experiments. * p<0.01.

5.3 Phenocopying loop activation

In order to confirm the involvement of the principal component of the loop identified in our system, we did an experiment with the adenoviral vector expressing for Akt-1 dominant negative (Akt-DN) and GSK-3β constitutively active (GSK-3β-CA). As previously described, cells were stained with JC-1 to evaluate the promotion of dye aggregates (red) by the high membrane potential. Figure 5.11 (upper panel) shows that cells grown in 5 mM glucose and infected with the Akt-DN or GSK-3β-CA adenoviral vectors had a higher membrane potential compared with non-infected cells grown in 5 mM glucose. It can also be seen that these infected cells had a similar membrane potential to non-infected cells exposed to 25 mM glucose for 6 hours.
Analogously, Figure 5.11 (lower panel) shows that the infected cells had comparable ROS levels with the non-infected cells grown in 25 mM glucose for 6 hours.

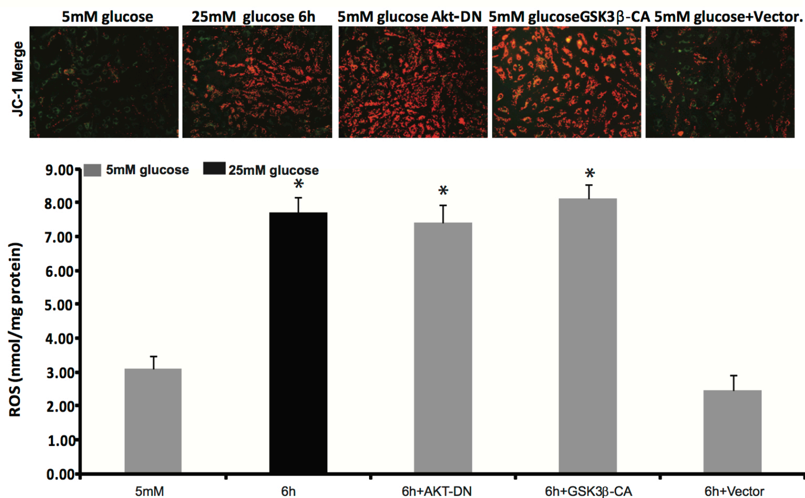


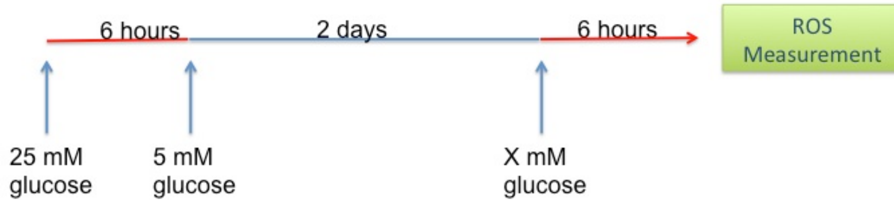
Figure 5.11 Phenocopy loop activation by adenoviral infection of Akt-1 dominant negative and GSK-3β costitutively active. Akt-DN and GSK-β-CA infected cells were grown in 5 mM glucose. Non-infected cells were grown either in 5mM or 25 mM glucose. Cells empy vector infected (mock) were grown in 5 mM glucose. Upper panel: Mitochondrial membrane potential measured by JC-1 probe. Lower panel: ROS levels measured by DCFH-DC. Data are the mean ± S.E.M from 5 independent experiments. * $p<0.01$.

5.4 Disruption of feedback loop components reverses the sustained effects of transient glucose stimulus

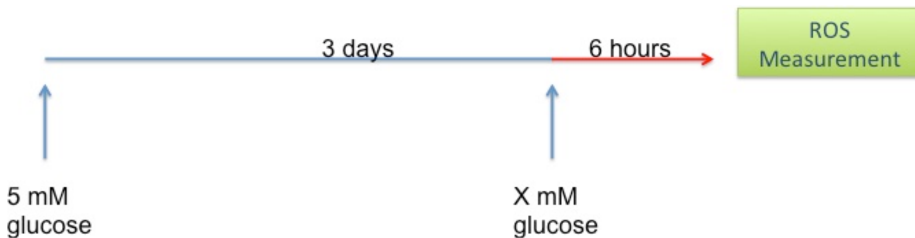
We identified a multi-component positive feedback loop induced by transient exposure to high glucose. The activation of this loop maintains persistently high ROS levels even when the cells were returned to normal glucose conditions. This results in the shift to the left of the glucose concentration-ROS generating curve in mitochondria.

HAECs were treated as indicated in the diagrams below. Figure 5.12 shows ROS levels as function of the glucose concentration of the second treatment (represented by the X treatment in the upper diagram). As shown before in Figure 5.4, the control cells (B condition-blu line) required 10 mM glucose, at least, to significantly increase the ROS levels, whereas the cells pre-treated with high glucose (A condition-red line) had higher levels of ROS even in glucose concentration below 5 mM. This resulted in a shift to the left of the glucose concentration ROS curve. Cells pretreated for 6h in high glucose (25 mM) plus two days in low glucose (5 mM) and then treated for 6h with different glucose concentration from 0 to 25 mM but in presence of a GSK-3β inhibitor (gold curve) recapitulated to the normal profile in ROS curve dose-response.

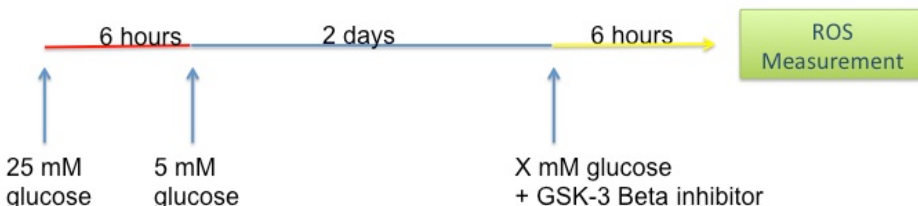
(A)



(B)



(C)



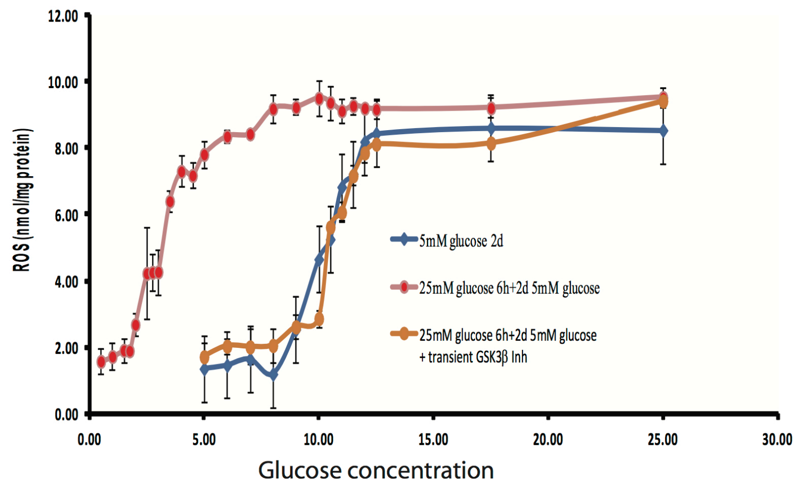


Figure 5.12 GSK-3β inhibitor reverted ROS. ROS production in HAECs pretreated for 6h in 25 mM glucose followed 2 days in 5 mM glucose (red line) and HAECs treated as control with 5 mM glucose (blue line). After these conditions, cells were treated for 6h with different glucose concentration (from 0 to 25mM with a 0.5 mM increase per experimental point) in presence (gold line) of GSK-3β inhibitor (10 mM).

Transient exposure to high glucose induces methylation of histones associated with the p65 promoter. Histone methylation is an important post-translational modification involved in fundamental processes such as transcriptional regulation and genome stability (Chen et al., 2009; Martin and Zhang, 2005). In particular, methylation of H3K4 (lysine 4 of histone 3) favors transcriptional activation (Kondo et al., 2003). To confirm the involvement of each single loop component in histone methylation we performed a ChIP assay for H3K4me1. HAECs were transient exposed to high glucose and then treated to perturb any of the elements in the feedback loop. The result was a complete restored system to its normal state, reversing persistent hyperglycemia-induced epigenetic changes as showed in Figure 5.13.

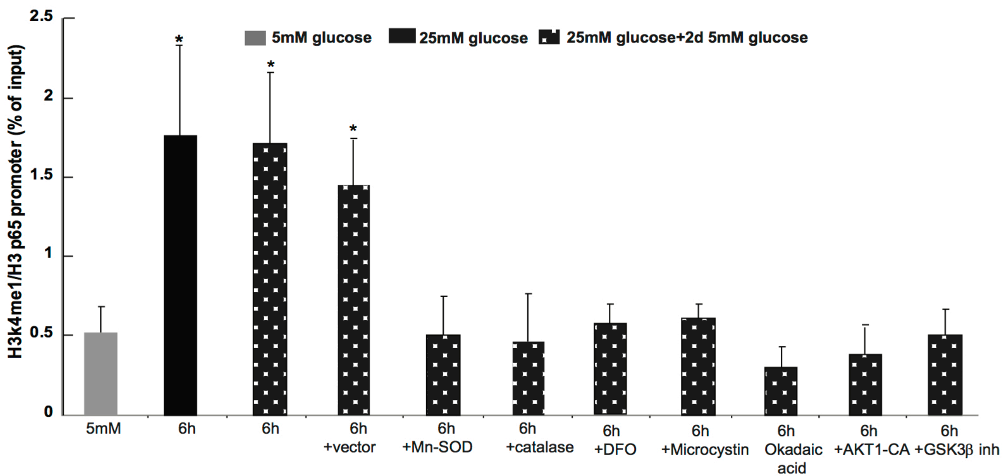


Figure 5.13 Transient normalization of loop components reversed H3K4me1. NF-κB p65 promoter H3K4me1 after transient hyperglycemia in HAECs. Data are the mean ± S.E.M from 5 independent experiments. * $p<0.01$.

The disruption of the feedback loop by acting on the loop components was able to revert the H3K4me1 increase in p65 proximal promoter and to revert the increase in p65 and NF-κB-dependent pro-inflammatory gene expression (Fig. 5.14).

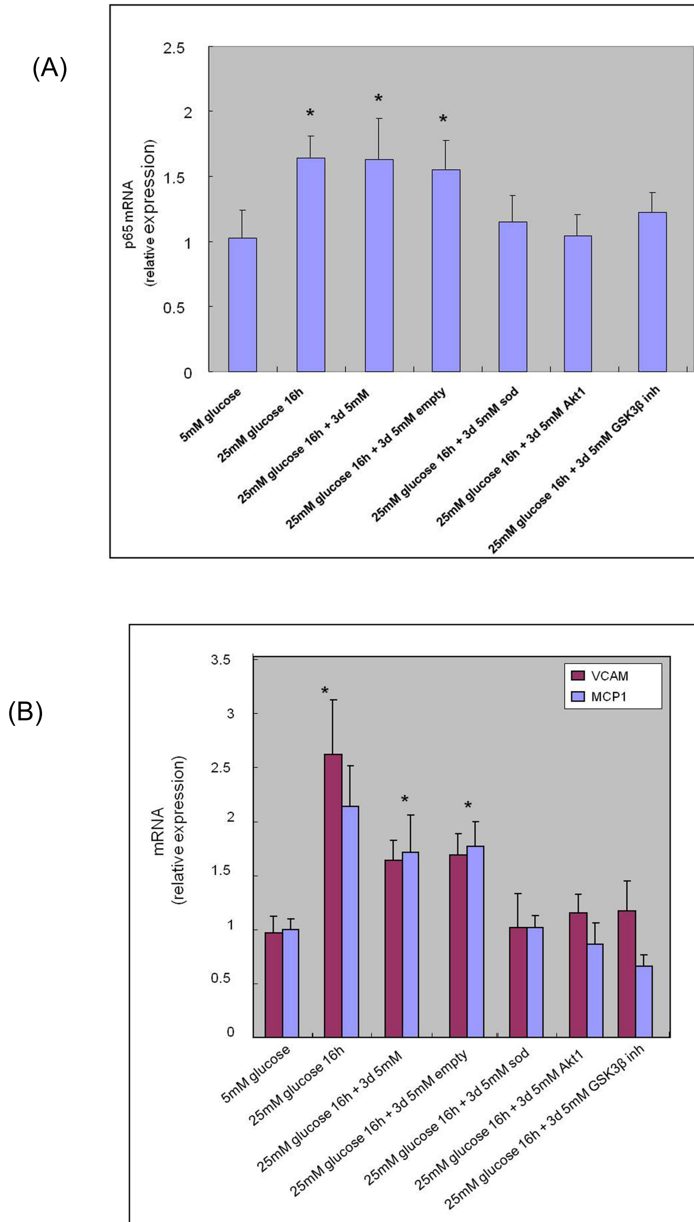


Figure 5.14 Transient normalization of MnSOD, Akt-1 and GSK-3β reversed p65 mRNA and gene expression. (A) p65 mRNA (B) VCAM and MCP1 mRNA measured by quantitative PCR.

CHAPTER 6

Discussion

In the present study we found how transient hyperglycemia induced the long-lasting activating epigenetic changes in the proximal promoter of the NF-κB subunit p65. Since in normal cells the epigenetic changes described are rapidly reversed by histone demethylases and methyltransferases we discovered how transient spikes of hyperglycemia cause persistent mitochondrial overproduction of reactive oxygen species (ROS), during subsequent periods of prolonged normal glucose, causing persistent epigenetic changes in the promoter of p65. We identified a multi-component positive feedback loop induced by transient exposure to high glucose, in human vascular endothelial cells, which maintains persistently increased ROS production in normal glucose condition by shifting the glucose concentration-ROS generating curve of mitochondria to the left (Figure 5.4). Transient disruption of any of the elements in the feedback loop rapidly restores the system to its normal state, including reversing persistent increase in ROS production, persistent epigenetic changes and persistent increase NF-κB-dependent pro inflammatory gene expression.

Our observations of metabolic memory are based on behavior of many biologic systems where sustained cellular responses can be seen after transient stimulus and are maintained by the continuous activation of feedback loops. These feedback loops function as bistable, hysteretic toggle switches. The term “bistable” means that the system can be in either of two alternative stable steady states, depending upon its history, and the term “hysteretic” means that once the system has been switched from one state to the other, it tends to stay there.

Long-lasting stress, even when glucose level is normalized, has been reported in various contexts, but the molecular mechanisms remain unknown (Ihnat et al., 2007; Paneni et al., 2012; Ceriello et al., 2007). In our work for the fist time we show the molecular mechanism underlying the sustained ROS production.

Overproduction of ROS by mitochondria is considered as a causal link between elevated glucose and the major biochemical pathways involved in the development of vascular complications in diabetes (Nishikawa et al., 2000). Moreover hyperglycemia-induced ROS formation causes the epigenetic changes found in the promoter of the NF-κB p65 subunit in both cultured human aortic endothelial cells and in non-diabetic mice (El Osta et al., 2008).

The epigenetic modifications allow cells/organisms to quickly respond to changing environmental stimuli, (Gluckman et al., 2007; Bjornsson et al., 2004; Whitelaw and Whitelaw, 2008) and also confer the ability for the cell to “memorize” these encounters once the stimulus is removed (Dolinoy and Jirtle, 2008; Morgan and Whitelaw, 2008).

Here we used transient hyperglycemia as a model to study persistent ROS generation and epigenetic changes caused by shift from one bistable state of system to another.

Our investigation was focused on the role of mitochondria in metabolic memory. Mitochondria are not static structure in the cell and are the major source of hyperglycemia-induced ROS (Nishikawa et al., 2000). Here we report how transient high glucose can dramatically change mitochondrial proprieties as showed by the ROS production curve shifted to the left even in glucose concentration lower than 5 mM (Figure 5.4).

Our data show that 2 hours high glucose exposure causes ROS production leading to oxidation of sensitive iron-sulphur (FeS) clusters (Rouault and Klausner, 1996), the major components of mitochondrial respiratory electron transport chain, resulting in loss of protein function and, at the same time, releasing of free iron.

The superoxide produced by electrons transport chain is converted by superoxide dismutase (MnSOD) in H_2O_2, which reacts with free iron in Fenton catalysis, generating hydroxyl radicals (Raha and Robinson, 2000).

The diagram in figure 6.1 shows the normal and high glucose conditions.

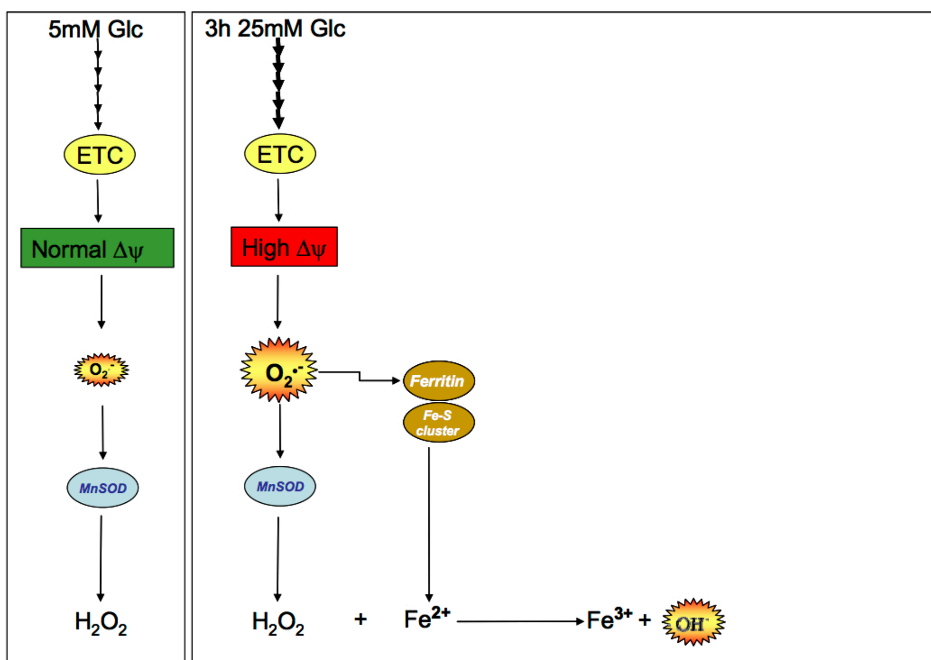


Figure 6.1 Schematic representation of ROS production in normal and high glucose conditions.

PP2A is one of the main intracellular serine/threonine phosphatase and plays important roles in maintaining endothelial cell physiological functions, including regulation of endothelial cell cytoskeletal structure, protection of the endothelial cell barrier, regulation of glucose metabolism and fat synthesis, and regulation of endothelial nitric oxide synthase phosphorylation status (Kabashima et al., 2003; Palanivel et al., 2004; Tar et al., 2006; Urbich et al., 2002; Zolnierowicz, 2000). PP2A has been showed to be ROS sensitive with controversial effects (Cicchillitti et al., 2003; Chen et al., 2009), however our results show that the hydroxyl radicals generated by Fenton reaction are able to increase PP2A activity as demonstrated

by overexpression of MnSOD, Catalase and employment of the iron chelator DFO which are all able to prevent increased PP2A activity. It is well known that PP2A controls negatively Akt-1 by dephosphorylation in Thr 308 (Seeling et al., 1999), and through this effect, PP2A is a physiologic negative regulator of insulin's metabolic signaling pathway (Ugi et al., 2004). As a result of PP2A over-activation we found a significant decrease in Akt-1 activity since 3 hours of high glucose treatment. Akt-1 has been shown to regulate hexokinase expression, activity, and mitochondrial interaction (Miyamoto et al., 2008; Aubert-Foucher et al., 1984; Vander Heiden et al., 2001) also through GSK-3β inhibition by dephosphorilation in serine 9 (Cross et al., 1995). The binding of HKs to the mitochondria may be dependent on the phosphorylation state of VDAC. Pastorino et al. demonstrated that GSK-3β can phosphorylate VDAC affecting HK binding to the mitochondria (Pastorino et al., 2005b). We found an increase in GSK-3β activity, due to Akt-1 inhibition, that lead to VDAC phosphorylation and as a consequence to VDAC-HK dissociation after 3 hours of high glucose treatment, furthermore VDAC-HK dissociation is persistent in normal glycemia after transient high glucose stimulus. It has been very well established that HKII activity is critical to set down $\Delta\Psi$ and moreover mitochondrial high $\Delta\Psi$ is associated with ROS formation (Raha and Robinson, 2000; da Silva et al., 2004; Korshunov et al., 1997). Thus, the persistent ROS production we found in normal glucose is due to VDAC-HK dissociation that causes increase in mitochondrial $\Delta\Psi$ leading to continuous ROS production. The use of a peptide, formed by N-terminal 15 aminoacids of HKII linked to a HIV-1 TAT sequence, was able to dissociate VDAC-HKII in 5 mM glucose causing $\Delta\Psi$ increase and ROS production confirming the involvement of VDAC-HKII in the continuous ROS formation in our system.

Our findings suppose the presence of a critical component that actives the loop to have ROS production in memory condition, indeed ROS formation turns up after 4 hours of transient high glucose treatment, persisting unchanged for two days in low glucose. After at least 4 hours of transient high glucose treatment, there is a “critical switch” turned on able to allow ROS production in low glucose conditions. It is difficult to identify the nodal control of the switch, but it is necessary the dissociation of HK from VDAC so that we can have persistent ROS production. The diagram in figure 6.2 shows a schematic representation of the feedback loop.

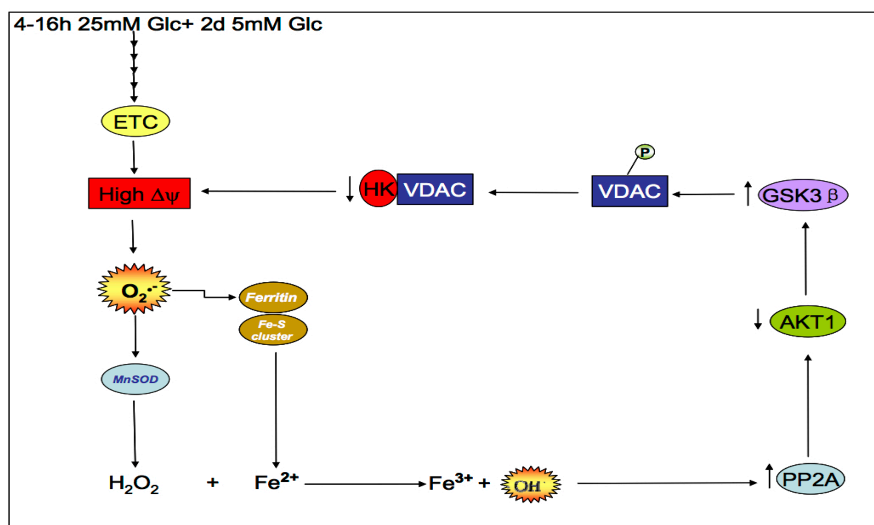


Figure 6.2 Schematic representation of feedback loop.

The most interesting part of our work was the possibility to act on the loop component and revert the damaging effects of transient high glucose stimulus. We were able to reset GSK-3β activity using a small peptide inhibitor resulting in ROS normalization as proved by the experiment in figure 5.12 (Chapter 5). The cells transient exposed to high glucose for 6 hours plus two days in low glucose exhibit persistent ROS production even after treatment with glucose concentration below 5 mM glucose. The treatment for 6 hours with GSK-3β inhibitor was able to restore the normal mitochondrial physiology represented by the curve comparable with the control. Following this, the persistent epigenetic changes induced by transient high glucose were completely reversed in these experiments normalizing each component of the feedback loop.

Data from the EDIC study, which followed patients with type 1 diabetes after they completed the DCCT, show that early chronic exposure to a moderately high level of hyperglycemia has prolonged effects on diabetic complications during subsequent periods of improved glycemia, showing clinically the "metabolic memory." For example, atherosclerotic changes not even present at the end of the DCCT appeared subsequently in the previously higher HbA1c group, followed by a two fold increase in myocardial infarction, strokes, and cardiovascular death. This occurred despite the fact that their HbA1c since the end of the DCCT was identical to that of the formerly intensive control group during the entire time that these arterial changes developed (Nathan D.M. et al., 2003; Nathan D.M., et al. 2005).

The transient hyperglycemia causes persistent atherogenic effects during subsequent normoglycemia by inducing long-lasting changes in chromatin remodeling. These persistent changes in gene expression are induced by spikes of hyperglycemia that have durations too short (6-16 hours followed by 6 days of normal glycemia) to influence HbA1C values (El Osta et al., 2008). These results highlight the dramatic and durable effects that short-term hyperglycemic spikes can have on vascular cells and suggest that transient spikes of hyperglycemia may be an HbA1c-indipendent risk factor for diabetes complications.

Increasing understanding about the mechanisms underlying glycemic variability and its potential deleterious consequences, and determining how better to reduce the magnitude and frequency of glycemic spikes, should become near-term priorities for translational and clinical type 1 diabetes research. Physicians will have to realize that much remains to be done in identifying important factors contributing to microvascular complications risk, which are not captured by the HbA1c.

In conclusion, our work introduce the identification of a mechanism that explain why microvascular complications progress also in presence of improved glycemic control and potentially may contribute in defining novel therapeutic target and additional markers for monitoring glycemic control and for designing better treatment methods to reduce the deleterious effect of hyperglycemic metabolic memory.

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