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MECHANISMS INVOLVED IN THE REPRESSION OF HUMAN INSULIN GENE PROMOTER ACTIVITY MEDIATED BY CHRONIC HYPERGLYCEMIA

By

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ABSTRACT

MECHANISMS INVOLVED IN THE REPRESSION OF HUMAN INSULIN GENE PROMOTER ACTIVITY MEDIATED BY CHRONIC HYPERGLYCEMIA

By

Maria Fernanda Pino

Type II diabetes is characterized by insulin resistance and failure of pancreatic β-cells to secrete sufficient amounts of insulin to overcome hyperglycemia. Once diabetes is established, chronic hyperglycemia has been postulated to cause adverse alterations in β-cell function, thus exacerbating the disease state. Some of the characteristics of hyperglycemia-induced β-cell damage include both suppression of insulin gene expression and glucose-induced insulin secretion. The later is associated with decreased insulin gene promoter activity. Mechanisms accounting for chronic hyperglycemia-induced suppression of insulin promoter activity still remain unclear. Studies in other cell systems suggest that stress associated with hyperglycemia such as oxidation could be involved in tissue damage. Therefore, experiments were performed to understand the role of c-Jun N-terminal kinase (JNK), a stress-activated kinase, in chronic hyperglycemia-induced insulin promoter repression.

These studies demonstrated that JNK activity is significantly increased, in parallel with increased AP-1 transcription factor activity, in INS-1 cells cultured in 16.7 mM glucose compared to 4.0 mM glucose. Over-expression of JNK repressed insulin promoter activity in INS-1 cells cultured in 4.0 mM glucose. Over-expression of MLK3,

an upstream activator of JNK, increased JNK activity, AP-1 transcription factor activity, and repressed insulin promoter activity in cells cultured in 4.0 mM glucose. These data show that activation of JNK either by high levels of glucose or upstream activators leads to functional activation of downstream JNK target proteins and causes reduced insulin promoter activity. These findings suggest that glucose-induced insulin promoter repression might be mediated by the JNK signaling pathway.

Pdx-1, an important regulatory transcription factor of insulin gene expression, partially mediates the repression of glucose-induced insulin promoter activity. Because over-expression of Pdx-1 in insulinoma cells cultured in high levels of glucose is insufficient to prevent insulin promoter repression, we further characterized other potential promoter targets. Truncation analysis and functional studies demonstrated that high levels of glucose mediate promoter repression between –327 and –261 nucleotides. Mobility shift assays showed that three glucose-sensitive complexes bind to the A5/Core, palindrome, and E3 elements, respectively within the insulin promoter. Site-specific mutations of all three elements in the insulin promoter gene partially prevented glucose-induced insulin promoter repression.

Collectively these studies suggest that elevated extracellular glucose mediates insulin promoter repression possibly through the JNK signaling pathway and through novel mechanisms that target the distal promoter region.

To my mom, sister, and family

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I. INTRODUCTION

Type II diabetes is characterized by the combination of insulin resistance and β-cell dysfunction. β-Cell failure can be ascribed, at least in part, to the adverse effects of chronic hyperglycemia on pancreatic β-cell function. One of the characteristics of β-cell failure is the loss of glucose-induced insulin secretion that is associated with loss of insulin gene expression (1). Reduced binding activity of two important transcription factors, Pdx-1 and C1 activator, is correlated with the loss of insulin gene expression and the loss of promoter activity (2-4). Studies performed to understand the role of Pdx-1 in glucose-induced repression of the insulin promoter suggest that Pdx-1 is not the only transcription factor affected by hyperglycemia (Olson, LK, unpublished data). The C1 activator has recently been cloned (5), and its role in repression of the insulin promoter has yet to be investigated. These results led us to hypothesize that high glucose concentrations mediate insulin promoter repression through other mechanisms besides affecting binding activity of Pdx-1 and the C1 activator.

Previous studies have suggested that glucose-mediated repression of insulin gene transcription is associated with increased oxidative stress (6-8). Glucose generates oxidative stress through several pathways including: polyol pathway flux, non-enzymatic glycation, increased advanced glycation end-product (AGE) formation, hexosamine pathway flux, and activation of protein kinase C (PKC) (9). Furthermore, β-cells cultured in high levels of glucose and treated with antioxidants partially prevented decreased insulin gene promoter activity and Pdx-1 binding (7). Even though reactive oxygen species (ROS) might mediate glucose-induced insulin promoter repression, the

exact mechanism remains unclear. Since ROS can activate stress-activated signaling pathways, this led us to hypothesize that stress-activated kinases, such as c-Jun N-terminal kinase (JNK) might mediate glucose-induced repression of insulin promoter activity.

Pdx-1, an important transcription factor that regulates insulin gene expression, partially mediates glucose-induced insulin promoter repression. Because over-expression of Pdx-1 is insufficient to prevent insulin promoter repression in cells cultured in high levels of glucose, we further characterized other potential promoter targets. Functional analysis of a truncated insulin promoter vector demonstrated that additional distal promoter elements might be involved in glucose-induced insulin promoter repression. Therefore we investigated the role of the distal promoter region including sequences from –327 to –269 in the repression of insulin promoter activity induced by chronic hyperglycemia.

II. LITERATURE REVIEW

1. Endocrine functions of the pancreas

The pancreas is an organ with exocrine and endocrine functions. It is derived from endodermal cells of the upper duodenal region of the foregut (10, 11) that leads to dorsal and ventral protrusions developing into mature pancreas (12, 13). The exocrine functions, which constitute more than 98% of the pancreas, consists of secretion of digestive enzymes and bicarbonate solutions by the pancreatic acini and ducts into the duodenum. The endocrine functions are performed by islets of Langerhans, which in humans consist of one to two million round clusters (islets) of cells. The islets are composed of alpha (α), beta (β), delta (δ), and pancreatic peptide (PP) cells. The α-cells, which constitute 20% to 25% of the islet, are located at the periphery. α-Cells secrete the hormone glucagon, which regulates carbohydrate metabolism by inducing glycogen breakdown, gluconeogenesis, and synthesis of ketones. Thus, the overall role of glucagon is to increase glucose and ketone plasma concentrations. β-Cells, which make up 60% to 70% of the islet, are located centrally in the islet and secrete the hormone insulin. Insulin, which is often seen as the most important controller of metabolism, maintains blood glucose levels within a narrow range of 80 to 130 mg/dl (4.4 to 7.2 mM) by inducing glucose uptake by muscle cells, adipose tissue, and the liver. δ-Cells, which constitute 10% of the islet, secrete the hormone somatostatin, which acts within the islets to inhibit both insulin and glucagon secretion. The remaining cells are PP cells, the source of pancreatic polypeptide, which has an unclear physiological function.

The islets are well vascularized which allows them to secrete hormones into the blood, monitor blood glucose levels, and ensure efficient paracrine regulation among the hormones. Each β- and α-cell has a basal (arterial) and an apical (venous) face. Between the lateral surfaces of neighboring β-cells run canaliculi that span the distance between the arteriolar and venous ends of the cell. These connections allow the cells to be exposed laterally to regulatory molecules, such as glucose.

2. Role of insulin in daily metabolic control

The main function of insulin is to regulate blood glucose levels, and it does so by regulating the metabolism of carbohydrates, lipids, and proteins. After the consumption of a meal, insulin is secreted. Insulin induces efficient storage of the excess nutrients while inhibiting the mobilization of endogenous nutrients. The stored nutrients can be made available during subsequent fasting periods to maintain required fuel for the body. The major targets of insulin action are the liver, adipose tissue, and muscle.

2.1 Carbohydrate metabolism

In the liver, insulin stimulates glucose oxidation and storage and at the same time inhibits the output of glucose. Insulin stimulates oxidation of glucose by inducing expression of glucokinase and its actions to phosphorylate glucose. Insulin then increases storage of glucose by inducing the glycogen synthase enzyme complex. At the same time, insulin inhibits the release of glucose by lowering the rate of glycogenolysis by inhibiting glycogen phosphorylase activity and by decreasing glycogen glucose-6-phosphatase levels (14). The hormone also lowers glucose production through

decreasing gluconeogenesis by inhibiting free fatty acids and amino acid mobilization from fat and muscle to the liver (15). Insulin also decreases the levels of enzymes involved in gluconeogenesis, such as pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose-1,6-diphosphatase. Insulin also decreases glucagon secretion by α-cells, thus decreasing hepatic glucose output (16).

Insulin stimulates glucose uptake in muscle and adipose tissue by increasing the net rate of translocation of glucose transporters (Glut-4) from intracellular compartments to the plasma membrane (17, 18). In muscle, depending on the concentrations of insulin, a certain percentage of glucose is metabolized through glycolysis and oxidation. The remaining glucose is stored as glycogen. Insulin induces glycolysis, mainly through regulation of hexokinase and 6-phosphofructokinase. It has been demonstrated that insulin increases the expression of hexokinase II, but not hexokinase I in rodents and humans (19). Studies in intact muscle have shown that insulin increases the flux of glucose-6-phosphate by increasing the activity of 6-phosphofructokinase (20). Insulin regulates glycogen synthesis by increasing the activity of glycogen synthase, which generates glycogen from uridine diphosphate glucose (UDP-glucose) (21, 22).

2.2 Fat metabolism

Fat metabolism and mobilization are highly sensitive to insulin. Overall, insulin enhances storage and blocks mobilization and oxidation of fatty acids. In adipose tissue, insulin inhibits hormone-sensitive lipase activity, thus inhibiting lipolysis (23, 24). Insulin also inhibits fat mobilization by retaining free fatty acids (FFA) and inducing reesterification of FFA to triglycerides (TG) within liver and adipose tissue (23, 25). By

suppressing of lipolysis and release of FFA, insulin inhibits the generation of ketoacids in the liver and induces the use of ketoacids in the peripheral tissues.

Triglycerides are transported by lipoproteins in the form of very low-density lipoprotein (VLDL) from the liver. Insulin inhibits hepatic VLDL secretion and promotes the hydrolysis of circulating VLDL to transfer FFA into adipose tissue (26). In the liver, insulin induces FFA synthesis from glucose-derived pyruvate, which is converted into acetyl-CoA and then into malonyl-CoA. Insulin also favors hepatic synthesis of cholesterol from acetyl-CoA.

2.3 Protein metabolism

Insulin is known to regulate nitrogen balance. The role of insulin in regulating nitrogen balance is best described in Type I diabetics because a lack of insulin creates lean tissue atrophy and hyperaminoacidemia. The mechanism by which insulin regulates nitrogen balance has been only recently elucidated. One mechanism is insulin enhances protein and amino acid sequestration into target tissues. Insulin also inhibits proteolysis (27, 28) and patients with diabetes have an increase in urinary nitrogen.

Insulin is also associated with regulation of protein synthesis (29, 30) and amino acid oxidation (31). Insulin regulates the initiation of skeletal muscle protein synthesis (32). Amino acid oxidation is control by the availability of amino acids, which is decreased due to the inhibition of proteolysis.

Insulin secretion is very important in controlling the metabolic responses of daily life, including feeding and exercising. The above review emphasizes the importance of insulin in regulating blood glucose levels and mentions that insulin is also involved in

lipid and protein metabolism. All these pathways can not be separated; instead, they feed back into and regulate one another.

3. Insulin biosynthesis

3.1 Insulin synthesis

Insulin is among the best understood of the polypeptide hormones, having been the first protein for which the complete amino acid sequence was determined as well as the first hormone whose gene was molecularly cloned. Pancreatic β-cells maintain a readily available pool of insulin that can be rapidly secreted in response to a stimulus, such as a rise in blood glucose concentration. Any increase in insulin release is compensated for by a corresponding increase in insulin biosynthesis, so that β-cell insulin stores are constantly maintained. Thus, insulin biosynthesis and processing is a highly regulated and a dynamic process.

Biologically active insulin consists of two polypeptide chains, the A-chain (21 amino acids) and B-chain (30 amino acids), joined by two interchain disulfide-linked bridges at A-Cys/B-Cys and another intrachain disulfide bridge between A-Cys/A-Cys (33). Insulin is initially synthesized as a precursor molecule preproinsulin (34, 35), which is composed of a 24-amino-acid N-terminal hydrophobic signal peptide, followed by the insulin B-chain, then the 31-amino-acid of the connecting peptide (C-peptide), and then the insulin A-chain (Fig. 1). Preproinsulin mRNA is transported from the nucleus to the cytoplasm where co-translation with ER takes place, and the newly synthesized preproinsulin is located at the rough endoplasmic reticulum (RER) (37). In the RER, signal peptidases, associated with the lumen side of the RER membrane, remove the

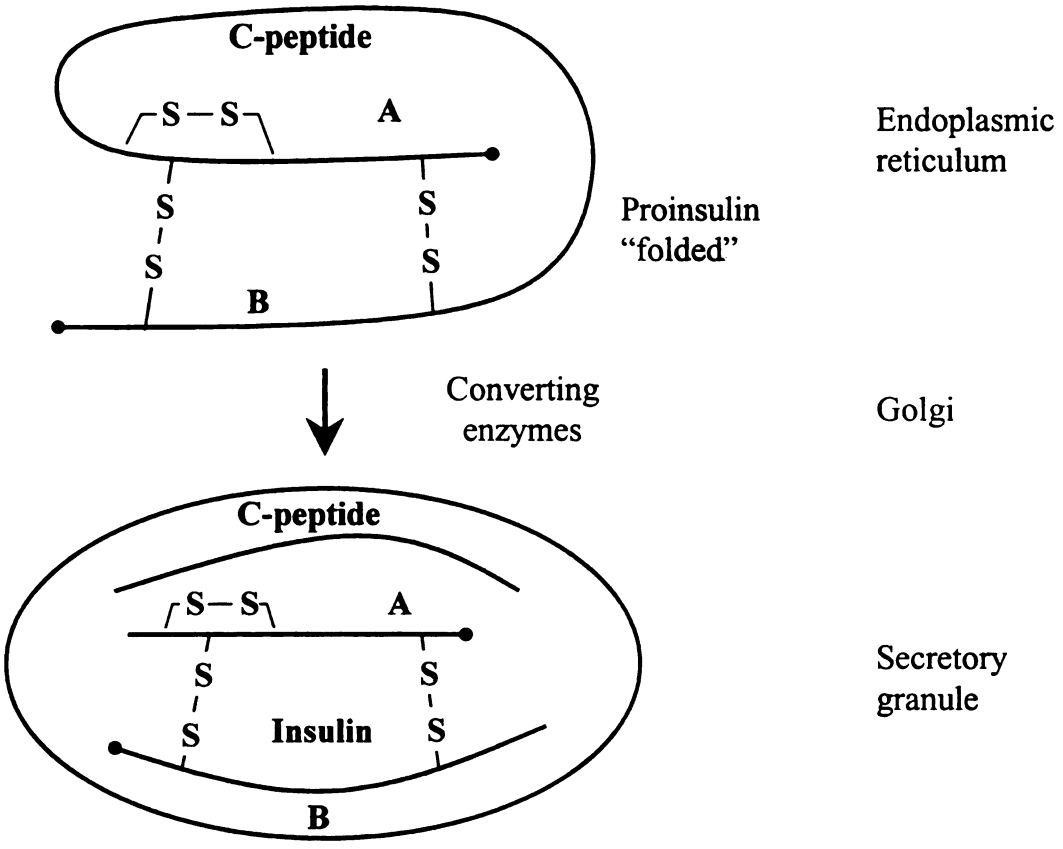

Figure 1. Proinsulin and insulin molecules. Insulin is first synthesized as a precursor molecule named preproinsulin, which is then converted into proinsulin in the rough endoplasmic reticulum (RER). Proinsulin then is converted into mature insulin by removing the C-peptide in the secretory granule. Pictured was modified from Berne *et al.* (36).

signal peptide and proinsulin forms in the RER lumen. Once proinsulin is correctly folded with the disulfide bonds, it is delivered to the *cis*-Golgi apparatus and continues to the *trans*-Golgi apparatus by vesicular transport. The *trans*-Golgi containing proinsulin is coated with clatherin, and leads to immature buds that mature to form insulin granules. In the insulin granules proinsulin becomes active insulin when the C-peptide is cleaved by endopeptidases PC2 and PC3. The mature granules contain insulin and soluble C-peptide (Fig. 1).

3.2 Insulin secretion

The major controlling factor for insulin secretion is the plasma glucose concentration. Insulin secretion is also regulated by other factors, such as nutrients, hormones, and acetylcholine. Because of the great importance of glucose metabolism to induce insulin secretion, this mechanism has been studied extensively. The following description reviews insulin secretion induced by glucose (Fig. 2).

When blood glucose levels rise, β-cells take up and metabolize the glucose, which induces insulin release. Glucose enters the β-cells through the low affinity-facilitated glucose transporter Glut2. The high Km (15 to 20 mM) and Vmax of Glut2 allow for rapid equilibrium of glucose across the β-cell membrane (38). Glucose is then phosphorylated into glucose-6-phosphate by glucokinase, which has a high Km (5 to 10 mM), ensuring that glucose phosphorylation rates are proportional to blood glucose levels. Glucose phosphorylation is thought to be the major rate-limiting step in glucose metabolism within the β-cell, thus playing an essential role in normal glucose-induced insulin secretion. Glucose-6-phosphate is then metabolized through glycolysis and the

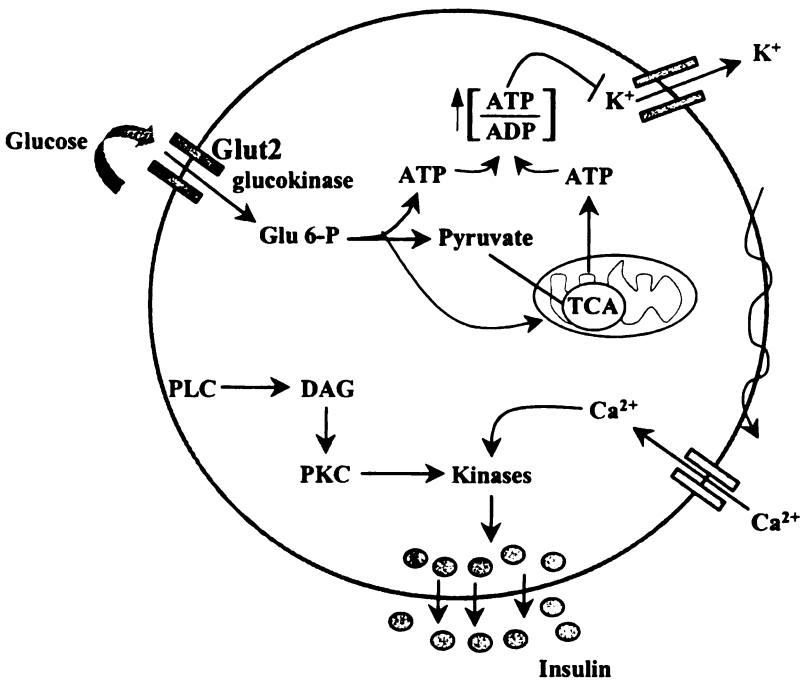

Figure 2. Schematic representation of glucose-induced insulin secretion. Glucose is transported into pancreatic β-cells through the facilitated transporter Glut2 and enters the glycolytic pathway and then the TCA cycle, resulting in an increase of ATP. The high ratio of ATP/ADP results in closure of the ATP-sensitive K^+ channels, membrane depolarization, and opening of voltage-dependent Ca^{2+} channels, which increases Ca^{2+} flux. This rise of intracellular Ca^{2+}, along with a variety of second messengers that lead to activation of PKC and other kinases, lead to insulin secretion. Picture was modified from McKinnon *et al.* (39) and Howell *et al.* (40).

Krebs cycle, raising the ratio of adenosine-triphosphate to adenosine-diphosphate (ATP: ADP). The resulting high ratio of ATP:ADP leads to ATP binding to ATP-sensitive K^+ channels, inducing inhibition (closure) of the channel (41, 42). Closure of ATP-sensitive K^+ channels leads to membrane depolarization and opening of voltage-dependent Ca^{2+} channels (43), leading to an influx of Ca^{2+}. The rise in intracellular Ca^{2+}, along with second messengers such as increased cAMP and breakdown of membrane lipids, all lead to mobilization of intracellular stores of Ca^{2+}. Increased intracellular Ca^{2+} activates Ca^{2+} / calmodulin kinases, resulting in insulin exocytosis (44, 45). A rise in intracellular Ca^{2+}, however, is not the only regulator of insulin exocytosis; G-proteins, phospholipase C, protein kinase C, and other protein kinase activities are also involved in insulin release (40, 46-49). Recently, Aspinwall *et al.* (50) demonstrated autocrine stimulation of insulin secretion by insulin in isolated mouse pancreatic β-cells and clonal β-cell cultures. Insulin binds to the insulin receptors on the surface of β-cells and activates tyrosine phosphorylation of insulin receptors and insulin receptor substrates, IRS-1, IRS-2, and PI3-Kinase (51-53). The activation of these proteins has been correlated with the increase in intracellular Ca^{2+} and the secretion of insulin (50). Overall, insulin secretion is very complex, and most likely involves the coordinated responses of several factors.

4. Regulation of insulin gene transcription

The human insulin gene is a small gene located on chromosome 11p15.5 (54), and it consists of three exons and two introns (55). In contrast to humans, rats and mice have two non-allelic insulin genes, which are coordinately expressed and regulated. The restriction of insulin expression to β-cells is at the level of transcription, which is

regulated by the insulin gene promoter (5' flanking sequences, -328 to +1). Mutational and deletion studies of the rat insulin I and II gene and the human insulin gene have identified multiple sequences along the insulin promoter that contribute positively and negatively to its activity: the A, E, C, cAMP-regulatory element (CRE), CRE/CCAAT, and the Negative Regulatory Element (NRE)/Z element (Fig. 3).

The A elements, or A boxes, consist of A1, A2, A3 and A5 elements, located in the human promoter at –79 to –84, -123 to –134, -210 to –216, and –313 to -319, respectively. All the A elements except the A2 element contain a core TAAT sequence, where several transcription factors bind including the highly specific homeodomain transcription factor, the pancreatic duodenal homeobox-1 (Pdx-1) (56). Pdx-1 is expressed in β and δ cells of the islets of Langerhans and in dispersed endocrine cells of the duodenum. It is involved in regulating the expression of a number of key β-cell genes in addition to insulin, such as Glut2 (57), glucokinase (58), the islet amyloid polypeptide (59), and somatostatin (60). Gene knock out experiments of Pdx-1 demonstrate that Pdx-1 is essential for development of the pancreas (61). Furthermore, heterozygous mutations of Pdx-1 are found in MODY 4, a form of Type II diabetes (62).

The homeodomain protein Nkx6.1, which is expressed in developing and mature β-cells, binds to the A3/A4 element of the rat I promoter (63). Mice deficient for Nkx6.1 display a dramatic reduction in β-cell numbers with a dramatic decrease in insulin expression, yet other endocrine and exocrine cell types are not affected. Nkx6.1 functions as a transcriptional repressor and most likely plays an important role in β-cell differentiation (63). Many other proteins can bind the A elements. For example, high mobility group (HMG) proteins can bind to the A3/A4 region of the rat I insulin promoter

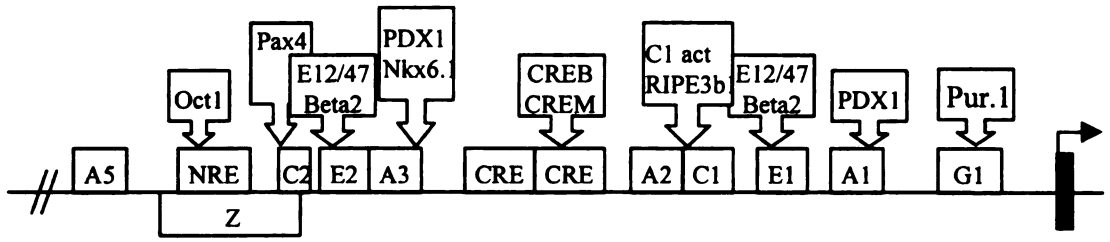

Figure 3. Human insulin promoter. Cis-acting regulatory elements along the promoter regions of the human insulin gene are boxed. Protein binding to these elements is indicated above in a highlighted box. The A1-5, E1-2, C1-2, and G1 elements are termed according to the nomenclature in (64). Pictured was modified from Molloul *et al.* (65).

(71). HMG I expression increases transcriptional synergy of Pdx1-bHLH heterodimers *in vivo* (71).

The E-box has a consensus sequence of CANNTG. There are two E elements in the human insulin gene; the E1 is located at –104 to –112 and the E2 is located at –230 to –238. Although the E1 element is highly conserved among mammalian insulin promoters, the E2 element is not well conserved. The E elements bind a ubiquitous class of the basic helix-loop-helix (bHLH) protein family, E12/E47, which heterodimerizes with the β-cell and the neuronal specific transcription factor Beta2. Beta2 is important for both insulin gene transcription (66) and pancreatic development (67). Homozygous knockout mice lacking Beta (i.e., $Beta2^{-/-}$) have fewer β-cells and develop diabetes (67). E12/47 and Beta2 control both insulin and glucagon gene expression. The heterodimer complex, however, has less binding affinity to the glucagon promoter than the insulin promoter (68). The E12/47/Beta2 dimer synergyzes with Pdx-1, and activate transcription of an E-A minienhancer in non-β-cells engineered to express these proteins (69). The human E2 element has also been reported to bind upstream stimulatory factor (USF) (70).

Some transcription factors have actually been demonstrated to interfere with the E12/E47 transactivation potential and induce insulin gene repression. The c-Jun transcription factor, a member of AP-1 family, inhibits the insulin gene by inhibiting the transactivation potential of E47 (72). Some proteins can compete for binding with E12/E47 and repress insulin gene expression. The Id proteins, a bHLH family that lacks

the DNA-binding domain, can heterodimerize with BETA2, resulting in a nonfunctional heterodimeric complex with decreased DNA binding activity (73).

The C1 element is a cytosine-rich sequence that lies between the A2 and the E1 element. The C1 element binds to the β-cell specific C1 activator/RIPE3b1 and to the RIPE3b2 in the rat insulin gene II (74). Recently the C1 activator was cloned and identified as a mammalian homologue of avian MafA/L-Maf (mMafA) (5).

The C2 element is a G/C rich conserved element located in the human insulin gene from -253 to –244 (75). The glucagon and somatostatin promoters also contain this conserved sequence, and all three genes, insulin, glucagon, and somatostatin, are regulated by the same islet specific factor, which was termed pancreatic "islet cell-specific enhancer sequence" (PISCES). The C2 rat I element binds the transcription factor paired-homeodomain PAX6, which is a transactivator (76). PAX6 is found in all islet cells, as well as in some neuroendocrine cells (76, 77). Mice homozygous for a mutation in the PAX6 gene die after birth due to cranial and facial defects, and the fetuses have a decrease in the number of islet cell types (76, 77). The remaining β-cells have decreased levels of insulin mRNA and insulin content (76). PAX6 binding to the human insulin promoter has not been demonstrated. Its role in the human insulin gene is not known. An additional factor that binds to the C2 element was characterized and named "D0". Interestingly, the binding of this transcription factor is sensitive to the redox-state of β-cells (75).

The cyclic AMP response element (CRE) binds the transcription factor CRE binding protein (CREB), which is regulated by the second messenger cyclic AMP (cAMP) through the phosphorylation of protein kinase A. Phosphorylated CREB

interacts with other transcription factors and induces transcripiton (reviewed in Ref. 75). There are four putative CREs in the human insulin gene, two in the promoter and two in the coding region (79). Glucose and other hormones stimulate cAMP accumulation, but this has only a modest effect on insulin gene transcription (80). There are several isoforms of CRE modulators that bind to CRE elements and can act as activators or repressors (81). CRE modulators named CREM have different isoforms by alternative splicing which can be activators or repressors. CREM activators interact with basal transcription machinery more efficiently than CREB. In contrast, the CREM repressor does not bind to the transcription machinery; instead, it competes for binding with CREM activators (82, 83). Interestingly, the Goto-Kakisaki (GK) rat, a Type II diabetic animal model, contains higher amounts of CREM repressor in islets than control rat islets. Therefore, CREM may have a role in the repression of insulin mRNA levels in this diabetic animal model (83).

The CRE element overlaps with a CCAAT motif, which binds the ubiquitous transcription factor NF-Y (84). This transcription factor leads to basal transcription activity and inhibits cAMP-induced transcription activity (84). The CCAAT/enhancer-binding protein β (C/EBPβ) binds to a downstream region of the CCAAT element, the A2 element (85, 86). It was demonstrated that C/EBPβ inhibits insulin gene expression by interfering with the transactivation domain of E47 (86). C/EBPβ expression is upregulated in β-cells chronically exposed to high glucose concentrations, such as in Zucker diabetes fatty rats and 90 % pancreatectomized rats, suggesting a possible role of this transcription factor in repressing insulin transcription in Type II diabetes (85).

The negative regulatory element (NRE) is located between –260 and –281 in the human insulin gene. It has been shown to inhibit insulin gene transcription in insulinoma cells (87, 88). The NRE has been suggested to act as a silencer because, when linked to a heterologous promoter, it represses transcription (88). The NRE, however, can be positively regulated when upstream sequences from NRE are linked to NRE in the heterologous promoter (88).

Recently, it was demonstrated that the NRE is not a silencer in all cell systems. In fact, removal of this insulin region causes a marked loss of activity in rat fetal islets (64). The NRE functions as a potent activator when placed upstream of a minimum rat promoter or a heterologous promoter in both fetal and adult islets. However, in the same constructs the NRE acted as a repressor in tumor β-cell lines, non-β-cells, and primary-cultured fibroblasts (89). Because of the positive effects of the NRE element in the islets, it was renamed the Z element (89). Several complexes bind to the Z element. In insulinoma cell lines, it has been demonstrated that both Oct 1 (88) and the glucocorticoid (90) receptor bind to the Z/NRE element. In contrast, these transcription factors were not contained in the complex that bind to the Z element in fetal islets (89). Further experiments are needed to clarify the role of Z/NRE in insulin gene transcription.

The G element is characterized by the GAGA sequence. It is located at positions –40 to –57 and is essential for gene transcription in fetal rat islets (91), and to a lesser degree in HIT-T15 cells (92). The ubiquitous zinc finger Purl binds to G1 element in the rat insulin I and II genes, and stimulates promoter activity (93). The role of the G1 element in the human insulin gene does not seem critical since its mutation does not affect promoter activity (94).

The complexity of the insulin promoter suggests that it is regulated by a combinatorial mechanism. The most studied cooperative interactions are between the E and A elements. Experiments performed in the rat insulin I gene promoter minienhancer, which contains just the E2 element juxtaposed to the A3/A4 sequence elements, demonstrated that deletion of either the E2 element or the A3/A4 element eliminate promoter activity, suggesting synergisms between these elements (95). It has been demonstrated that transcription factors that bind to the A3/A4 elements, such as Pdx1, act through a protein-protein interaction domain to recruit multiple proteins, including E47, BETA2, and HMG I, to the E2A3/A4 minienhancer (71). Interactions between Pdx-1 and E47 can also recruit co-activators such as p300 to induce rat I minienhancer promoter activity (96).

Studies of the human insulin promoter have demonstrated that the E1 element interacts with the A1 element and A2/C1 elements (64). The A2/C1-E1-A1 region alone, however, does not explain all of the activity of the human insulin promoter, suggesting that other transcription factors and DNA elements are required for full promoter activity.

5. Diabetes mellitus

Diabetes mellitus used to be considered a disease of minor significance for global health, but is now one of the largest threats to human health. Changes in the human environment and human lifestyle have led to large increases in both obesity and diabetes. This disease now affects 151 million people worldwide and 14.2 million Americans (97).

There are two main forms of diabetes mellitus, insulin dependent-diabetes (IDDM or Type I) and non-insulin dependent diabetes mellitus (NIDDM or Type II).

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5.1 Type I diabetes mellitus

Type I diabetes, generally observed in children, is primarily due to autoimmune-mediated destruction of pancreatic β-cell islets, resulting in an absolute insulin deficiency. The etiologic agents that induce this disease are not well understood. Environmental factors such as virus infections are most likely to be involved in the destruction of β-cells. There is also Type I diabetes that is not related to an autoimmune-mediated destruction and is most likely due to an inherited predisposition that does not involve the histocompatibility genes. People with Type I diabetes must take exogenous insulin for survival. This form of diabetes has a very low frequency compared with Type II diabetes. A comprehensive review of Type I diabetes is beyond the scope of this thesis.

5.2 Type II diabetes mellitus

Glucose homeostasis is maintained by a balance between glucose production by the liver and glucose utilization by insulin-dependent tissues (e.g., fat and muscle) and insulin-independent tissues (e.g., brain and kidney). Glucose utilization is regulated primarily by insulin and glucagon. Glucose utilization generally depends on three factors: 1) the ability of the body to secrete insulin both acutely and in a sustained fashion, 2) the ability of insulin to inhibit hepatic glucose output and to promote glucose disposal, and 3) and the ability of glucose to enter cells that do not require insulin. Type II diabetic patients are generally characterized by two pathological defects. One is decreased insulin action in peripheral tissues that need to increase the uptake glucose or to inhibit hepatic glucose output, a phenomenon known as insulin resistance (98). The

other is the inability of β-cells to secrete sufficient amounts of insulin necessary to compensate for insulin resistance (99).

These two primordial defects in the pathogenesis of Type II diabetes are caused by a combination of genetic and nongenetic factors. The genetic factors are not well characterized, while the nongenetic factors include increased age, high caloric intake, obesity, central adiposity, sedentary lifestyle, and low birth weight. Type II diabetics may control their blood glucose levels with a balanced diet and exercise, pharmacological therapies, and sometimes with exogenous insulin. This type of diabetes accounts for 90% of total global diabetes cases.

5.2.1 β-Cell failure during the progression of diabetes

The inability of β-cells to secrete sufficient amounts of insulin necessary to compensate for insulin resistance is generally termed β-cell failure. Studies performed on Zucker diabetic fatty (ZDF) male rats and 90% pancreatectomized rats, both Type II diabetic animal models, suggest that there are four phases that lead to β-cell deterioration (reviewed in Ref. 97, 98). These studies associate changes in islet morphology, insulin secretion, and gene expression with the hyperglycemic state. The description of the following phases is hypothetical, and results that lead to these hypotheses can be found in the above-mentioned citations.

<u>β-Cell compensation and dysfunction in the progression of insulin resistance and Type II diabetes</u>:

Following there is a general description of the characteristics of the Zucker diabetic fatty (ZDF) male rats and the 90% pancreatectomized rats models, and their relative

controls, which are used in the description of the four phases of β-cell failure during the development of diabetes. Zucker fatty diabetic male rats have a genotype of homozygous *fa/fa* leptin receptor. They develop insulin resistance and glucose intolerance between 3 and 8 weeks of age (prediabetic at 7 weeks old) and usually become overtly diabetic between 8 and 10 weeks. Lean heterozygous +/*fa* or homozygous +/+ normal leptin receptor littermates develop neither insulin resistance nor diabetes, and they are called Zucker lean controls (ZLC). A β-cell function analysis was performed in ZLC, prediabetic, and diabetic rats. The serum glucose levels of prediabetic ZDF rats were higher than in controls, but the difference was not significant. The serum triglyceride (TG) levels of prediabetic animals were normal, in contrast with the diabetic animals, which had a high level of TG. The body weight of the prediabetic animal was only 14 % higher than that of the lean animal at 7 weeks old, as well as the diabetic rat compared to ZLC 9-12 week old. Both prediabetic and diabetic animals are insulin resistant, with insulin levels that are 8 to 12-fold higher than those of controls.

In the 90 % pancreatectomized (Px) animal model, 85 to 95 % of the pancreas is removed. Regeneration of islets lasts no longer than ~ 10 days after surgery, but there is a stable mature population of islets exposed to hyperglycemia from the first week post-Px. The animals gain normal weight and are metabolically stable without needing special diets or hypoglycemic therapies that might affect the β-cells.

Phase I: β-cell adaptation for insulin resistance. To compensate for insulin resistance, the β-cells of prediabetic ZDF animals and Px animals increase in mass with increased neogenesis (regeneration of islets from precursor cells) and replication. The β-cells also become hypertrophic with multiple irregular projections into the surrounding

exocrine pancreas. There is significantly increased insulin secretion at each glucose concentration tested, with a left shift in the dose-response curve relating glucose concentration and insulin secretion. The β-cells have defects in the normal oscillatory pattern of insulin secretion, indicating impairment of the normal feedback mechanism between glucose and insulin secretion.

Phase II: decompensation of β-cells exposed to mild hyperglycemia. The β-cells lose the ability to secrete insulin in response to acute glucose, but there is preservation of insulin secretion to other secretagogues such as arginine. There are changes in important gene expression, such as decreased Glut2, glucokinase, pyruvate carboxylase, genes involved in ion channels, and transcription factors (Pdx-1, HNFs, Nkx6.1, and Pax6). There is increased expression of lactate dehydrogenase (LDH), glucose-6-phosphatase, and the transcription factor c-Myc. There are some differences in gene expression between both models, such as an increase in hexokinase in β-cells of Px, in contrast to the hexokinase repression in β-cells of the ZDF rat.

Phase III: decompensation of β-cells exposed to severe hyperglycemia. β-Cells show total loss of insulin response to glucose and impairment of insulin response to other non-glucose secretagogues. There is β-cell degranulation, which is correlated with decreased insulin mRNA. There are alterations in metabolic genes, such as further decreased expression of Glut2, glucokinase, and glycerol phosphate dehydrogenase, as well as decreased expression of potassium channel Kir6.2 and calcium ATPase. In the β-cells of Px, there is a marked increased in the expression of nitric oxide synthase (iNOS), antioxidant genes such as heme oxygenase-1, Mn-superoxide, and glutathione peroxidase, and antiapoptotic genes such as gene A20. These phenotypic changes can be

beneficial or detrimental to β-cells. For example, the induction of these protective genes may be a compensatory mechanism that promotes survival of β-cells. In contrast to β-cells of Px, β-cells of ZDF rat have a marked decreased in iNOS expression. This contradictory result can be explained if the isolated islets from Px were contaminated with ductal cells, which could led to an increase in iNOS expression. In addition, it has been demonstrated that NO can impair glucose-induced insulin secretion (102). Even though all these alterations occur, some β-cells still remain intact and are able to maintain some insulin secretion and prevent more profound β-cell deterioration.

Phase IV: decompensation with structural damage. Insulin granules also contain amylin peptide, which is co-released with insulin. The granule content of amylin is only 1 to 2 % those of insulin. Amylin tends to polymerize, and amylin fibrils accumulate extracellularly within the islets of Type II diabetics. The mechanisms of amylin fibril formation are not well understood, but they can have destructive effects on β-cell function. In this phase, glycogen deposits and lipid droplets are also observed, and investigators have correlated these accumulations of glycogen and lipids to lipid toxicity. Finally β-cells go through apoptosis, a process that has been difficult to quantify since in a chronic situation it is short-lived.

As the 90 % pancreatectomized rat and the Zucker fatty rat models demonstrate, there are many phenotypic changes in β-cells that lead to the loss of glucose-induced insulin secretion, and eventually to β-cell apoptosis.

6. Pathology of β-cells in Type II diabetes

Type II diabetes results from the failure of β-cells to compensate for the increased insulin demand due to increased insulin resistance. It has been demonstrated that once diabetes is established, chronic hyperglycemia and hyperlipidemia can induce deleterious effects on β-cell function, referred to as glucose toxicity and lipotoxicity, respectively. This section will focus on *in vitro* and *in vivo* Type II diabetes models where it has been shown that gluco-lipo toxicity induces β-cell dysfunction.

6.1 Glucotoxicity

As early as 1948, Dohan and Luckens (103) suggested that high levels of glucose induce some changes in β-cell phenotype. They administered large doses of glucose to normal cats, inducing permanent hyperglycemia and degeneration of islet of Langerhans, and ketonuria was observed. Dohan and Luckens (103) proposed that hyperglycemia could play a role in the pathogenesis of diabetes. In the last two decades, the hypothesis that hyperglycemia could be responsible, at least in part, for β-cell dysfunction has received considerable attention, and studies in different Type II animal models have been performed.

6.1.1 Studies in Type II diabetic patients

Studies in the mid-1970s demonstrated that normalization of blood glucose levels for short periods of time restored glucose-induced insulin secretion in Type II diabetics (104). Brunzell *et al.* (105) demonstrated that the first-phase of glucose-induced insulin secretion is lost in humans with high plasma glucose concentrations. Vogue *et al.* (106)

showed that decreased glucose-induced insulin secretion is partially restored after 20 hrs of insulin infusion in hyperglycemic Type II diabetes patients. These studies clearly point to the important role that hyperglycemia plays in the defects of β-cell function associated with Type II diabetes and demonstrate that insulin response to glucose is partially restored upon normalization of plasma glucose levels.

6.1.2 *In vivo* studies of animal models of Type II diabetes and *in vitro* studies of isolated islets and insulinoma cells.

In the late 1980s, *in vivo* Type II diabetic models suggested that hyperglycemia leads to decreased glucose-stimulated insulin secretion. Leahy J.L *et al.* (107, 108) infused normal rats *in vivo* with various concentrations of glucose for 48 hrs and then measured insulin response to glucose (2.0 mM and 16.7 mM) in the *in vitro* isolated perfused pancreas. During the infusion period, the animal became hyperglycemic. Glucose-induced insulin release was blunted after 48hrs of glucose infusion, and insulin content was decreased. In contrast, in a second protocol, when phlorizin (a reagent used to decrease plasma glucose levels by inhibiting renal tubular reabsorption of glucose) was added during a second 48hrs of infusion, glucose-induced insulin release was completely restored. These results indicate that high levels of glucose decrease glucose-induced insulin release and possible biosynthesis.

Studies in the 90 % pancreatectomized rat model have provided further evidence that hyperglycemia induces loss of glucose-induced insulin release (109). The ninety percent pancreatectomized rats showed moderate hyperglycemia 4 weeks after surgery. Insulin response to glucose was blunted but arginine-induced insulin response was intact

in intraperitoneal and intravenous glucose tolerance tests and intravenous arginine challenge given 6-7 weeks after surgery. Similarly, when the pancreatic remnant was perfused *in vitro*, insulin release was markedly reduced after challenge with high glucose concentrations and remained the same after challenge with arginine. These data suggest that chronic stimulation of β-cells can lead to loss of glucose stimulation of insulin secretion.

Glucose toxicity on β-cell function was demonstrated in additional *in vivo* studies by transplanting islets grown in high glucose levels under the kidney capsule of syngenic mice that were made diabetic with streptozotocin (110). The islets grown in media containing high levels of glucose (22.0 mM glucose) failed to restore blood sugar levels of diabetic animals. In contrast, diabetic animals became normoglycemic with islets grown in normal media (5.0 mM glucose).

Investigators have demonstrated that altered glucose-induced insulin secretion in diabetes is associated with reduced glucose transporter Glut2. Perifused islet studies in Zucker fat diabetic rats show impairment in the ability of the islets to secrete insulin in respond to a glucose stimulus (101). Studies in this animal model demonstrated that loss of glucose-induced insulin secretion is accompanied by a marked reduction of Glut2 protein (111), which is associated with reduced levels of Glut2 mRNA (112). Reduced Glut2 expression and mRNA were also observed in islets of the 90% pancreatectomized rat model (113). Whether Glut2 contributes to the secretory impairment of diabetes is unclear. Some reduction of Glut2 transport capacity has been demonstrated in Zucker diabetic fatty rats (111, 114), but the changes may not be sufficient for transport to become rate limiting for glucose metabolism. The rate-limiting step in glucose

metabolism is the phosphorylation of glucose by glucokinase, and glucose transport capacity of β-cells exceeds that of glucose phosphorylation by 10-fold (115). In addition, De Vos *et al.* demonstrated that human β-cells have very little Glut2 but abundant Glut1. Studies have demonstrated that Glut2 interacts with glucokinase to increase glucose phosphorylation and thus to enhance insulin secretion (116, 117). Nevertheless, loss of Glut2 expression, at least in rodents, may be involved in β-cell dysfunction in diabetes.

In vitro studies of cultured human pancreatic islets, isolated rat islets, and insulinoma cell lines have also demonstrated that hyperglycemia mediates β-cell dysfunction. Eizirik *et al.* demonstrated that prolonged exposure of human pancreatic islets to high levels of glucose *in vitro* impairs β-cell function (118). Isolated islets from adult cadaveric organ donors were cultured for seven days in media containing 5.6, 11, or 28 mM glucose. Insulin content was decreased in islet cultured in 11 or 28 mM glucose, compared to 5.6 mM glucose. The isolated human islets were submitted to a 60-min stimulation with a low (1.7 mM) followed by a high (16.7 mM) concentration of glucose. The islets cultured in the high concentration of glucose (28 mM) had a reduced insulin secretion compared to the islets incubated in 5.6 mM glucose. The rates of insulin biosynthesis, glucose oxidation, and total protein biosynthesis were significantly lowered in islets cultured in 28 mM glucose, compared to 11 and 5.6 mM glucose. There was no change in islet DNA content in the three treatments. In addition, Briaud *et al.* (119) demonstrated that isolated rat islets exposed to high levels of glucose for 6 weeks showed decreased insulin mRNA levels. These results suggest multiple mechanisms by which hyperglycemia may induce β-cell dysfunction.

Studies with insulinoma cell lines have also demonstrated some of the mechanisms involved in hyperglycemia-induced β-cell dysfunction. The insulinoma HIT-T15 cell line, a Syrian hamster pancreatic islet transfected with SV-40 large T antigen, shows similar characteristics of β-cell dysfunction as observed in *in vivo* experiments when cells are incubated in high levels of glucose (11.1 mM) for prolonged periods of time (6 months). Insulin content, insulin mRNA, and insulin stimulated secretion are reduced in HIT-T15 cells incubated serially in high levels of glucose (120). These phenotypic changes in HIT-T15 cells were reversible when cells were cultured in low levels of glucose (120). To understand the molecular basis for the reduction in insulin mRNA, Olson *et al.* transiently transfected HIT-T15 cells with a chloromphenicol acetyl transferase (CAT) reporter gene controlled by the 5'regulatory sequences of the human insulin gene (INS(-327)CAT) and demonstrated that cells serially cultured in 11.1 mM glucose have decreased insulin promoter activity (121). In addition, decreased insulin promoter activity was associated with a reduction in binding activity of two important insulin gene transcription factors, Pdx-1 and C1 activator (RIPE3b1) (3, 121). Olson *et al.* also demonstrated that high levels of glucose reduce Pdx-1 mRNA by a post-transcriptional mechanism (3). These studies suggest that β-cells exposed to chronic hyperglycemia decrease glucose-induced insulin secretion, in part, because of reduced insulin mRNA levels, and this correlated with reduced insulin promoter activity.

Subsequent work by Harmon *et al.* (122) assessed the temporal loss in binding activity of Pdx-1 and C1 activator in HIT-T15 cells cultured in high levels of glucose. HIT-T15 cells serially cultured in high levels of glucose reduced insulin promoter activity by passage 80 to 85. The C1 activator binding activity was reduced by passage 81; in

contrast, Pdx-1 binding activity was reduced by passage 106. These results suggest that the loss of C1 activator may play a dominant role in glucose toxicity of β-cells. Studies analyzing whether reconstitution of Pdx-1 would restore insulin promoter activity in HIT-T15 cultured in high levels of glucose have indicated that Pdx-1 partially reconstitutes insulin promoter activity (122). Experiments reconstituting insulin promoter activity with C1 activator have not been yet performed because it was not cloned until recently. These studies suggest that high levels of glucose affects insulin gene transcription by decreasing Pdx-1 and C1 activator binding activity.

Similar effects of glucose toxicity on β-cell function were observed when βTC-6 cells, a mouse β-cell transformed with the large T antigen of SV40 driven by the rat insulin II promoter (123, 124), were incubated in elevated glucose for prolong periods of time (125). β-TC-6 cells, incubated for up to 41 weeks in 11.1 mM glucose, had decreased insulin content and insulin mRNA levels compared to cells incubated in 0.8 mM glucose (125). Insulin promoter activity was also decreased and this was associated with a loss in C1 activator binding activity (125). Interestingly, no change in Pdx-1 binding activity was observed in βTC6 cells cultured serially in high levels of glucose. These results suggest that chronic exposure of βTC-6 cells to high levels of glucose concentrations decreases insulin gene transcription, in part, by reducing C1 binding activity.

Studies in INS-1 cells, another insulinoma cell line, also demonstrated that high levels of glucose decrease insulin mRNA, and that decreased insulin mRNA is recovered when cells are cultured in low levels of glucose (126). The investigators also

demonstrated that reduced insulin mRNA is associated with reduced Pdx-1 and C1 binding activities.

Lu *et al.* (86) demonstrated that chronically exposing HIT-T15 cells and INS-1 cells, both insulinoma cells lines, to high levels of glucose leads to increase in the CAAT/enhancer-binding protein β (C/EBPβ) gene expression, suggesting that glucose toxicity in β-cells may be related to increased levels of C/EBPβ. Interestingly, over-expressed C/EBPβ represses rat I insulin promoter activity by interacting with E47 transcription factor and inhibiting dimerization and DNA binding of E47 (86). *In vivo* studies in the Zucker diabetic fatty (ZDF) rat and the 90 % pancreatectomized rat models demonstrated similar findings in the regulation of C/EBPβ expression. Seufert *et al.* (85) demonstrated that Pdx-1 expression is downregulated while C/EBPβ is upregulated in ZDF rats and in 90 % pancreatectomized rats. These results suggest that hyperglycemia-induced repression of insulin expression may be mediated by upregulation of C/EBPβ.

c-Myc transcription factor has also been shown to be involved in the glucose-induced repression of insulin gene expression (4). c-Myc expression is induced in diabetic rats following partial pancreatectomy and in rats made hyperglycemic with glucose clamps (4). Over-expression of c-Myc represses insulin gene promoter activity in insulinoma cells and in primary rat islets by inhibiting Neuro D/BETA2-mediated transcriptional activation (127). Furthermore, Kaneto et al. demonstrated that glucose-induced c-Myc expression is activated by PKC β2 in primary rat islets (127). Other potential roles of induced c-Myc during diabetes have been correlated to cell replication. c-Myc is involved in cell cycle progression, differentiation and apoptosis (128-130). β-

cell hypertrophy is a compensatory mechanism induced by hyperglycemia, as described in the section of β-cell adaptation to diabetes. Recently, Laybutt *et al.* (131) demonstrated that the acetyl-CoA carboxylase gene, a gene involved in fatty acid oxidation, is upregulated in the 90 % pancreatectomized rat. Acetyl-CoA carboxylase gene promoter has c-Myc binding sites, and thus increased c-Myc could upregulate acetyl-CoA carboxylase expression. c-Myc may be involved in a variety of phenotypic changes in β-cell dysfunction during diabetes.

As described so far, hyperglycemia causes a variety of changes in β-cells that trigger β-cell dysfunction. This idea was further explored by Jonas *et al.* (4) using the *in vivo* 90 % pancreatectomized animal model. The authors showed that hyperglycemia decreases gene expression involved in glucose-induced insulin release in parallel with the reduction of transcription factors necessary for β-cell development and differentiation. At the same time, hyperglycemia induced the expression of genes that are only minimally expressed in β-cells, such as lactate dehydrogenase A and hexokinase I. These changes occurred in parallel with increased β-cell hypertrophy, which is a typical characteristic of β-cells compensating for insulin resistance. The β-cell changes were specific to hyperglycemia, because normalizing of blood glucose levels with phlorizin prevented phenotypic changes. In conclusion, chronic hyperglycemia leads to β-cell dysfunction and β-cell hypertrophy by a variety of alterations in gene expression that regulate β-cell development and differentiation.

6.2 Lipotoxicity

Increased incidence of type II diabetes is associated with obesity, and in many patients, weight loss can significantly control the condition. There are high levels of plasma free fatty acids (FFA) and increased fat oxidation in many obese diabetics. Increased plasma FFA has been associated with increased insulin resistance and increased liver gluconeogenesis (132). In obese Type II diabetic animal models there is excessive FFA within the islets and this has been suggested to contribute to β-cell dysfunction, known as lipotoxicity. Prolonged exposure of islets to FFA stimulates basal insulin secretion (133, 134) and inhibits glucose-induced insulin secretion (133, 135). Elks *et al.* (135) demonstrated that isolated rat islets chronically perfused with palmitate suppress glucose-stimulated insulin release. Islets were perfused with 1 mM palmitate for up to 4 hrs and then perfused with 3 or 17 mM glucose for 20 min. The results showed that chronic exposure of islets to palmitate suppresses first and second phase insulin release. Inhibitors of fat oxidation including α-bromostearate and methyl-3-tetradecylglycidate reversed this suppression. *In vitro* studies by Gremlich *et al.* (136) demonstrated that isolated rat islets chronically exposed to palmitate decreased insulin gene transcription in the presence of 30 mM glucose by inhibiting Pdx-1 mRNA and protein expression. Palmitate also inhibited Pdx-1 binding activity to insulin and Glut2 gene promoter, as well as Glut2 and glucokinase mRNAs and protein expression. These experiments suggest that the second phase of glucose-induced insulin secretion is inhibited by palmitate through negative regulation of insulin and glucose sensing genes. Thus, free fatty acids can contribute to β-cell dysfunction as observed in diabetes.

As previously discussed, both hyperlipidemia and hyperglycemia induce β-cell dysfunction. The toxic effects of both conditions on β-cell function have been controversial. The following review will provide evidence that suggests that hyperglycemia is required for hyperlipidemia to damage β-cells. *In vitro* experiments by Jacqueminet *et al.* (137) demonstrated that inhibition of insulin gene expression by chronic exposure of isolated rat islets to palmitate requires the presence of elevated glucose concentrations. Isolated rat islets were incubated for a week in the presence of 2.8 or 16.7 mM glucose with or without 0.5 mM palmitate. Insulin mRNA was increased from 2.0 to 16.7 mM glucose and palmitate inhibited this glucose-induced increase in insulin mRNA. The authors associated palmitate reduction in insulin mRNA to reduction in insulin promoter activity through studies using HIT-T15 cells. The conclusion of these experiments was that long-term exposure to palmitate coupled with the presence of high levels of glucose, represses insulin mRNA. *In vivo* experiments have demonstrated that a rise in TG and FFA causes β-cell dysfunction in Zucker diabetic fat rats. These animals have increased FFA and TG in the prediabetic phase and increased islet triglyceride content immediately before the hyperglycemia state (138). In addition, diet restriction before hyperglycemia appears to reduce hyperlipidemia, hypertriglyceridemia, and accumulation of fat in islets, as well as prevent of hyperglycemia and β-cell dysfunction (138). These results indicate that increased TG and FFA in Type II diabetic rats induce β-cell dysfunction independently.

Recently, Harmon *et al.* (139) investigated the roles of hyperglycemia and hyperlipidemia on β-cell dysfunction in the Zucker diabetic fatty rat (ZDF). The ZDF rats were treated with bezafibrate, a lipid-lowering drug that does not affect plasma

glucose levels, or phlorizin, a drug that reduces plasma glucose without affecting lipids levels, after they had become hyperglycemic and hyperlipidemic. Treating rats with bezafibrate lowered plasma TG levels but did not prevent the rise of TG levels in the islets and did not prevent the decrease in insulin mRNA. In contrast, treating ZDF rats with phlorizin lowered plasma glucose levels and lowered islet TG content as well as preserved insulin mRNA levels. These results indicate that high levels of glucose, and not high levels of lipids, induces β-cell dysfunction such as decreased insulin mRNA and elevated islet TG content. In addition, Briaud *et al.* (140) investigated the role of hyperlipidemia and hyperglycemia on β-cell dysfunction in the Goto-Kakizaki (GK) rat, a lean Type II diabetic animal model. Feeding GK rats with a high fat diet for 6 weeks led to an increase in epididymal fat weight, plasma TG, and FFA levels. High-fat fed GK rats showed impaired insulin secretion, decreased insulin mRNA, and low insulin content in contrast to high-fat fed Wister rats, a non-diabetic lean rat. Islet TG content and islet glucose oxidation were not affected by the high fat diet. High-fat fed GK rats treated with insulin prevented reduction of glucose-induced insulin secretion. These results indicate that hyperlipidemia-induced β-cell dysfunction requires the presence of hyperglycemia. Thus, glucotoxicity and lipotoxicity are closely interdependent, in the sense that lipotoxicity does not exist without hyperglycemia. This is consistent with the clinical observation that hyperlipidemic individuals are not necessarily diabetic, and their β-cell function is typically normal. These results are very important for understanding glucose toxicity on β-cell function in the Zucker diabetic fatty rat model, since high levels of lipids are always present in these animals.

One of the mechanisms by which lipotoxicity induces β-cell dysfunction is through generation of reactive oxygen species and uncoupling respiratory chain reactions. Carlsson *et al.* (141) demonstrated that isolated pancreatic islets exposed to palmitate have decreased glucose-induced insulin secretion, decreased ATP levels, decreased islet cell mitochondria membrane potential, and increased, yet, uncoupled respiration. They also observed an increase in β-cell mitochondrial volume in islets exposed to palmitate. Use of a mitochondrial uncoupler reagent (carbonyl *p*-phenylbydrazone) at concentrations that decreased mitochondrial membrane potential to a similar level as palmitate reduced glucose-induced insulin secretion. In addition, islets exposed to palmitate increased the generation of reactive oxygen species but not of nitric oxide. These results indicate that islets exposed to fatty acids mediate β-cell dysfunction, in part, through oxidative stress and uncoupling oxidative phosphorylation. Interestingly, chronic hyperglycemia also induces β-cell dysfunction, in part, through reactive oxygen species, which will be explained in the next section. Taken together these findings suggest that chronic hyperglycemia and hyperlipidemia interdependently may mediate β-cell dysfunction through oxidative stress.

6. Mechanisms of hyperglycemia-induced damage

7.1 Oxidative stress.

Several experiments have demonstrated that oxidative stress may be a mechanism through which hyperglycemia causes β-cell dysfunction. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidant levels

(142). High levels of glucose increase ROS levels, leading to oxidative stress. ROS are eliminated by scavenger proteins such as catalases, superoxide dismutases, and glutathione peroxidases, as well by antioxidants like glutathione and vitamins (143). It has been demonstrated that hyperglycemia can lead to an increase of both activity and mRNA levels of antioxidant enzymes such as Cu, Zn-superoxide dismutase, catalase, and glutathione peroxidase (144). Thus, over-expression of antioxidant enzymes could be a mechanism to compensate for glucose-induced oxidative stress. Three main mechanisms are involved in the development of oxidative stress in the presence of hyperglycemia: 1) protein glycation and formation of advanced glycation end products (AGEs), 2) glucose autoxidation, and 3) the polyol pathway.

A. Protein glycation and advanced glycation end products

Protein glycation results from the formation of covalent binding between the aldehyde glucose group and the amino group of proteins. This binding is generated through non-enzymatic glycation reactions, known as Maillard reaction (145). In the presence of transition metals such as copper and iron, glycated proteins can donate an electron to oxygen, leading to oxygenated free radicals (146, 147). Glycated proteins can undergo irreversible modifications leading to advanced glycosylated end products (AGEs); this is a slow process and AGEs are formed only on long-lived macromolecules (142, 145).

B. Glucose autoxidation

Glucose can oxidize when catalyzed by trace amounts of transition metals; this process generates superoxide anions and carbonyl compounds. Superoxide anions can

generate hydrogen peroxide, which in the presence of transition metals produces reactive hydroxyl radicals (149, 150). The rate of glucose oxidation is very slow, but it has been demonstrated in diabetes that collagen breakdown occurs during autoxidative glycation (151).

C. Polyol pathway

Glucose is converted to sorbitol by aldose reductase, which is then converted to fructose by sorbitol dehydrogenase; this pathway is known as the polyol pathway (152). Aldose reductase activity requires NADPH. Therefore, increased activity of the polyol pathway can result in depletion of intracellular NADPH, which is also required for antioxidant enzymes to eliminate ROS (153). In addition, enhancement of the polyol pathway leads to an increased concentration of intracellular sorbitol due to its slow diffusion rate. Therefore, hyperglycemia induces the accumulation of sorbitol inside the cell, which induces osmotic stress, as observed in lens (154). Enhanced polyol pathway leads to the generation of fructose, which can readily go through non-enzymatic glycosylation, generating more ROS.

7.1.1 Oxidative stress in diabetes

Many investigators have reported that chronic exposure to hyperglycemia causes tissue damage through oxidative stress (155-158). Increased glycated tissues such as kidney, liver, brain, and lung are observed in diabetic conditions (159, 160). Glycated proteins such as albumin, lens crystalline, and hemoglobin are also observed in diabetic

individuals, as well as advanced glycated end products (AGEs) in LDL (161-163). Increased glycated and AGE proteins can further generate ROS, which can exacerbate diabetic conditions. In addition to other tissue damaged through ROS, pancreatic β-cells are very susceptible to ROS because they have low expression of antioxidants such as glutathione and catalases (164).

7.1.1.1 Oxidative stress in diabetic individuals and whole animals

Oxidative markers such as 8-hydroxy-2'deoxyguanosine (8-OHdG) have been identified in the urine and blood of patients with Type II diabetes (165, 166). 8-OHdG is a mutation induced by ROS that results in C:G to A:T transversions during DNA replication. In addition, Ihara *et al.* (157) demonstrated increased levels of 8-OHdG in pancreatic β-cells of the Goto-Kakizaki (GK) rat, which develops diabetes spontaneously. Increased 4-hydroxy-2-neonal (HNE)-modified proteins are also observed in pancreatic β-cells of GK rats (157). HNE is an α,β-unsaturated aldehyde formed by lipid peroxidation, which is induced by ROS. The authors showed further induction of 8-OHdG and HNE when the GK rats were fed a 30 % sucrose solution for 4 weeks. In contrast, 8-OHdG and HNE were reduced when GK rats were fed with voglibose, a postprandial blood glucose suppressor that delays carbohydrate digestion and reduces the rate of glucose absorption by inhibiting intestinal α-glucosidase.

Since hyperglycemia causes oxidative stress, one can hypothesize that antioxidants could prevent hyperglycemia's toxic effects on different organs and β-cells. Effects of

antioxidants were analyzed in Type II diabetic rat models. Tanaka *et al.* (7) demonstrated that Zucker fat rats treated with antioxidants, N-acetyl-L-cysteine (NAC) or aminoguanidine (AG), prevented a rise of 8-OHdG and HNE in the blood, and partially prevented hyperglycemia, changes in glucose tolerance, defective insulin secretion, and insulin content. Importantly, the authors also demonstrated that nitric oxide is not involved in β-cell dysfunction, since an inhibitor of nitric oxide synthase had no beneficial effects on β-cell dysfunction. This distinction is important because nitric oxide is involved in cytokine-induced β-cell apoptosis observed in Type I diabetes.

Further studies by Kaneto *et al.* (6) demonstrated that antioxidants could improve diabetic conditions. Diabetic C57BL/KsJ-db/db mice are an obese Type II animal model where hyperglycemia is induced because of increased insulin resistance and subsequent insufficient β-cell compensation. Mice were treated with NAC for 4 to 10 days after they had become hyperglycemic. Mice treated with NAC showed improved glucose-stimulated insulin secretion and an improved intraperitoneal glucose tolerance test. Antioxidant treatment also preserved insulin content and insulin mRNA levels, as well as improved detection of Pdx-1 in nuclei of islet cells. Overall, these results indicate that hyperglycemia-induced β-cell dysfunction may be mediated by oxidative stress.

7.1.1.2 Oxidative stress in isolated islets and insulinoma cell lines

Under diabetic conditions, oxidative stress is also produced through the polyol pathway (167, 168). Fructose is a reduced sugar and can be readily glycated to amine groups, a process that generates ROS and AGEs. Kaneto *et al.* (169) demonstrated that treating HIT-T15 cells with 50 mM fructose or 25 mM D-ribose, a reduced sugar, induces

oxidative stress and apoptosis. In this study, β-cell apoptosis was prevented in cells incubated with NAC. These results indicate that reducing sugars triggers oxidative stress, which may explain β-cell deterioration in diabetes.

In support of the hypothesis that high levels of glucose induces β-cell dysfunction through oxidative stress, Tajiri *et al.* (170) demonstrated that in isolated rat islets, glucose-induced repression of insulin secretion is restored by aminoguanidine (AG), an inhibitor of glycation and formation of AGEs. Isolated islets were cultured in 38 mM glucose with or without AG for 6 weeks, and then had a wash-out period of continued culture at 11 mM glucose for 24 hrs without AG. Islets were subject to analysis of insulin secretion at 3.3 or 27 mM glucose. AG treatment enhanced insulin response to 27 mM glucose two-fold compared to cells treated without AG. These results indicate that inhibition of glycosylation reactions might prevent hyperglycemia-induced β-cell dysfunction.

Effects of antioxidants on hyperglycemia-induced repression of insulin gene expression and insulin promoter activity have been analyzed. Matsuoka *et al.* (158) demonstrated that insulin promoter activity was decreased in HIT-T15 cells cultured in 40 mM D-ribose for three days, and this decrease was associated with reduced Pdx-1 binding activity. Reduced insulin promoter activity and Pdx-1 binding activity were prevented in HIT-T15 cells incubated in 10 mM NAC or 1mM AG. Further experiments by Tanaka *et al.* (7) also demonstrated that high levels of glucose reduce insulin gene expression through ROS. HIT-T15 cells were cultured serially with 11.1 mM glucose and antioxidants, NAC or AG. Insulin mRNA level, insulin content, insulin promoter

activity, Pdx-1 binding activity, and glucose-induced insulin secretion were all moderately increased by NAC or AG. Overall, these studies indicate reduced insulin gene expression by hyperglycemia is mediated through oxidative stress.

Glucose can also modify and activate transcription factors by *O*-linked glycosylation through the hexosamine pathway. In the hexosamine pathway, *O*-linked glycosylation is catalyzed by O-linked N-acetylglucosamine transferase (OGT), which attaches the N-acetylglucosamine monosacharides (GlcNAc) to the hydroxyl group of serine or threonine residues of intracellular proteins. Glucose is converted to N-acetylglucosamine through several reactions, and one of the enzymes involved is glutamine: fructose-6-phosphate aminotransferase (GFAT), which converts fructose-6-phosphate to N-acetylglucosamine-6-phosphate. Recently, Kaneto *et al.* (171) investigated whether β-cell dysfunction, as induced by hyperglycemia, can also be observed by artificially inducing the hexamine pathway. Isolated pancreatic β-cells were infected with adenovirus-mediated over-expression of GFAT or treated with glucosamine. Both treatments impaired glucose-stimulated insulin secretion and reduced the expression levels of insulin, Glut2, and glucokinase, as well as reduced Pdx-1 binding activity. Importantly, glucosamine increased hydrogen peroxide levels, and the phenotypic β-cell changes induced by GFAT were prevented by treatment with N-acetyl-L-cysteine (NAC) but not with an inhibitor of *O*-linked glycation. These results demonstrated that GFAT induces β-cell dysfunction as observed in β-cells treated with high levels of glucose and that these changes are prevented by antioxidants. These results suggest that hyperglycemia-induced β-cell dysfunction may be mediated through the hexosamine pathway by inducing oxidative stress.

7.1.2 Mechanisms of oxidative stress-induced β-cell damage

Several investigators have demonstrated that hyperglycemia induces β-cell dysfunction through reactive oxygen species (ROS), although the exact mechanism of action for ROS-induced damage in β-cells is not known. There are at least four mechanisms by which ROS can damage β-cells. First, ROS can modify DNA by forming 8-OHdG (157), which is a pro-mutagenic lesion. Second, ROS can modify membrane lipids, inducing lipid peroxidation and further modifications to form aldehydes such as HNE (157). Third, ROS can modify mitochondrial membrane potential, which eventually interrupts the coupling of glucose metabolism to insulin secretion, as demonstrated by Maechler *et al.* (172). Maechler *et al.* showed that pancreatic β-cell dysfunction is induced in isolated rat islets and INS-1 cells (an insulinoma cell line) treated with 200 μM H_2O_2 for 10 min. Insulin secretion was inhibited when isolated rat islets were pretreated with H_2O_2. In contrast, H_2O_2 did not affect the secretory response induced by plasma membrane depolarization. Similar results were obtained in INS-1 cells treated with H_2O_2. In addition, H_2O_2 treatment induced depolarization of INS-1 mitochondrial membrane but inhibited the hyperpolarization induced by glucose. H_2O_2 also inhibited glucose-induced increase of mitochondrial Ca^{2+}. Consequently, H_2O_2 inhibited the increase of cytosolic ATP. These experiments demonstrate that H_2O_2 targets mitochondria and leads to inhibition of glucose-induced insulin secretion. Fourth, Kaneto *et al.* (8) demonstrated that ROS induces β-cell dysfunction by activating c-Jun N-terminal kinase (JNK). Insulin mRNA levels and DNA binding activity of Pdx-1 were reduced in isolated rat islets treated with 50 μM H_2O_2 for 48 hrs. Furthermore, H_2O_2 activated p38 kinase and protein kinase C. H_2O_2 -induced insulin mRNA repression was

prevented in rat islets infected with an adenovirus-mediate expression of a dominant-negative form of JNK. In contrast, inhibition of p38 kinase or protein kinase C did not prevent repression of insulin mRNA by H_2O_2. Islets treated with N-acetyl-L-cysteine (NAC) prevented H_2O_2 induced repression of insulin mRNA. These results indicate that H_2O_2 induces β-cell dysfunction by activating the JNK pathway, suggesting that high levels of glucose might mediate β-cell dysfunction by activating the JNK pathway.

The toxic effects of chronic hyperglycemia on β-cell function are caused, in part, by oxidative stress. A compensatory mechanism to this oxidative stress is to enhance expression of antioxidant and antiapoptotic genes. Using the 90 % pancreatectomized rat model, Laybutt *et al.* (173) showed that the expression of antioxidant genes such as glutathione peroxidase, and antiapoptotic genes such as A20 increased within the first week of surgery. The changes in gene expression were reversed when blood glucose levels were reduced by treatment with phlorizin. In conclusion, chronic exposure of β-cells to hyperglycemia increases antioxidant and antiapoptotic gene expression and this is likely a mechanism to compensate for induced oxidative stress and to protect against apoptosis.

7.2 Protein Kinase C activation

Protein kinase C (PKC) is an important mediator of signal transduction in response to several cellular signals (174), such as in glucose-induced insulin secretion (175, 176). In the diabetic state, PKC is induced in a variety of tissues including aorta, retina, heart,

renal, brain, and peripheral nerve (177). Some PKC isoforms activities are increased by hyperglycemia and associated with abnormalities observed in diabetes, especially in cardiovascular, retinal, and renal tissues (178),(179, 180). Recently, Kaneto *et al.* (127) demonstrated that PKC activity is increased in isolated pancreatic β-cell cultured in high glucose concentrations. The authors showed that PKC β is involved in glucose-induced c-Myc expression, which has been shown to repress insulin gene expression and alter the expression of other β-cell genes.

7. Protein kinase regulation of β-cell function

8.1 Growth

A small percentage of normal adult pancreatic β-cells go through mitogenesis (181, 182). This mitogenesis is about 0.5 % of the population of β-cells in a pancreatic islet (181). Glucose and other secretagogous can induce proliferation (182). Interestingly, β-cell growth is also observed during β-cell compensation for insulin resistance in the prediabetic phase (100). It has been postulated that transient and mild hyperglycemia induces β-cell growth (100). However, as the hyperglycemia and hyperlipidemia states increase, β-cell growth is not sufficient to compensate for insulin resistance (183). Therefore, understanding the molecular mechanisms of β-cell growth and maintaining this state have been of great interest to prevent loss of β-cell mass during the development of Type II diabetes.

It has been demonstrated that physiological glucose concentrations induce β-cell proliferation, which is mediated by protein kinase A (PKA), Ca^{2+}/calmadulin, and PKC (45, 184). This glucose-induced mitogenesis seems to be a result of a glucose metabolite, since pyruvate and mitochondrial fuels also promote β-cell proliferation (185).

Recently, Hügl *et al.* (186) demonstrated that insulin-like growth factor I (IGF-I) induces β-cell growth in a glucose-dependent manner. This study showed that INS-1 cells, an insulinoma cell line, incubated in 15 mM glucose and 10 mM IGF-1 increase cell proliferation more than 50 –fold. IGF-1-induced β-cell proliferation was mediated by the insulin receptor substrate (IRS) family, PI3K, and the 70-kDa S6 kinase ($p70^{s6k}$). The activation of IRS proteins by IGF-I or insulin receptors leads to activation of PI3K and subsequently to activation of $p70^{s6k}$. IGF-1 mitogenesis activity does not require the activation of the Janus kinase-2/Signal transducer and activator of transcription-5 (JAK2/STAT5) signaling pathway, nor activation of JNK, or p38 MAP kinases (Cousin, SP; Hügl, SR & Rhodes, C, unpublished observations).

Growth hormone (GH) is another potent inducer of glucose-dependent β-cell proliferation via JAK2/STAT5 with no cross talk with IRS proteins (187). It appears that PI3K also is required for GH to provide glucose-dependent β-cell proliferation (187).

Interestingly, prolonged exposure of β-cells to high levels of glucose or FFA induces apoptosis, causing a reduction in β-cell mass (188, 189). The signaling pathway involved in FFA reduction of IGF-1/ glucose-dependent proliferation is by activation of protein kinase B (PKB) and chronic activation of atypical PKC zeta (185). Interestingly, FFA induces insulin resistance through PKB and certain isoforms of PKC (190, 191).

Thus, it seems that the same signaling pathways mediate FFA inhibition of β-cell growth and induction of insulin resistance, two fundamental pathologic characteristics of Type II diabetes.

Recently, Lingohr et al. (192) demonstrated that prolonged (>24 hrs) activation of extracellular-regulated kinase-1/2 (Erk-1/2) and PI3K signaling pathways are important to induce glucose and IGF-1-induced β-cell proliferation. They also demonstrated that transforming growth factor-α (TGF) and epidermal growth factor (EGF) transiently activated Erk1/2 (< 20 min) and this activation was not correlated with β-cell proliferation. Importantly, it can be postulated that hyperglycemia and hyperlipidemia can induce β-cell proliferation-dependent on prolonged activation of specific signaling pathways. The prolonged activation of certain pathways not only induces β-cell proliferation but also might induce other signaling pathways and eventually lead to inhibition of glucose-induced insulin secretion. Thus, these kinases might also be involved in reduced insulin secretion and decreased insulin gene transcription during the hyperglycemia and hyperlipidemia states. Future studies need to be performed to elucidate the roles of IRS proteins, PKC, and PKB in glucose-induced repression of insulin gene expression.

8.2 Insulin gene transcription

Insulin is essential to maintain blood glucose levels within a narrow range. In turn, glucose regulates insulin secretion. This process requires regulation not only of

insulin exocytosis but also of insulin translation and transcription (193, 194). It is commonly believe that in the short-term (< 20 min), glucose regulates insulin at postranslational and postranscriptional levels, and in the long-term (> 2 hrs), glucose regulates insulin gene at transcriptional levels (195). It has been recently demonstrated that glucose also has an immediate regulatory function at the transcriptional level (196). In addition, glucose metabolism is required for regulation of insulin transcription and insulin promoter activity (52). Recently, studies have demonstrated that insulin increases insulin mRNA (197, 198) and insulin promoter activity (199, 200). Insulin also induces an increase in binding activity of Pdx-1 (200). Insulin regulates insulin gene transcription via β-cell insulin receptors and its downstream targets, the IRS-2/PI-3 kinase/p70 s6k and calmodulin kinase signaling pathways (52). p38 kinase has also been shown to regulate Pdx-1 phosphorylation and its translocation from the cytoplasm to the nucleus (201). However, these results have been challenged by studies in which over-expression of an upstream p38 kinase and use of a p38 kinase inhibitor did not support the involvement of this kinase in the regulation of insulin mRNA (202). In contrast, over-expression of PI3K and use of a PI3K inhibitor suggested that this kinase mediates Pdx-1 activation and translocation (202).

One can postulate that prolonged exposure of β-cells to high levels of glucose can alter the activation of many of kinases and eventually lead to decreased insulin gene expression. In the first stages of β-cell dysfunction, increased insulin secretion is observed, a phenomenon termed "hyperinsulinemia". The prolonged exposure of β-cells to high levels of insulin might also alter insulin gene regulation.

Glucose-induced insulin secretion is associated with a small stimulation of mitogen-activated protein (MAP) kinase (203). In addition, glucose causes transcriptional activation of AP-1 family members such as c-fos, *jun* B, and c-jun (204). The accumulation of these genes is dependent on cAMP, which by itself has only a small effect on the stimulation of c-fos, *jun* B, c-jun genes, and glucagon-like peptide-1 (GLP-1). The accumulation of c-jun and c-fos occurs at physiological glucose concentrations (3 to 11 mM) and requires one to two hrs. The authors conclude that the accumulation of AP-1 family members induced by the synergy of glucose and GLP-1 may be involved in facilitating induction of insulin gene, and may play an important role in the adaptive process of β-cells to hyperglycemia. It has also been documented that over-expression of c-Jun represses cAMP-induced activation of human insulin promoter activity (79). If c-Jun represses insulin promoter activity then glucose-induced accumulation of c-fos and *jun* B might prevent insulin gene repression by sequestering c-jun through dimerization and consequently facilitate glucose/cAMP induction of insulin gene promoter (204).

8.3 Cytokines involved in β-cell cytotoxicity

It is well document that certain cytokines are involved in the destruction of pancreatic β-cells in Type I diabetes (205, 206). Culturing islets with IL-1β induces inhibition of glucose-induced insulin secretion, impairs islet oxidation of glucose, and increases apoptosis (207-210). The cytotoxic effect of IL-1β on β-cells is mediated, in part, by expression of nitric oxide synthase (NOS) and overexpression of nitric oxide (NO) (208). Recently it was demonstrated that IL-1β increases JNK activity, and

phosphorylation of c-Jun and ATF-2 in RINm5F β-cells, an insulinoma cell line (211, 212). INS-1 cells treated with IL-1β for two days leads to apoptosis (213). Bonny *et al.* (213) demonstrated that IL-1β-induced apoptosis is mediated by increased JNK expression and decreased JNK-interacting protein-1 (JIP-1) content. A series of experiments either by over-expressing JIP-1 protein or inhibiting JIP-1 protein with an inducible JIP-1 antisense RNA led to the conclusion that JIP-1 protein is an anti-apoptotic agent that can protect cells from the cytotoxic effects of IL-β (213). It has also been demonstrated that p38 MAP kinase and ERK1/2 pathways are involved in IL-1β-induced NO in isolated pancreatic islets and RINm5F cells (214). Overall, these experiments demonstrated that cytokines induce β-cell apoptosis by activating p38 MAP kinase and JNK pathways, which increases the expression of iNOS and production of NO.

9. INS-1 cell model

INS-1 cells are a rat insulinoma cell line that was derived from cells isolated from an x-ray-induced rat transpantable insulinoma (215). INS-1's cell growth is dependent on seeding density, and the optimal conditions are 3.5 X 10^4 cells/ cm^2. Cell growth depends on 2-mercaptoethanol (2-ME, 50 μM). Removal of 2-ME causes a 15-fold drop in total cellular glutathione levels (216). Thus, 2-ME maintains high levels of total glutathione, which probably prevents cell damage from oxidative stress since it has been reported that β-cells contain low levels of antioxidants (164). Trypsinization and replating are required for continuous propagation.

Morphological studies of INS-1 cells show that the cells are very similar to normal β-cells. INS-1 cells contain several granules that exhibit a central dense core and a peripheral clear halo, although the degree of granularity varies between cells (215). Immunoflourescence staining demonstrated that most of the cells contain cytosolic insulin but the staining varies between cells. No glucagon, somatostatin, or pancreatic polypeptide was detected in INS-1 cells, indicating that only pancreatic β-cells are contained in this cloned (215). INS-1 cells contain high levels of insulin (~ 8 – 10 μg per million cells), which can be maintained for at least two and a half years of continuous culture (215). Although, INS-1 cells have high amounts of insulin compared to other insulinomas, it has ~ 4 times less than normal β-cells (40 μg/ 10^6 cells). INS-1 cells synthesize both insulin I and II peptides. However, glucose does not stimulate the incorporation of amino acids into newly synthesized hormone, a characteristic that is observed in normal β-cells. This could indicate that there is abnormal packaging, storage, or secretion of insulin in INS-1 cells compared to normal β-cells.

INS-1 cells secrete insulin in response to glucose (215). Insulin secretion studies were analyzed by static incubation of cells clusters in suspension or attached to plastic culture dishes, and by perifusion (215). In static analysis, cells were preincubated in a Krebs-Ringer-bicarbonate-Hepes-buffer (KRBH) for 30-60 min. Cells were then washed and incubated for 30 or 60 min in KRBH buffer containing 2.8, 11.2, or 16.7 mM glucose. In the perifusion studies, INS-1 cells were detached from culture flasks by trypsinization and then spun for 3 hrs in a culture flask containing regular media. Cells were then transferred into perifusion chambers and perifused with KRBH containing same concentrations of glucose as in the static incubations for 45 min. INS-1 cells have a

high insulin response at 11.2 mM of glucose compared to no glucose or 2.8 mM glucose, and a slight but not significant decrease of insulin secretion at 16.7 mM glucose compared to 11.2 mM glucose. Thus, INS-1 cells secrete insulin in response to glucose during an acute challenge. However, this secretion is much reduced compared to normal β-cells (217). INS-1 cells are also sensitive to other secretagogous such as arginine, leucine, and KCl, and inhibitory substances including somatostatin, epinephrine, and diazoxide (an ATP-sensitive K^+ channel opener).

INS-1 cells also have the capacity to induce membrane depolarization, which is a prerequisite for glucose-induced insulin secretion (215). Glucose (1 mM) depolarizes INS-1 cell's membrane and higher glucose concentrations led to an extended depolarization. The depolarization was correlated with increased cytosolic Ca^{2+}concentrations when challenge to glucose (215). These results suggest that INS-1 cells are able to metabolize glucose and this leads to membrane depolarization most likely by closure of ATP-sensitive K^+ channel and subsequently an increase in cytosolic Ca^{2+} and insulin secretion.

One important player in sensing high levels of blood glucose is the glucose transporters in β-cells. β-Cells express the high K*m* (~ 17 mM) Glut2 and to a lesser degree of Glut1 expression (218). INS-1 cells exhibit high levels of the Glut2 transporter (219). Studies understanding Glut2 regulation by glucose have been performed in INS-1 cells (219). These studies demonstrated that INS-1 cells incubated in 20 mM glucose for 24 hrs increased Glut2 mRNA levels compared to 2 mM glucose. These results are in agreement with studies performed in HIT-T15 cells incubated in 22.2 *versus* 11.1 mM

glucose for 24 hrs (220), and in rat islets cultured in 11.1 *versus* 5.5 mM glucose for 24 hrs (221). These results suggest that Glut2 expression and regulation in INS-1 cells may be similar to normal β-cells.

Glucose-induced insulin secretion is markedly enhanced by glucagon-like peptide-1 (GLP-1) (reviewed in Ref. 219, 220). GLP-1 is secreted by the enteroendocrine L-cells in response to nutrient intake and acts on β-cells as a potent insulin secretagogue (222, 223). GLP-1 also up-regulates other genes involved in β-cell function such as insulin gene expression and promoter activity (224), and Pdx-1 gene expression (225). Some of the characteristics of GLP-1 in enhancing glucose-induced insulin secretion have been identified in INS-1 cells (226). INS-1 cells treated with stimulatory GLP-1 concentrations induce insulin promoter activity (226). These results also imply that INS-1 cells express GLP-1 receptors as observed normal β-cells.

One of the key components linking glucose metabolism to insulin secretion is the ATP-sensitive potassium channel (K_{ATP}). Upon glucose metabolism, K_{ATP} channels are closed in response to an increase in ATP/ADP ratio, resulting in membrane depolarization, which opens voltage-dependent Ca^{2+} channels leading to a increase in intracellular Ca^{2+} levels and subsequent insulin secretion (227). K_{ATP} channels are composed of a sulfonylurea receptor-1 (SUR1), a domain for ATP binding, and a potassium channel (Kir6.2), which forms the pore of the channel (227). Studies understanding the regulation of K_{ATP} channels have been performed in INS-1 cells (228). These studies indicate that INS-1 cells expressed the SUR1 and Kir6.2. In addition Moritz *et al.* (228) demonstrated that INS-1 cells incubated in high concentrations of

glucose for more than 24 hrs lead to downregulation of SUR1 and Kir6.1 mRNA levels. Studies in Zucker diabetic fatty rats (229), in 90 % pancreatectomized rats (4), and in isolated rat islets exposed to high levels of glucose for 24 hrs (230) also showed decreased expression of Kir6.2 mRNA levels. These studies show that INS-1 cells have some similar characteristics observed in Type II diabetic models.

INS-1 cells have also been used as a model to study regulation of essential genes in the glycolytic pathway by hyperglycemia (231). INS-1 cells express phosphofructokinase-1 (PFK1), which phosphorylates fructose-6-phosphate to fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which provides cytosolic NADH, and L-pyruvate kinase (L-PK), which catalyzes the formation of ATP. In agreement with previous *in vivo* and *in vitro* studies (99, 232), INS-1 cells cultured in 25 mM glucose for three days led to an increase in insulin secretion at low (2-5 mM) glucose and did not respond to higher glucose concentrations (231). The prolonged incubation of cells led to an increase in gene expression of PFK1, GAPDH, and L-PK compared to cells cultured in 5 mM glucose (231). In contrast, glucokinase (GK) and 6-phosphofructo-2-kinase transcripts remain unchanged. INS-1 cells incubated for prolong periods in high levels of glucose also accumulate glycogen, like islet tissues (233) and the ZDF animal model (7). The authors conclude that the increase of these enzymes and accumulation of glycogen are involved in the compensatory mechanisms of β-cells exposed chronically to hyperglycemia. These compensatory mechanisms are a high rate of insulin secretion at low glucose concentrations, hypertrophy, and hyperplasia, which have been observed *in* Type II diabetic models (100, 101).

Important for our studies, INS-1 cells exhibit other characteristics observed in Type II diabetes such as decreased insulin gene expression. Studies in INS-1 cells have demonstrated that insulin mRNA and insulin promoter activities are reduced in cells exposed to high levels of glucose for 24 to 48 hrs (126). Reduced insulin promoter activity is correlated to reduced Pdx1 binding activity in INS-1 cells (126). These results have also been shown *in vivo* in 90 % pancreatectomized rat (113) and Zucker diabetic rat models (101). When INS-1 cells are returned from high glucose levels to low glucose levels or when Zucker rats blood glucose levels are normalized with phlorizin, insulin mRNA levels is no longer repressed (113, 126).

INS-1 cells have also been used as a model for hyperlipidemia-induced β-cell dysfunction studies. Hyperlipidemia is associated with β-cell dysfunction because Type II diabetic patients frequently exhibit high triglycerides and free fatty acid blood levels (234) and display reduced glucose-induced insulin secretion (235). Studies in INS-1 cells demonstrated that prolonged exposure of cells to fatty acids markedly represses glucose-induced insulin secretion (236).

Pancreatic β-cells are known to be very susceptible to oxidative stress because of their low expression levels of antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase in comparison to other tissues (161, 234). RINm5F, a cell line derived from the same islet tumor as INS-1 cells (238, 239), express low levels of the above-mentioned antioxidant enzymes (164). These results suggest that INS-1 cells might also express low levels of antioxidant enzymes, making the cells vulnerable to reactive oxygen species. INS-1 cells express peroxiredoxins (240), another

group of antioxidant enzymes that catalyze reduction of both hydrogen peroxide and alkyl peroxides to water or corresponding alcohol by using thioredoxin as a hydrogen donor (241, 242). In addition, peroxiredoxins can also protect against nitrogen radicals (243). Studies in INS-1 cells demonstrated that oxidative stress induces peroxiredoxin levels (240). INS-cells treated with cytokines including IL-1β, IFN-γ, and TNF-α for 48 hrs led to an up-regulation of peroxiredoxins, which has also been observed in isolated pancreatic islets (240). These results indicate that the induction of peroxiredoxin expression might be a compensatory response to oxidative and nitrosative stress. Other studies have used INS-1 cells as a model to investigate whether over-expression of antioxidant enzymes protects the cells against the cytotoxic effects of nitric oxide donors (244). Over-expression of Cu/Zn superoxide dismutase protected INS-1 cells against nitric oxide cytotoxicity.

Overall, INS-1 cells behave in a similar manner as pancreatic β-cells. They metabolize glucose and secrete insulin through the same mechanism as normal β-cells. Importantly for our studies, INS-1 cells secrete insulin at physiological glucose concentrations and have similar phenotypic characteristics as Type II diabetic models. Thus, INS-1 cells appear to be a good model to investigate mechanisms involved in the repression of glucose-induced insulin secretion as observed in Type II diabetes.

INS-1 cells, however, do not completely mimic all the responses observed in β-cells exposed to hyperglycemia. First, glucose toxicity on β-cells is thought to occur after a prolong exposure of cells to high glucose concentrations that leads to a progressive loss of β-cell differentiation. Isolated pancreatic islets incubated in high (> 11 mM)

glucose levels for 7 days show reduced glucose-induced insulin secretion, islet content of insulin, and insulin mRNA levels (245). In contrast, INS-1 cells cultured for 24 to 48 hrs shows reduced insulin mRNA levels, which are associated with reduced insulin promoter activity (126). The discrepancy in the behaviors of both isolated islets and INS-1cells is not well understood. One can speculate that isolated islets contain in addition to β-cells, α-cells and δ-cells, which can positively affect β-cell function. In addition, INS-1 cells require 2-ME for growth and replication, in contrast, isolated islets are typically cultured without 2-ME. This could indicate that INS-1 cells might have even lower levels of antioxidant enzymes than isolated islets. Thus, INS-1 cells may be even more vulnerable to oxidative stress than isolated islets, thus allowing for shorter periods of time to observe phenotypic changes induced by oxidative stress.

III. MATERIALS AND METHODS

1. Materials

Cell culture media and Lipofectamine were purchased from Invitrogen (Gaitherburg, MD). Luciferase (LUC) assay kits were from Promega (Madison, WI). Anti-JNK1 (C17) antibodies, anti-phosphorylated-JNK (G-7) antibodies, glutathione S-transferase (GST)-c-Jun fusion protein and Protein-A agarose were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Nitrocellulose membranes were from BioRad (Hercules, CA). Chemiluminescence detection kits were from Pierce (Rockford, IL). [γ-^{32}P] ATP and [^{14}C]chloramphenicol were from NEN Life Science Products (Boston, MA). Ligase enzyme was from Invitrogen Corp (Carlsbad, CA). Taq DNA polymerase was from Invitrogen Corporation (Carlsbad, CA). Vent-DNA polymerase was from New England Biolabs-Inc (Beverly, MA). DNA purification kit, Geneclean-Turbo, was purchased from Bio101 (Vista, CA). Quick spin columns B-50 sephadex was purchased from Roche, as well as the inhibitors aprotinin and leupeptin (Indianapolis, IN). The substrate for CAT assays, butyryl CoA, was from Sigma (St. Louis, MO). pRK, MLK3, MLK3K144A, and anti-MLK3 antibody were provided by Dr. Kathleen Gallo, Michigan State University (MSU). The 2XTRE-LUC reporter gene was provided by Dr. Maduker, MSU. JNK1α1 and JIP-1 expression vectors were provided by Dr. Roger Davis, University of Massachusetts Medical School and Howard Hughes Medical Institute, Worcester, MA. pMMTVCAT vector was provided by Dr. Richard Mikcisek, MSU. PK(-197)CAT vector was provided by Dr. Howard C. Towle, University of Minnesota,

MN. The Z, X, Y, Za, and Zb minienhancer reporter vectors were provided by Dr. Michael German, University of San Francisco, CA.

2. INS-1 cell culture

INS-1 cells (215) (kindly provided by Dr. C. Wollheim) were routinely cultured in 5% CO_2-95% air at 37 °C in RPMI-1640 media containing 11.1 mM glucose and supplemented with 10% fetal-bovine serum, 1 mM pyruvate, 10 mM Hepes, 50 μM 2-mercaptoethanol, 100 units penicillin/ ml and 100 μg streptomycin/ ml. Cells were passed weekly by trypsin-EDTA detachment. All experiments were performed on INS-1 cells between passages 70 and 85.

3. Plasmid DNA constructs

The plasmid INSCAT contains the chloromplenicol acetyltransferase (CAT) gene under transcriptional regulation by the human insulin gene sequences –327 to +30 as previously described (121). The –230 INSCAT (INS(-230)CAT) plasmid contains the CAT gene under transcriptional regulation by the human insulin gene sequences –230 to +30. The mC1INS(-230)CAT vector, which contains mutations in the C1 element, the mA1A3 INS(–230)CAT, which contains mutations in both A1 and A3 elements, the mA1A3C1 INS(-230)CAT, which contains all three mutated elements, were generated by PCR amplification using INS(-230)CAT, mA1INS(–230)CAT, and mA3A1INS(-230)CAT as a template, respectively. Oligonucleotide primers used to generate these specific mutant elements are listed in Table 1. mA1INS(-230)CAT was previously made by Dr. Olson where the A1 element was mutated from CCCTAATGGG to CCGCGCGCGG. The mutations in the A3 element were TAAT to TCCT and the

mutations in the C1 element were GCCTCA to GCTGCA. Truncated insulin promoter vectors were also generated by PCR amplification. The template used in the PCR reactions was INS(-327)CAT and the respective primers are shown in Table 2. INS(-250)CAT was previously made by Dr. Olson. The mA5/Core/Pal/E3 INSCAT vector was generated by first making mPalINSCAT vector using INS(-327)CAT as a template. Then, mPalINSCAT was used as a template to generate mPal/E3INSCAT, which was finally used as a template to produce mA5/Core/pal/E3 INSCAT vector. Primers used to generate the mA5/Core/pal/E3 INSCAT vector are shown in Table 3. The plasmid 2XTRE-LUC contains the luciferase gene under transcriptional regulation of the prolactin promoter from –36 to +37 and two copies of the alpha collagenase AP-1 promoter enhancer (246). pCDN3-JNK1α1 and pCMV5-flag-JIP1 expression plasmid have been previously described by Derijard *et al.* (247) and Dickens *et al.* (248), respectively. pRK-MLK3 and pRK-MLK3K144A expression plasmids have been previously described by Gallo *et al.* (249). MLK3 was also cloned into pCR3.1 vector because transfections containing the pRK vector significantly reduced basal INSCAT activity. To clone MLK3 cDNA into pCR3.1 vector, MLK3 was amplified by PCR from the pRK-MLK3 vector, and then ligated into pBluescript II KS vector. MLK3 cDNA was then cut out of the pBluescript II KS vector and ligated into the pCR3.1 vector. The multimer $(E1)_3$RSV103CAT plasmid was made by ligating three hybridized E1 element sequences, which contain BamH1 restriction site at both 5'and 3'ends (5' GAT CCG GGG TCG GCA GAT GGC TGG GGG CG 3') upstream to the minimal promoter (only the TATA box) of Rous sarcoma virus driving CAT expression. All vectors were verified by sequencing on an ABI Prism 3700 DNA Analyzer.

4. PCR reactions

In general, all mutated and truncated insulin promoter vectors were generated by PCR using primers containing Xba I or Xho I restriction sites. The insert was then ligated into poCAT vector that contained these sites in the multilinker region.

In general, PCR reactions contained 2 ng/μl template, 20 pmol of appropriate oligonucleotide primers, 10 mM dATP, 10 mM dGTP, 10 mM dTTP, 10 mM dCTP, and buffer and polymerase according to manufacturer's instructions (Vent polymerase was used for mutated INS(-230)CAT vectors, and Taq polymerase was used for truncated INSCAT vectors and mA5/Core/Pal/E3 INSCAT vector). The thermal cycle profile employed a 1 min of denaturation at 94 °C followed by 20 to 25 amplification cycles (1 min of denaturation at 94 °C, 30 sec annealing at 58 °C, and 30 sec extension at 68 °C) and a extension step for 4 min at 68 °C. The mA1C1A3 INSCAT and mA5/Core/Pal/E3 INSCAT vectors were generated by PCR amplification using a "PCR-bridge reaction". The "PCR-bridge reaction" consisted of two PCR products that both contained the same mutated element, and both were used as templates to generate a PCR product with the mutated element. One PCR product contained sequences from 5' end, which had a XbaI restriction site, to the mutated element. The other PCR product contained sequences from the mutated element to the 3' end, which had a XhoI restriction site. The PCR "bridge reaction" contained 5 μl to 10 μl of each PCR template, 20 pmol of the appropriate primer that contained the Xba I or Xho I restriction site, and the additional components of the PCR reaction as described above. The mutated insulin promoter cDNA inserts were then digested with respective restriction enzymes. Following

digestion and verification of sizes by 1% agarose gel, the cDNA inserts were ligated into linearized poCAT vector that had Xba I and XhoI sites.

To generate pCR3.1-MLK3, MLK3 was amplified by PCR from 20 ng pRK-MLK3 vector by using 20 pmol primer that contained the replication start sequence and a BamH1 restriction site, and another 20 pmol primer that contained the replication stop sequence and a EcoRI restriction site (see Table 1 for sequences). The PCR reaction also contained 1 µl of DMSO to disrupt disulfide bonds, 10 mM of each dNTP, 5 µl buffer, and 1 µl of pfu DNA polymerase. The thermal cycle profile employed 1 min of denaturation at 94 °C followed by 25 amplification cycles (1 min of denaturation at 94 °C, 1 min of annealing at 69 °C, and 6 min of extension at 72 °C) and an extension step of 10 min at 72 °C. The MLK3 cDNA insert was digested with EcoRI and BamHI restriction sites and ligated into linearized pBluescript II KS vector.

5. Ligation reactions and purification of DNAs

The general reaction had a volume of 15 µl containing 0.1 pmol of digested vector, 0.3 pmol, 0.6 pmol, or 0.9 pmol of cDNA insert, and buffer and ligase according to manufacturer's instructions. Reaction was then incubated overnight at 16 °C. cDNA vectors were amplified by competent *E. coli DH 5α*. Vectors were then purified by using Qiagen kit and vectors were further purified by cesium chloride method.

6. Transfections

For all reporter gene studies, INS-1 cells were subcultured for two days before transfection at a density of 1.5 x 10^6 cells per well (diameter 3.5 cm) in RPMI-1640

media supplemented as described above. Cells were transfected for 5 hrs according to manufacture protocols using a ratio of 1 μg plasmid to 2 μl Lipofectamine and were then incubated in RPMI-1640 media containing 4.0 or 16.7 mM glucose (as indicated in figure legends). In experiments designed to test the effect of JNK1α1 or MLK3 on promoter activity, 1 μg of reporter plasmid was used and increasing concentrations of expression plasmid (as indicated in figure legends). All cells were transfected with equal amounts of DNA by use of control expression plasmids containing no inserts. Cells were harvested either 24 or 48 hrs after transfection (as indicated in figure legends) and CAT or luciferase activity was assayed.

7. CAT assays.

After treatment, cells were washed twice with 1X PBS. CAT assays were performed as follows. In general, 1 ml of TEN (40 mM Tris, 150 mM and 1 mM EDTA) was added to cells for 5 min on ice. Cells were then scraped and transfer to Eppendorf tube, then spun at 5000 rpm for 30 sec. TEN was aspirated and 100 μl of 250 mM Tris pH 7.5 was added. Cells were then lysed by three cycles of freezing and thawing. Broken cells were spun down at 14,000 rpm for 5 min at 4 °C. Lysates were aliquoted into new Eppendorf tubes and stored at -20 °C.

In general, CAT assays were performed by combining the appropriate amount of extracts and 250 mM Tris to a 50 μl total volume. Then 100 μl of CAT reaction (250 mM Tris, 2 μl /rxn of ^{14}C-Chloromphenicol (54 mCi/mmol, 0.05 mCi/ml), and 3 μl of 0.01 M Butyl CoA) was added. The reaction was incubated usually for 2 hrs at 37 °C. Amount of extracts and incubation times were determined in such a way that the CAT

activity was in the linear range of the reaction. Extraction of labeled butyl CoA was performed as follows: first, 300 µl xylene was added, then vortexed for 30 sec, and spun for 3 min at 14000 rpm. Second, xylene (upper layer) was removed and added to an Eppendorf tube that contained 300 µl of 250 mM Tris, then vortexed for 30 sec, and spun for 3 min at same speed as before. Then 150 µl of the xylene (upper layer) was added to a scintillation counter vial containing 5 ml of scintillation cocktail. CAT units were normalized to protein concentrations. All experiments were carried out in duplicate.

8. Luciferase assays

After treatment, cells were washed twice with 1X PBS. Luciferase activity was measured according to the protocol from Promega. In general, 200 µl of luciferase lysis buffer was added per well and incubated for 15 min at room temperature. Cells were then scraped and frozen at –20 °C. Cells were then spun down and supernatant was transferred to a new Eppendorf tube. 20 µl of supernatant was assayed with 100 µl luciferase reaction buffer. A Turner TD 20E luminometer (Turner Designs) was used to measure luciferase activity. All experiments were carried out in duplicate.

9. JNK kinase assay

INS-1 cells were subcultured for 2 days at a density of 5×10^6 cells per plate (diameter 6.0 cm) in RPMI-1640 media supplemented as described above. In experiments designed to test the effect of MLK3 on JNK activity, cells were transfected with 3 µg expression plasmid and 6 µl Lipofectamine. Cells were then incubated for 24 hrs in RPMI-1640 media containing either 4.0 mM or 16.7 mM glucose and the

supplements as described above. Cells were then lysed for 5 min on ice in 1 ml lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM Na_4PP_i, 100 μM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM PMSF, and 0.15 U/ml aprotinin). Lysates were centrifuged at 4 °C for 20 min at 4,000 rpm in an Eppendorf centrifuge. Lysates (200 μg protein) were incubated at 4° C for 90 min with 1 μg of anti-JNK-1 antibody bound to 20 μl of Protein -A-agarose. JNK was then precipitated by centrifugation and washed three times with HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton-X-100, 10% glycerol) containing 1 M LiCl, three times with HNTG buffer, and twice with kinase reaction buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $MnCl_2$, 0.1 mM Na_3VO_4). Immunoprecipitates were resuspended in 20 μl of kinase reaction buffer containing 5 μCi per reaction [γ-^{32}P]-ATP (3000 Ci/mmol), 50 μM ATP and 1 μg GST-c-Jun protein and then incubated at room temperature for 30 min. JNK enzymatic reactions were terminated by the addition of an equal volume of 2 x SDS sample buffer (100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 100 mM DTT, 1% β-mercatoethanol, 50 mM EDTA). Proteins were then separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and quantitated on a PhosphoImager (Molecular Dynamics). All nitrocellulose membranes were then immunoblotted with a 1:1000 dilution of anti-JNK antibodies to ensure that equal levels of JNK were immunoprecipitated and transferred.

10. Nuclear extracts

Nuclear extracts were made from INS-1 cells according to the method of Schreiber et al. (250). In general, cells were washed with 10 ml cold PBS. Cells were

then scraped and pelleted at 1500 g for 5 min in a 15 ml tube. Pellets were resuspended in 1 ml Tris-buffered saline, transferred to Eppendorf tube and pelleted for 15 sec at 4 °C. Pellets were resuspended in 800 µl buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 0.2 µg/µl leupeptin, 0.2 µg/µl aproptinin) by gently pipeting up and down. Cells were allowed to swell on ice for 15 min. Then, 50 µl of 10 % NP40 was added and samples were vortexed for 10 sec. Lysates were spun for 30 sec at 14000 rpm at 4 °C. The supernatants were removed and the nuclear pellets were resuspended in 100 µl buffer B (20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 0.2 µg/µl leupeptin, 0.2 µg/µl aproptinin). Nuclear pellets were rocked vigorously for 15 min on ice. Nuclear debris was spun down for 5 min at 4 °C, and finally nuclear extracts were aliquoted and stored at -70 °C.

11. Phosphorylated JNK analysis

Proteins (30 µg) were resolved on 10% SDS-PAGE, transferred to nitrocellulose membranes, and phosphorylated-JNK was detected using a 1:1000 dilution of an anti-phosphorylated-JNK antibody. Protein levels were visualized by chemiluminescence.

12. Electrophoretic mobility-shift assays

Nuclear extracts were prepared as described above. Oligonucleotides were hybridized as follow: 1.2 µM oligonucleotides, 5.0 µl React 2, and 20 µl water were heated for 10 min at 68 – 72 °C, and then cooled slowly at room temperature. Oligonucleotides were either ^{32}p-5'-end-labeled or ^{32}p-3'-end-labeled-incorporation. The reaction to generate ^{32}p-5'-end-labeled oligonucleotides contained 5 µl [γ-^{32}p]ATP (3000

Ci/mmol), 1 μl T4 polynucleotide kinase, and 0.1 μl hybridized oligonucleotide in a 20 μl total volume. The reaction to generate ^{32}p-3'-end-incorporation-labeled oligonucleotides contained 2.0 μl [α-^{32}p]dCTP (3000 Ci/mmol), 3 μl 10 mM dNTP (except dCTP), 0.5 μl hybridized oligonucleotide, and 1.0 μl Klenow Fragment of *E. coli* polymerase I in a 50 μl reaction volume. Reactions were incubated for 30 to 45 min at room temperature for Klenow Fragment or at 37 °C for T4 kinase. For the reaction with Klenow Fragment, 1 μl dCTP was added to complete the filling reaction and incubated for 10 min at room temperature. The reactions were then incubated at 68 °C for 10 min and cooled slowly to room temperature. Unincorporated nucleotides were removed by using a Quick spin column according to manufacture protocol. 1 μl of probe was counted by using a Scintillation counter. Labeled oligonucleotides were mixed with nuclear extracts in a reaction that contained 10 μg nuclear extract protein, labeled oligonucleotides (30,000 cpm), 3.5 μl of 10 X binding buffer (50 % glycerol, 10 mM EDTA, 500 mM NaCl, 100 mM Tris pH 7.5, 10 mM DTT), 2 μg/μl polydIdC in a 25 μl total volume. The reaction was incubated for 30 min at room temperature. For super-shift assays, 1 μl or 2 μl antibody was added to labeled oligonucleotide before addition of proteins and binding buffer, and was incubated for 20 min on ice. After incubation, the binding reactions were separated by a 5 % nondenaturing polyacrylamide gels in 1X TGE buffer (40 mM Tris, 384 mM glycine, 2 mM EDTA, pH 8.3). Gel was dried and nuclear protein binding was visualized and quantitated by PhosphoImaging (Molecular Dynamics).

Name	5'end	Sequence
-230INS/ 5'XbaI	-230	GCGTCTAGACCCCTGGTTAAGACT
-230INS/ 3' XhoI	+30	GCGCTCGAGCTCTTCTGATGCAGC CTGTC
mA3/Xba I/ forward	-230	GCGTCTAGACCCCTGGTTAAGACT CTCCTGACCCGCTGG
mC1/Pst I/ forward	-126	CCGGAAATTGCAGCTGCAGCCCCC AGCCATCTG
mC1/Pst I/ reversed	-96	CAGATGGCTGGGGGCTGCAGCTGC AATTTCCGG

Table 1. Sequences of oligonucleotide primers. This table shows the primers used to generate the mA3A1(-230)INSCAT, mC1(-230)INSCAT, and mA3C1A1(-230)INSCAT vectors. -230INS/ 5' Xba I is the wild type 5' primer containing a Xba I restriction site. -230INS/ 3' Xho I is the wild type 3' primer containing a Xho I restriction site. mA3/Xba I is the forward primer that contains mutations in the A3 element. mC1/Pst I is the primer that contains mutations in the C1 element and a Pst I restriction site.

Name	5'end	Sequence
INS(-292)CAT	-292	GCCTCTAGAGGCTTTGCTCTCCTGGAGACA
INS(-270)CAT	-279	GCCTCTAGATGGAGACATTTGCCCCCAGCT
INS(-261)CAT	-261	GCCTCTAGAGCTGTGAGCAGGGACAGGTCT
INS(-250)CAT	-250	GCCTCTAGAGGACAGGTCTGGCCACCGGGC

Table 2. Sequences of oligonucleotide primers used to generate truncated insulin promoter vectors. Each primer contains six extra nucleotides upstream of the 5' end with a Xba I restriction site.

Name	5'end	Sequence
Za	-292	GGCTTTGCTCTCCTGGAGACATTTGCCCCCA
Zam-271	-292	GGCTTTGCTCTCCTGGAGACAGTTGCCCCCA
Zam-273	-292	GGCTTTGCTCTCCTGGAGAGATTTGCCCCCA
Zam-282/3	-292	GGCTTTGCTAGCCTGGAGACATTTGCCCCCA
Zd	-289	TTTGCTCTCCTGGAGACATTTGCCCCCAGCTG TGAGC
Palindrome	-289	TTTGCTCTCCTGGAGACATTT
mPalindrome	-289	TTTGCTAGCCTGGAGACATTT
E3	-278	GGAGACATTTGCCCCCAGCTGTGAGC
mE3	-278	GGAGAGCGTTGCCCCCAGAGTTGAGC
A5/Core/E4	-327	TCTCCTGGTCTAATGTGGAAAGTGGCCCAGG TGAGGGCT
A5/Core	-323	CTGGTCTAATGTGGAAAGTG
mA5/Core	-323	CTGGTCTACGTTGGAAAGTG
mA5/Core/Xba I	-323	GCCTCTAGACTGGTCTACGTTGGAAAGTG
E4	-306	GTGGCCCAGGTGAGGGCT

Table 3. Oligonucleotide sequences used for mobility-shift assays and used to generate mutations in the A5/Core, palindrome, and E3 elements. mA5/Core/Xba I, mPalindrome, and mE3 oligonucleotides contain mutations in the respective elements, which are underlined. These oligonucleotide primers were used to generate mA5/Core/Pal/E3 INS(-327)CAT vector. All the oligonucleotides shown except mA5/Core/Xba I were used for mobility-shift assays.

IV. INCREASED C-JUN N-TERMINAL KINASE (JNK) ACTIVITY IN INS-1 CELLS EXPOSED TO ELEVATED GLUCOSE CONCENTRATIONS MAY MEDIATE THE REPRESSION OF INSULIN PROMOTER ACTIVITY

1. Abstract

Chronic exposure of pancreatic islets and β-cell lines to elevated glucose concentrations causes β-cell dysfunction including reduced insulin gene transcription. Studies using antioxidants indicate that elevated glucose levels reduce insulin gene transcription by mechanisms involving reactive oxygen species (ROS), thus suggesting a role for stress-activated signaling pathways. This study was designed to investigate the possible involvement of c-Jun N-terminal kinase (JNK), a stress-activated kinase, in mediating the reduction of insulin gene transcription observed in β-cells incubated in elevated glucose concentrations. We have reported that exposure of INS-1 cells to 16.7 mM glucose for 24 to 48 hrs leads to a 70 % reduction in insulin promoter activity. This study demonstrated that incubation of INS-1 cells in 16.7 mM glucose for 24 hrs significantly increased JNK activity compared to cells incubated in 4.0 mM glucose. Treatment of cells with 16.7 mM glucose for 48 hrs also increased AP-1 reporter gene expression, indicating that glucose-induced activation of JNK functionally activates downstream signaling components such as AP-1 transcription factors. Transient transfection of cells with increasing concentrations of a JNK1 expression plasmid reduced insulin promoter activity in cells cultured in 4.0 mM glucose and this repression was more pronounced in cells cultured in 16.7 mM glucose. Over-expression of MLK3, an upstream activator of JNK, led to an increase in JNK activity in cells incubated in both

4.0 and 16.7 mM glucose. Over-expression of MLK3 also led to an increase in AP-1 reporter gene expression and a reduction in insulin promoter activity in cells cultured in both 4.0 and 16.7 mM glucose. These data show that activation of JNK, either by high levels of glucose or upstream activators, leads to functional activation of downstream JNK target proteins and causes reduced insulin promoter activity. Truncation and mutational analysis indicated that JNK might reduce insulin promoter activity through a generalized target such as a co-activator. In conclusion, increased JNK activity in INS-1 cells exposed to high glucose levels may mediate glucose-induced repression of insulin promoter activity.

2. Introduction

Type II diabetes is characterized by insulin resistance and failure of pancreatic β-cells to secrete sufficient levels of insulin necessary to overcome hyperglycemia. Once diabetes is established, chronic hyperglycemia has been postulated to further damage β-cells, thus exacerbating the disease state. Adverse effects of hyperglycemia on β-cells include suppression of insulin gene expression and glucose-induced insulin secretion (2-4, 100, 101, 109, 113, 121, 126). Reduction in insulin gene expression has been correlated with decreased insulin gene promoter activity and reduced binding activity of two important transcription factors, Pdx-1 and C1 activator (2-4, 121). Studies in INS-1 cells have demonstrated that insulin promoter activity is markedly repressed in cells cultured in 16.7 mM glucose for 48 hrs, and this coincides with a reduction in Pdx-1 and C1 activator binding (126).

Although some mechanisms involved in glucose-induced repression of insulin gene expression have been described, e.g. regulation of key transcription factors, the causative pathway remains unknown. Three possible mechanisms have been implicated in glucose-induced tissue damage in a diabetic state. First, glucose has been shown to modify proteins through non-enzymatic glycosylation, which generates reactive oxygen species (ROS) and advanced glycosylated end products (AGEs) (251). Autoxidative glycation has also been observed in diabetes, which further increases oxidative stress state (156). Glycated proteins are present in normal tissues but some tissues are more sensitive to this phenomenon. For example, increased glycosylation in tissues such as kidney, liver, brain, and lung are observed in diabetic conditions (159, 160). Second, hyperglycemia can increase protein kinase C activity, which has been associated with

diabetic vascular complications in the retina (252), aorta (253), and renal glomeruli (254). Third, glucose can be converted to sorbitol by aldose reductase through the polyol pathway (152). Aldose reductase uses NADPH as a co-factor and an increased activity of this enzyme can deplete intracellular NADPH levels, which is detrimental to cell function (153). For example, decreased NADPH levels inhibit the ability of reduced glutathione to eliminate reactive oxygen species (153). In addition, because sorbitol is not readily exported and has a low rate of conversion to fructose, sorbitol accumulates within the cell creating osmotic stress, and leading to swelling and rupture of the lens fiber cells (255).

Many investigators have studied the hypothesis that reactive oxygen species are involved in the toxic effects of glucose on β-cell function (7, 157, 169, 170). β-Cells are very vulnerable to ROS since they have low levels of glutathione, superoxide dismutase, and catalase (164). Some of the mechanisms by which ROS might induce β-cell dysfunction include DNA modifications such as 8-hydroxy-2'deoxyguanosine (8-OHdG) (256), and membrane lipid peroxidation, which forms aldehydes such as 4-hydroxy2-neonal (HNE) (257). 8-OHdG and HNE are oxidative markers and 8-OHdG has been identified in the urine and blood of patients with Type II diabetes (165, 166). Ihara *et al.* (157) demonstrated that β-cells of Goto-Kakizaki (GK), a Type II diabetic rat model, have high levels of 8-OHdG. In addition, after GK rats were fed with a diet high in sucrose, increased oxidative markers were observed, suggesting that chronic hyperglycemia might be involved in the oxidative stress-related dysfunction of GK rats β-cells. ROS have also been shown to inhibit glucose-induced increases in mitochondria and plasma membrane potential, which are required for metabolic signaling for insulin secretion (172, 258). Changes in β-cell function induced by hyperglycemia, reduced

sugars, or H_2O_2 has been shown to be partially or fully prevented by antioxidants such as N-Acetyl-L-cyteine (NAC) or aminoguanidine (AG) (7, 169, 170). Overall, these experimental results address the important role of oxidative stress in mediating adverse changes in β-cell function observed in the diabetic state.

Stress-activated protein kinases (SAPK) are a group of serine/threonine specific kinases that are activated by dual phosphorylation on threonine and tyrosine residues in response to a variety of extracellular stress factors such as UV, ROS, osmolarity, heat shock, and treatment of cells with cytokines. Two main groups of SAPK, c-Jun N-terminal kinase (JNK) (247, 259, 260) and p38 MAP kinase (261, 262) regulate cell proliferation, differentiation, and apoptosis. There are 12 isoforms of JNK encoded by three genes (JNK-1, 2 and 3) (263). Targets of JNKs are mostly transcription factors, including c-Jun, activating transcription factor-2 (ATF-2), and ETS-containing factors such as Elk1. JNK has natural regulators named IB1/JNK-interacting proteins (JIP), which are scaffolding proteins that interact with upstream components of JNK signaling pathway. There are three isoforms of JIP (JIP1-3). JIP1 and JIP2 are closely related proteins mainly expressed in pancreatic β-cells and in neurons (reviewed in 260). Although JIP1 is now understood to mediate JNK activation (264), it was originally identified as a cytosolic anchor protein that binds specifically to JNK (248). Over-expression of JIP1 in insulin-producing cells prevented JNK-mediated activation of transcription factors such as c-Jun, ATF2, and Elk1 (248, 265). Interestingly, missense mutations in the *JIP1* gene have been correlated with Type II diabetes (266). Studies by Bonny *et al.* (213) demonstrated that JIP1 is involved in β-cell apoptosis. These data suggest that the JNK signaling pathway maybe involved in β-cell pathology. JIP3 protein

is structurally unrelated to JIP1 and JIP2 and is ubiquitously expressed. The other SAPK, p38 MAP kinase, has four different isoforms and shares common targets with JNK such as ATF-2, as well as specific targets such as heat shock protein 25 (Hsp 25) (263).

The formation of ROS under hyperglycemic conditions suggests a possible role for SAPK in mediating β-cell dysfunction. In agreement with this hypothesis, Kaneto *et al.* (8) recently demonstrated that ROS suppresses insulin gene expression and secretion in isolated rat islets. The repression of insulin gene expression was mediated through JNK. Kaneto *et al.* (8) also demonstrated that adenovirus-mediated JNK expression in isolated rat islets represses insulin mRNA. In contrast, adenovirus-mediated dominant-negative JNK expression prevents ROS-induced repression of insulin mRNA. These results suggest that high levels of glucose might mediate β-cell dysfunction through JNK activity.

Herein, we demonstrated that high levels of glucose increased JNK activity and this may mediate glucose-induced repression of insulin promoter activity in β-cells.

3. Results

3.1 Exposure of INS-1 cells to elevated glucose concentrations increases JNK activity

Recent studies report that antioxidants can partially prevent β-cell dysfunction associated with chronic exposure to high glucose concentrations (6, 7, 158, 170). These studies raise the possibility that stress-activated protein kinases such as JNK may mediate β-cell dysfunction caused by high glucose concentrations. To determine whether exposure of INS-1 cells to elevated glucose affects JNK activity, INS-1 cells were incubated in 4.0 mM or 16.7 mM glucose for 24 hrs and JNK activity was then assayed.

Incubation of INS-1 cells in 16.7 mM glucose led to a 4.5 ± 0.4-fold (n=4) increase in JNK activity compared to cells incubated in 4.0 mM glucose (Fig. 4, Panel A). Western blot analysis for JNK demonstrated that glucose did not increase JNK activity by elevating JNK protein levels. As observed with JNK activity, incubation of INS-1 cells in 16.7 mM glucose led to increased phosphorylation of JNK protein levels in nuclear extracts compared to cells grown in 4.0 mM glucose (Fig. 4, Panel C). These results indicate that incubation of INS-1 cells in an elevated glucose concentration caused a significant increase in JNK activity.

3.2 Exposure of INS-1 cells to elevated glucose concentrations increase AP-1 transcription factor activity

In many cells types, activation of JNK is associated with activation of AP-1 transcription factors. To test whether the increased JNK activity observed in INS-1 cells in response to elevated glucose leads to functional changes in AP-1 activity, INS-1 cells were transfected with a luciferase reporter gene regulated by two consensus TREs (AP-1 consensus binding sites) inserted upstream of the rat prolactin minimal promoter (2XTRE-LUC) (246). Incubation of INS-1 cells in 16.7 mM glucose for 48 hrs led to a 3.6 ± 0.6 fold (n=3) increase in 2XTRE-LUC expression compared to cells treated with 4.0 mM glucose (Fig. 5). In contrast, incubation of INS-1 cells in 12.7 mM mannitol plus 4.0 mM glucose did not increase the expression of 2XTRE-LUC (data not shown). These data suggest that the mechanism whereby 16.7 mM glucose increased 2XTRE-LUC expression was not due to osmotic stress. Moreover, these data indicate that glucose-induced activation of JNK functionally activates downstream signaling

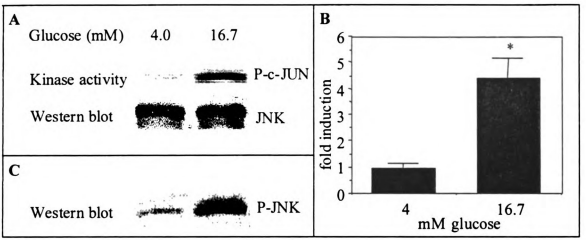


Figure 4. Incubation of INS-1 cells in high glucose concentration increases JNK activity. INS-1 cells were incubated in 4.0 or 16.7 mM glucose for 24 hrs, then JNK activity and phosphorylation were measured. Panel A (upper top) shows a representative JNK kinase assay performed as described in the Materials and Methods. Panel A (lower part) shows JNK protein levels in the cytosolic extracts used for the JNK assay depicted above. Panel B, shows the mean ± SE of JNK activity in four independent experiments (n=4). Asterisk indicates that JNK activity was significantly different between 4.0 mM and 16.7 mM glucose ($p < 0.001$). Panel C, shows a Western blot of phosphorylated JNK in nuclear extracts derived from INS-1 cells.

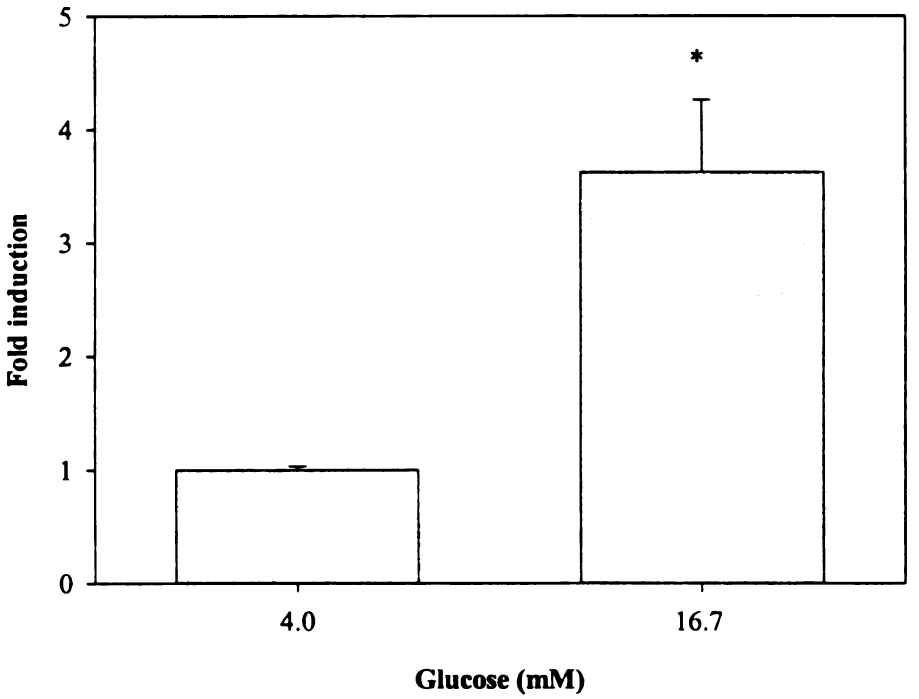


Figure 5. INS-1 cells cultured in high levels of glucose have increased AP-1 transcription factor activity. INS-1 cells were transfected with 2XTRE-LUC expression plasmid and incubated for 48 hrs in 4.0 or 16.7 mM glucose. Luciferase activity was measured according to the Materials and Methods. The data shown are the mean ± SE of three independent experiments done in duplicates (n=3). Asterisk indicates that AP-1 activity was significantly different between 4.0 and 16.7 mM glucose ($p < 0.03$).

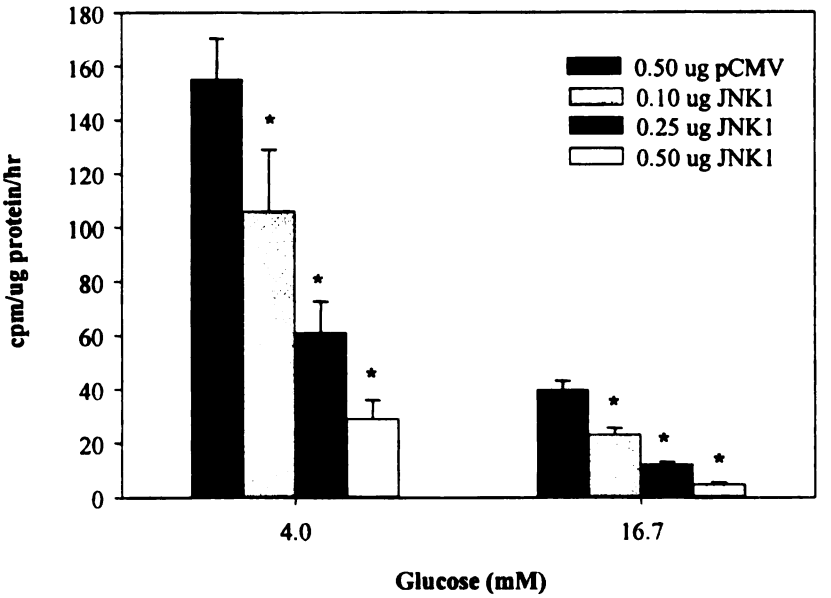
components such as AP-1 transcription factors.

3.3 Expression of JNK in INS-1 cells represses insulin promoter activity

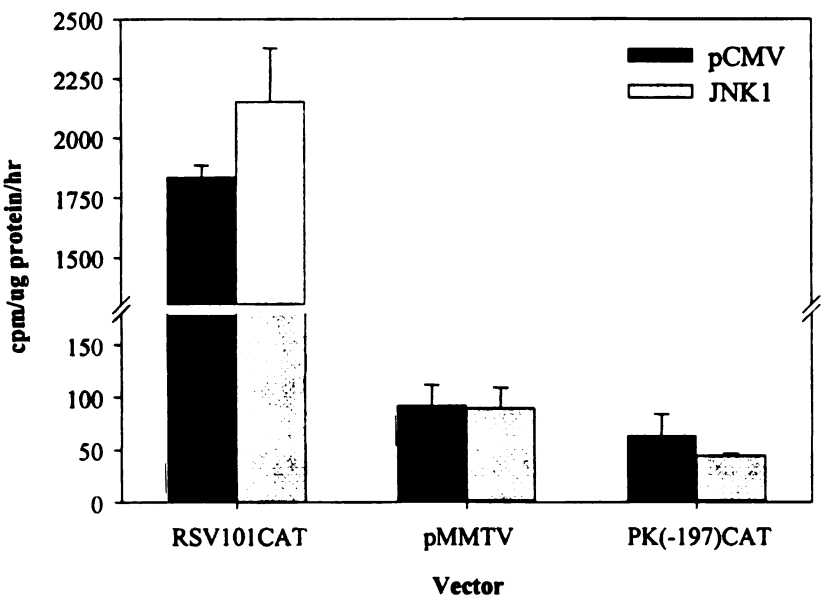
Incubation of INS-1 cells in high glucose concentrations causes a pronounced decreased in insulin mRNA and insulin promoter activity (126). The mechanism by which high glucose represses insulin promoter activity has not been fully determined and may involve glucose-induced activation of JNK. To test whether JNK activation is sufficient to repress insulin promoter activity, INS-1 cells were co-transfected with a human insulin promoter-regulated CAT reporter gene (INSCAT) and increasing concentrations of JNK1 expression plasmid (0, 0.1, 0.25, or 0.5 μg). As previously reported, incubation of INS-1 cells in 16.7 mM glucose caused a 74.4 % reduction in INSCAT expression compared to cells incubated in 4.0 mM glucose (Fig. 6A). Expression of JNK1 for 48 hrs reduced insulin promoter activity up to 78.4 % (n=6) in cells cultured in 4.0 mM glucose (Fig. 6A). The ability of JNK to reduce INSCAT expression was also observed when cells were incubated in 16.7 mM glucose concentrations (Fig. 6A), suggesting that elevated glucose concentrations can activate the over-expressed JNK leading to further suppression of the insulin promoter. To analyze whether JNK-induced repression of insulin promoter activity was specific, INS-1 cells were transiently co-transfected with different reporter vectors controlled by promoters from the Rous Sarcoma Virus (RSV101), Mouse Mammary Tumor Virus (MMTV) or liver pyruvate kinase (PK(-197)) and JNK (0.1 μg) expression vector. Expression of JNK did not significantly repress RSV101CAT, MMTVCAT or PK(-197)CAT expression

Figure 6. Over-expression of JNK represses insulin promoter activity. A, cells were co-transfected with INSCAT and pCMV (control vector) or increasing concentrations of JNK expression plasmid (as shown in the figure). After transfection, cells were incubated for 8 hrs in 4.0 mM glucose to allow for JNK expression. Cells were then incubated in 4.0 or 16.7 mM glucose for 48 hrs. Cells were then harvested and CAT activity was measured. Values are the mean ± SE of three individual experiments done in duplicates (n=6). Asterisk indicates that over-expressed JNK values are significantly different compared to control for the given glucose concentration ($p < 0.05$). B, INS-1 cells were co-transfected with RSV101CAT vector, pMMTVCAT vector or PK(-197)CAT vector and pCMV or JNK1 (0.1 μg) expression plasmid. Cells were then cultured for 48 hrs in 4.0 mM glucose. Values represent mean ± SE of two independent experiments done in duplicates (n=4).

A


180
160
140
120
100
80
60
40
20
0
cpm/ug protein/hr
0.50 ug pCMV
0.10 ug JNK1
0.25 ug JNK1
0.50 ug JNK1
*
*
*
*
*
*
4.0
16.7
Glucose (mM)

B


2500
2250
2000
1750
1500
150
100
50
0
cpm/ug protein/hr
pCMV
JNK1
RSV101CAT
pMMTV
PK(-197)CAT
Vector

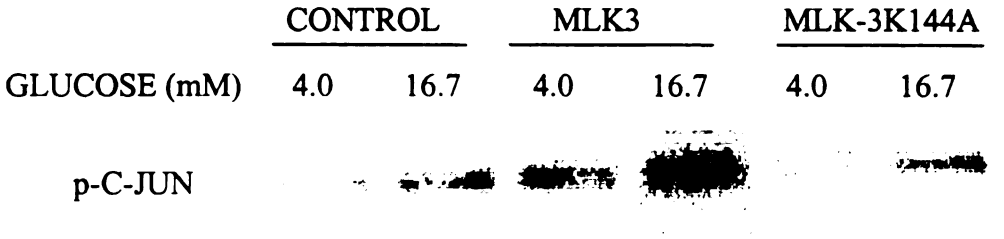
(Fig. 6B) and indicates that repression of the insulin promoter by JNK is not through a global, non-specific mechanism. Overall, these results support the involvement of JNK in the repression of insulin promoter activity in INS-1 cells expose to high glucose concentrations.

3.4 Expression of MLK3 in INS-1 cells increases both basal and glucose-stimulated JNK activity, and AP-1 transcription factor activity.

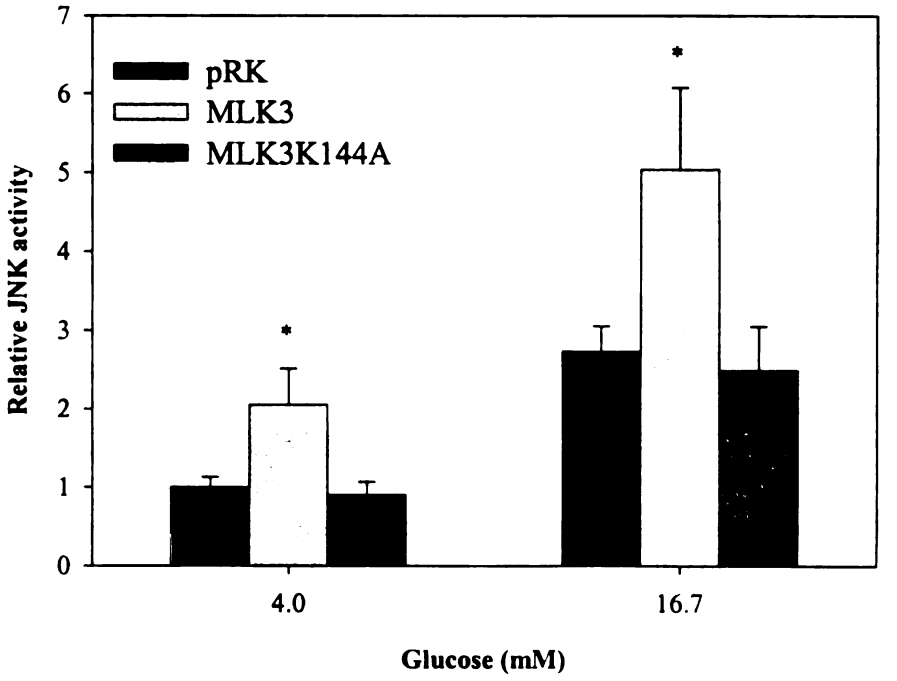
Activation of JNK by high glucose may be associated with decreased insulin promoter activity. If this is true, then direct upstream activators of JNK should also have the capacity to suppress insulin promoter activity. To test this hypothesis, INS-1 cells were transfected with an upstream activator of JNK termed MLK3. MLK3 is a serine/threonine kinase that belongs to the mixed-lineage protein kinase family (249). MLK3 has been shown to activate both MKK4 (267) and MKK7 (268), which can directly phosphorylate and activate JNK (269). Expression of MLK3 for 24 hrs led to a 2.1 ± 0.5 fold increase in JNK activity in INS-1 cells incubated in 4.0 mM glucose (Fig. 7). MLK3 further increased JNK activity up to 5.0 ± 1.0 fold in cells cultured in 16.7 mM glucose compared to cells cultured in 4.0 mM glucose. In contrast, a kinase inactive form of MLK3 termed MLK3K144A (249) was not able to activate JNK. To assess whether MLK3-mediated activation of JNK leads to functional changes in downstream targets such as AP-1 transcription factor activity, INS-1 cells were co-transfected with 2XTRE-LUC and MLK3 or MLK3K144A for 24 hrs. MLK3 led to a 2.2 ± 0.2 fold (n=3) increase of 2XTRE-LUC expression in cells incubated in 4.0 mM glucose (Fig. 8).

Figure 7. Over-expression of MLK3 in INS-1 cells activates JNK activity. INS-1 cells were transfected with pRK (control vector), MLK3 or MLK3K144A for 24 hrs in 4.0 or 16.7 mM glucose. Cells were harvested and JNK kinase activity was assayed as described in the Materials and Methods. Panel, A shows a representative JNK kinase assay. Panel B, shows the mean ± SE of JNK activity from five independent experiments (n=5). The asterisk indicates that values were significantly increased between 4.0 and 16.7 mM glucose when compared to control for the given glucose concentration ($p <$ 0.04). Data are normalized to the level of JNK activity measured in cells treated with 4.0 mM glucose. One experiment was performed by B. Böck, Department of Physiology, Michigan State University.

A


CONTROL
MLK3
MLK-3K144A
GLUCOSE (mM)
4.0
16.7
4.0
16.7
4.0
16.7
p-C-JUN

B


pRK
MLK3
MLK3K144A
Relative JNK activity
0
1
2
3
4
5
6
7
*
*
4.0
16.7
Glucose (mM)

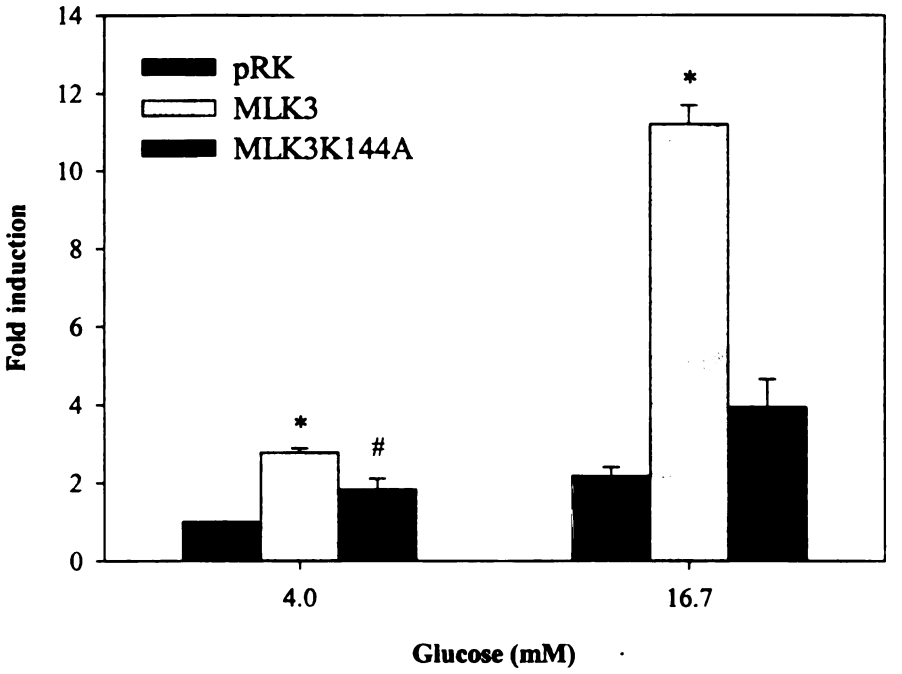


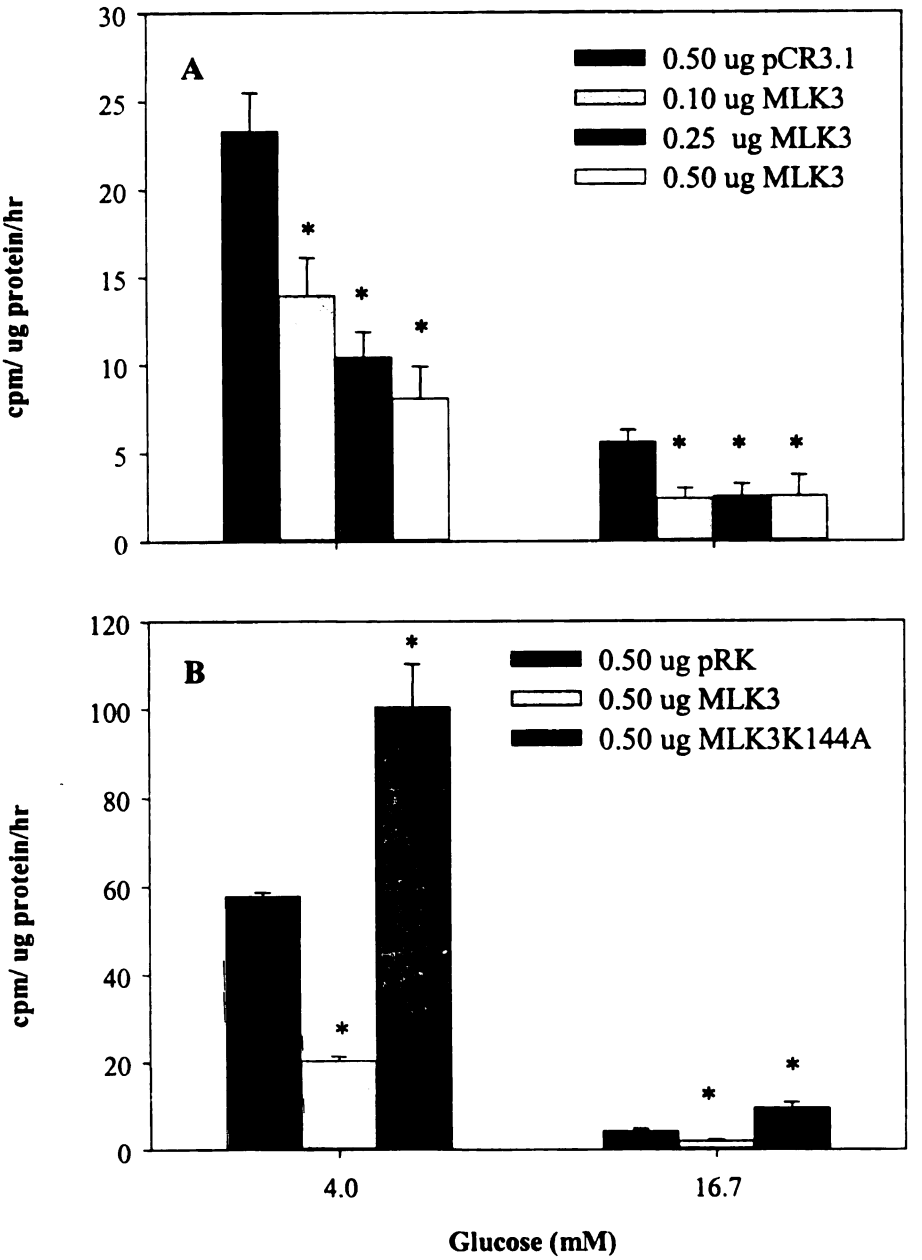
Figure 8. Over-expression of MLK3 increases AP-1 transcription factor activity. INS-1 cells were co-transfected with 2XTRE-LUC (1.0 μg) and pRK (control vector, 0.5μg), MLK3 or MLK3K144A expression plasmid. Cells were then incubated in 4.0 or 16.7 mM glucose for 24 hrs. Cells were harvested and luciferase assay was performed. Data are normalized to the level of control reporter gene expression measured in cells treated with 4.0 mM glucose. Values are the mean ± SE from three independent experiments done in duplicates (n=3). Asterisk indicates that values were significantly different between 4.0 and 16.7 mM glucose when compared to control for the given glucose concentration ($p < 0.002$). # indicates that values were significantly different compared to pRK values of the given glucose concentration ($p < 0.05$).

Addition of 16.7 mM glucose increased MLK3-induced 2XTRE-LUC expression 4.0 ± 0.4 fold (n=3) over that observed in cells treated with 4.0 mM glucose. In contrast MLK3K144A only marginally increased 2XTRE-LUC expression in cells incubated in either 4.0 or 16.7 mM glucose. These data demonstrate that MLK3 activates JNK which, in turn, leads to functional changes in signal pathways dependent upon activation of AP-1 transcription factor activity. Thus, expression of MLK3 can be used as a tool to activate JNK and investigate the potential role of JNK in the repression of insulin promoter activity.

3.5 Expression of MLK3 in INS-1 cells represses insulin promoter activity

If JNK is involved in repression of insulin promoter activity when INS-1 cells are chronically incubated in high glucose, then activation of JNK by MLK3 should also repress insulin promoter activity. To test this hypothesis, INS-1 cells were co-transfected with INSCAT and MLK3 or MLK3K144A expression plasmids and incubated for 48 hrs in 4.0 or 16.7 mM glucose. As described above, incubation of INS-1 cells in 16.7 mM glucose led to a marked decrease in INSCAT expression (Fig. 9A). Transfection of INS-1 cells with increasing concentrations of MLK3 expression plasmid reduced insulin promoter activity up to 64.4 ± 3.4 % (n=3) in cells cultured in 4.0 mM glucose (Fig. 9A). As observed with JNK expression, MLK3-mediated repression of insulin promoter activity was greater when cells were incubated in 16.7 mM glucose (Fig. 9A). In contrast, expression of kinase inactive MLK3, MLK3K144A, increased INSCAT expression in cells incubated in either 4.0 or 16.7 mM glucose (Fig. 9B). These data

Figure 9. Over-expression of MLK3 represses insulin promoter activity. Panel A, INS-cells were co-transfected with INSCAT vector and pCR3.1 (control) and increasing concentrations of MLK3 expression plasmid. Panel B, cells were co-transfected with INSCAT vector and pRK (control), MLK3 or MLK3K144A. Data shown are the mean ± SE from three independent experiments done in duplicates (n=3). Asterisk indicates that the values are significant different compared to control for the given glucose concentration ($p < 0.02$).


A
0.50 ug pCR3.1
0.10 ug MLK3
0.25 ug MLK3
0.50 ug MLK3
cpm/ ug protein/hr
30
25
20
15
10
5
0
B
0.50 ug pRK
0.50 ug MLK3
0.50 ug MLK3K144A
cpm/ ug protein/hr
120
100
80
60
40
20
0
4.0
16.7
Glucose (mM)

support the hypothesis that increased JNK activity induced by either chronic glucose exposure or expression of MLK3 can suppress insulin promoter activity.

3.6 Expression of JIP1 in INS-1 cells does not prevent glucose-induced insulin promoter repression

JIP1 protein is a specific JNK scaffolding protein that was first identified as a cytoplasmic inhibitor of JNK (248). Over-expression of JIP1 causes retention of JNK in the cytoplasm and prevents its signaling activity (248). To further investigate whether JNK mediates glucose-induced insulin promoter repression, we tested whether over-expression of JIP1 can prevent repression of insulin promoter activity. INS-1 cells were co-transfected with INSCAT and increasing concentrations of a plasmid encoding JIP1 protein. After transfection, cells were incubated in 4.0 mM glucose for ~ 8 hrs to allow expression of JIP1 under low glucose conditions. Transfection of cells with JIP1 expression plasmid (0.25 μg to 1.0 μg) did not affect INSCAT expression in cells cultured in 4.0 mM glucose for 48 hrs (Fig. 10). In contrast, cells transfected with 1.0 μg of JIP1 expression plasmid had reduced expression of INSCAT when cultured in 16.7 mM glucose (Fig. 10). In conclusion, over-expression of JIP1 protein in INS-1 cells cultured in 16.7 mM glucose was insufficient to prevent glucose-induced repression of insulin promoter activity. The inability of JIP1 to prevent repression of insulin promoter activity by glucose could be explained by three possibilities. First, JIP1 protein may not be expressed to levels necessary for complete inhibition of JNK. Second, JIP1 protein is actually an activator of JNK, thus over-expressed JIP1 protein leads to further repression of insulin promoter activity in cells cultured in 16.7 mM glucose where JNK pathway is

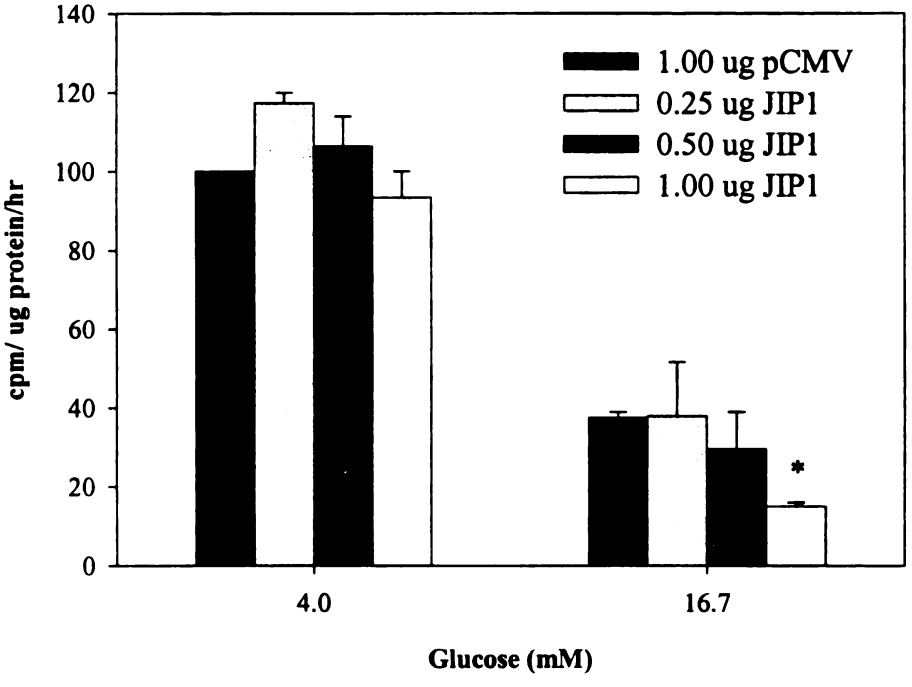


Figure 10. Expression of JIP1 protein does not reverse glucose-induced repression of insulin promoter activity in INS-1 cells. Cells were co-transfected with INSCAT vector and pCMV (control) or JIP1 expression plasmid. After transfection, cells were incubated for 8 hrs in 4.0 mM glucose and then for 48 hrs in 4.0 or 16.7 mM glucose. Data shown are the mean ± SE from two independent experiments done in duplicates (n=2). Asterisk indicates that the value is significant different compared to control for the given glucose concentration ($p < 0.003$).

activated. Third, other signaling pathways in addition to JNK are involved in insulin promoter activity repression.

To test whether over-expression of JIP1 protein effectively inhibits JNK activity in INS-1 cells, we analyzed whether glucose-induced activation of AP1 transcription factor activity is inhibited by JIP1. To accomplish this, INS-1 cells were co-transfected with 2XTRE-LUC and JIP1 expression plasmids. Over-expression of JIP1 protein only partially prevented increased 2XTRE-LUC expression in cells cultured in 16.7 mM glucose (Fig. 11). The inability of JIP1 protein to fully prevent glucose-increased 2XTRE-LUC expression could be explained by two possibilities. First, glucose activates 2XTRE-LUC expression through additional signaling pathways. Second, insufficient levels of JIP1 protein are being expressed for complete inhibition of JNK.

p38 MAP kinase is another stress-activated protein kinase that is able to phosphorylate and activate AP-1 family members such as ATF2. ATF2 can heterodimerized with AP-1 transcription factors and bind to TRE elements (270). Thus, p38 MAP kinase may be involved in glucose-induced increase of 2XTRE-LUC expression. To test this hypothesis, INS-1 cells were transiently transfected with 2XTRE-LUC and then treated for 24 hrs with or without a p38 MAP kinase inhibitor, SB203580 (10 μM) (271). p38 MAP kinase inhibitor did not affect 2XTRE-LUC expression in cells incubated in 4.0 or 16.7 mM glucose (Fig. 11), indicating that p38 MAP kinase is not likely to be involved in glucose activation of AP-1 in INS-1 cells.

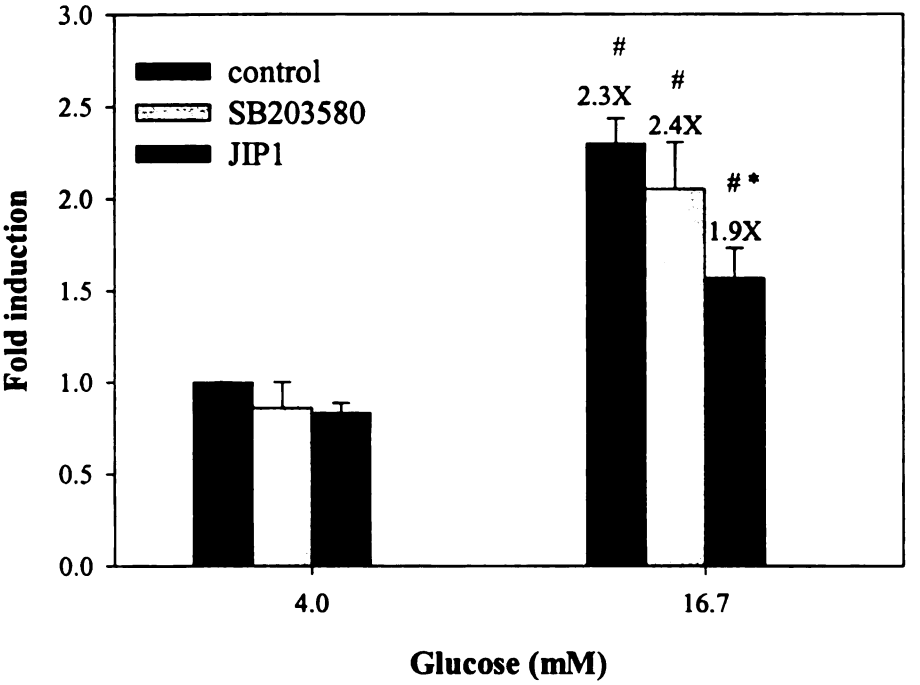


Figure 11. Effect of JIP1 over-expression and p38 MAP kinase inhibitor on glucose-induced AP1 transcription factor activity. INS-1 cells were co-transfected with 2XTRE-LUC and 2 μg pCMV (control) or 2 μg JIP1 expression plasmid. After transfection, cells were treated with DMSO (control and over-expressed JIP1 cells) or SB203580 (10 μM) in 4.0 or 16.7 mM glucose for 24 hrs. Cells were harvested and luciferase activity was measured. Values represent mean ± SE from three independent experiments done in duplicates (n=3). The asterisk indicates that expression of JIP1 significantly reduces AP1 activity compared to control in 16.7 mM glucose ($p < 0.01$). # indicates that the values are significantly increased compared to control in 4.0 mM glucose ($p < 0.05$).

3.7 Over-expression of JIP1 and inhibition of p38 MAP kinase did not prevent glucose-induced repression of the insulin promoter.

p38 MAP kinase might mediate insulin promoter repression since it has been shown to regulate insulin gene expression (272). Thus, a combination of p38 MAP kinase and JNK activity may mediate glucose-induced repression of the insulin promoter. To test this hypothesis, INS-1 cells were co-transfected with INSCAT and /or 2.0 μg JIP1 expression plasmid, then treated with or without SB203580 (10 μM). Expression of JIP1 protein or treatment with p38 MAP kinase inhibitor significantly reduced INSCAT expression when cells were cultured in both 4.0 and 16.7 mM glucose (Fig. 12). Expression of JIP1 in combination with the p38 MAP kinase inhibitor did not prevent INSCAT repression in cells cultured 16.7 mM glucose (Fig. 12).

3.8 JNK-induced repression of insulin promoter activity does not map directly to the A1, A3, or C1 elements.

Next, insulin promoter element(s) involve in JNK-induced insulin promoter repression were investigated. To overcome the complexity of interactions within the intact promoter (INSCAT that contains from –327 bp to + 30 bp), a truncated version of the insulin promoter linked to CAT, termed INS(-230)CAT (containing –230 bp to +30 bp) was tested. INS-1 cells were co-transfected with INS(-230)CAT and 0.1 μg JNK1 expression plasmid. Over-expression of JNK1 repressed INS(-230)CAT when cells were cultured in 4.0 mM glucose (Fig. 13). These data indicate that promoter elements contained within the –230 promoter are sufficient for JNK-induced repression.

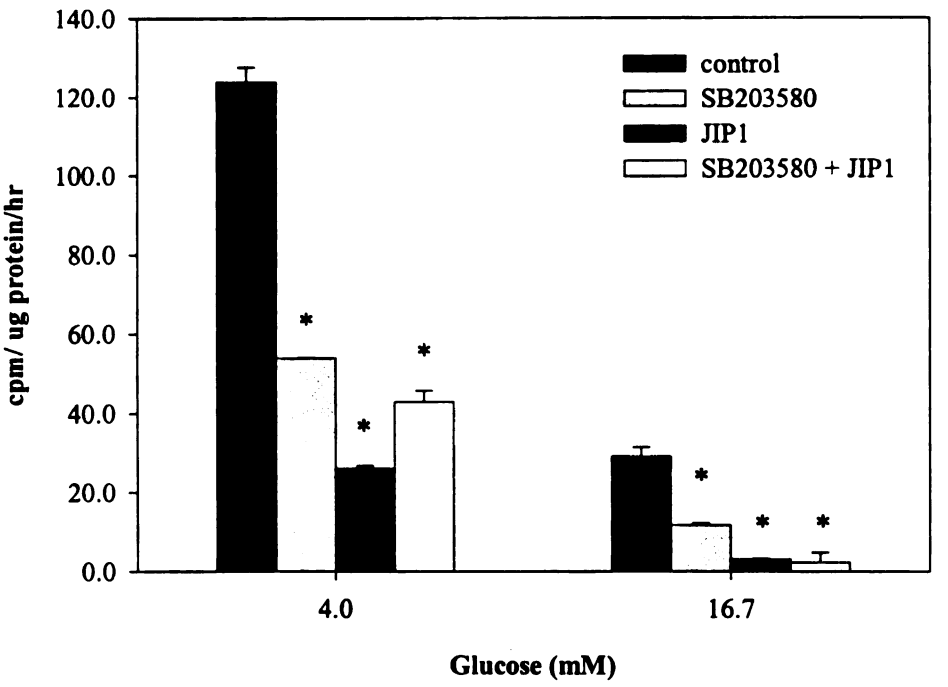


Figure 12. Over-expression of JIP-1 and inhibition of p38 MAP kinase does not prevent glucose-induced repression of insulin promoter activity. INS-1 cells were co-transfected with INSCAT vector and 2 μg pCMV (control vector) or 2 μg JIP1 expression plasmid. Cells were incubated for 8 hrs in 4.0 mM glucose to allow for JIP1 protein expression. Cells were then incubated with DMSO or 10 μM SB203580 in 4.0 or 16.7 mM glucose for 48 hrs. Values represent the mean ± SD of one experiment performed in duplicate (n=2). Asterisk indicates that the values are significantly different compared to control for a the given glucose concentration ($p < 0.02$).

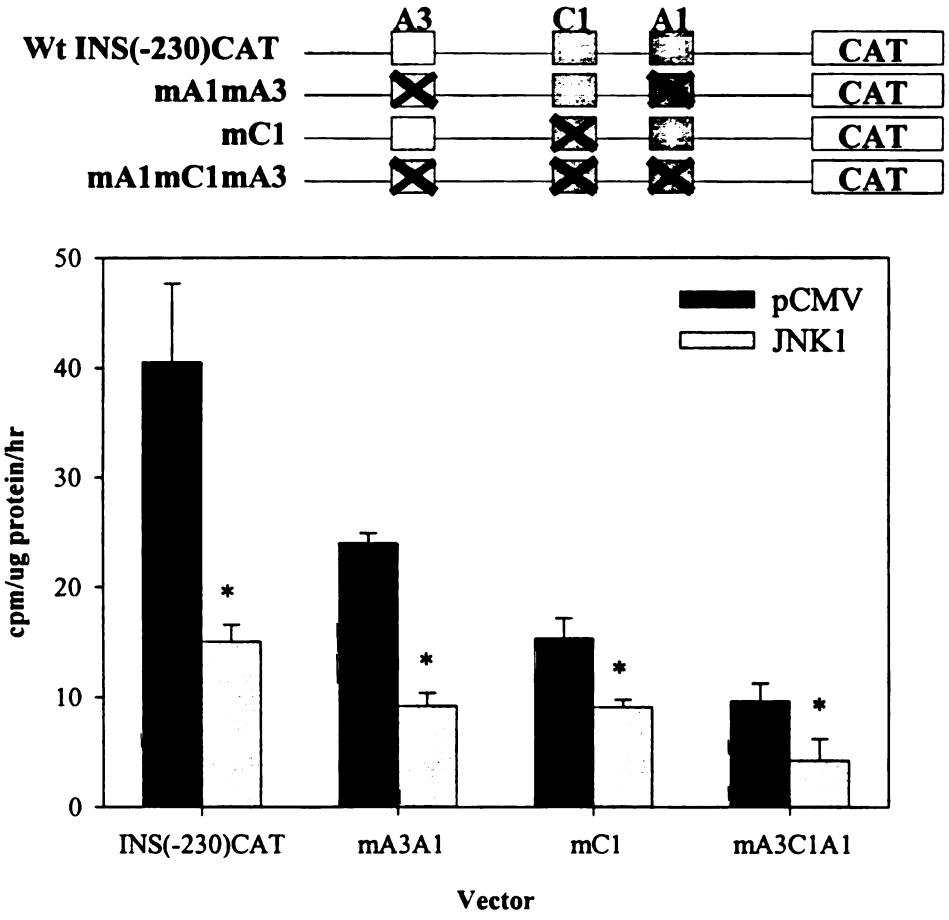


Figure 13. Repression of insulin promoter activity does not map solely to the A1, A3, and C1 elements. INS-1 cells were co-transfected with INS(-230)CAT, mA3A1(-230)INSCAT, mC1(-230)INSCAT, or mA1C1A3(-230)INSCAT and 0.1 μg pCMV (control) or 0.1 μg JNK expression vector. Cells were then incubated for 48 hrs in 4.0 mM glucose. Values represent means ± SE of three independent experiments done in duplicates (n=6). Asterisk indicates that values were significantly different compared to control ($p < 0.04$).

The –230 bp promoter region contains four important regulatory elements, the A1 and A3 elements where Pdx-1 binds, the E1 element where E12/E47 and Beta2 bind, and the C1 element where C1 activator binds (273). To further investigate whether the A1, A3 and C1 elements are involved in JNK-induced repression, the A1, A3, and/ or C1 elements were mutated. mA1A3-INSCAT contains mutated A1 and A3 elements, mC1-INSCAT contains mutated C1 element, and mA1C1A3-INSCAT contains all three A1, A3, and C1 mutated elements (mutations are described in the Materials and Methods). INS-1 cells were co-transfected with INS(-230)CAT, mA1A3-INSCAT, mC1-INSCAT or mA1C1A3-INSCAT, and 0.1 μg JNK expression plasmid. The basal activity of all the mutated promoter vectors was decreased compared to wild type (Fig. 13). Surprisingly, expressed JNK significantly repressed the activities of all the mutated insulin promoters (Fig 13). These results can be interpreted in two possible ways. First, the A1, A3, and C1 elements are not involved in JNK-induced repression of insulin promoter activity, suggesting that other elements such as E1 or CRE within the –230 bp promoter may mediate the repression. The E1 element can be a possible target of JNK, since this element is markedly regulated by acute glucose (91) and c-Jun inhibits the transactivation potential of E47, which binds to E1 element (72). The second possibility is that JNK can affect a general target such as a transcriptional co-activator.

Previous experiments have demonstrated that the E1 element is a very important regulatory element of insulin promoter activity, and deletion of this element causes a marked reduction in promoter activity (87, 91). To analyze whether JNK represses the insulin promoter through the E1 element, a multimer of three E1 elements linked to the RSV-TATA box (RSV103CAT) reporter vector was constructed. INS-1 cells were co-

transfected with the multimerized E1 ($(E1)_3$RSV103CAT) vector or RSV103CAT (control), and 0.1 μg JNK expression plasmid, and incubated in 4.0 or 16.7 mM glucose. In contrast to INSCAT, $(E1)_3$RSV103CAT expression was not repressed in cells cultured in 16.7 mM glucose (Fig. 14). These results are in agreement with previous results that show that glucose induces binding of factors to the E1 element (126). Expression of JNK protein repressed RSV103CAT (control) expression in cells cultured in 16.7 mM glucose. In contrast, expression of JNK protein did not significantly inhibit $(E1)_3$RSV103CAT expression in cells cultured in 4.0 and 16.7 mM glucose (Fig. 14). These data indicate that over-expression of JNK protein does not repress the activity of multimerized E1 element, suggesting that E1 element does not mediate JNK-induced repression of the insulin promoter.

4. Discussion

The role of JNK, a stress-activated protein kinase, in mediating repression of insulin promoter activity in INS-1 cells exposed to high concentrations of glucose was studied. INS-1 cells cultured in 16.7 mM glucose markedly increased JNK activity in parallel with increased AP-1 transcription factor activity. Over-expression of JNK1 protein repressed insulin promoter activity in INS-1 cells cultured in 4.0 mM glucose. These results suggest that the JNK signaling pathway might mediate hyperglycemic toxic effects on the insulin promoter. Our results agree with previous reports that demonstrated that over-expression of c-Jun, a downstream target of JNK, can repress insulin promoter activity in HIT cells (79). Over-expression of c-Jun was shown to inhibit insulin gene

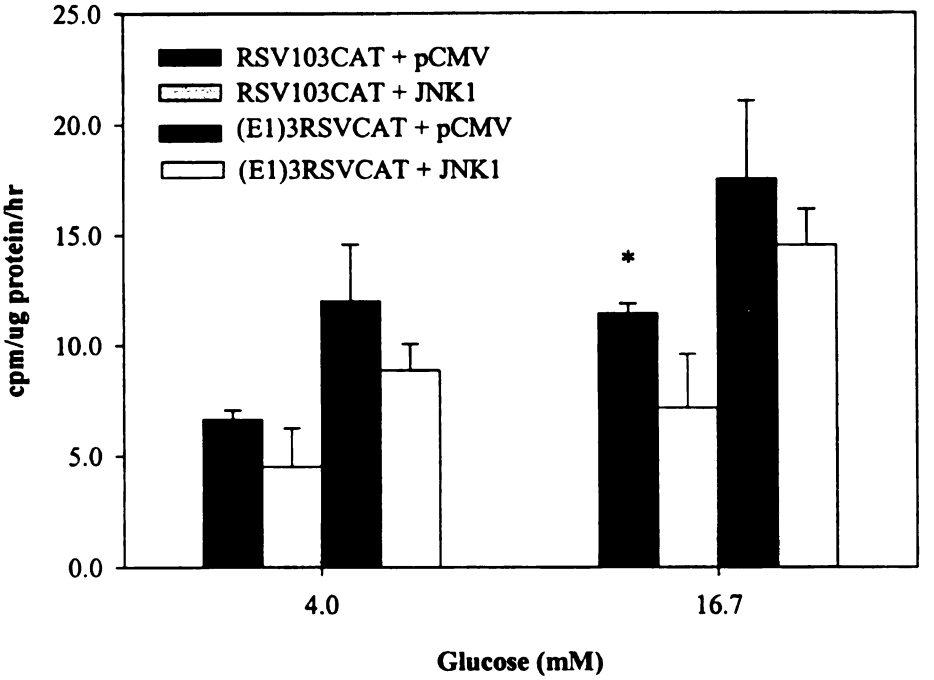


Figure 14. Expression of JNK does not repress the multimerized E1 element activity. INS-1 cells were co-transfected with $(E1)_3$RSV103CAT and pCMV (0.1 μg) or JNK1 expression vector. Then cells were incubated for 48 hrs in 4.0 or 16.7 mM glucose. Values represent means ± SE of three independent experiments done in duplicates (n=3). Asterisk indicates that the value was significant different compared to control in 4.0 mM glucose ($p < 0.0007$).

transcription by reducing the transactivation potential of the E12/E47 transcription factors which bind to the E-box (72). Recently, Kaneto *et al.* (8) demonstrated the involvement of JNK in suppressing insulin gene expression in isolated rat islets treated with H_2O_2, a strong activator of oxidative stress. The authors showed that H_2O_2 treated isolated islets had increased JNK phosphorylation and repressed insulin mRNA levels. In addition, over-expression of JNK lowered insulin mRNA levels in the absence of H_2O_2 and over-expression of a dominant negative form of JNK helped to maintain insulin mRNA levels from the toxic effects of H_2O_2. Many investigators have demonstrated that high levels of glucose increase ROS in β-cells (7, 157, 158). Furthermore, treatment of β-cells with antioxidants provides some protection against β-cell dysfunction such as partial recovery of Pdx-1 activity and insulin mRNA expression (7). Overall these reports highly suggest that hyperglycemia increases reactive oxygen species which, in turn, activate the JNK signaling pathway and this may mediate repression of insulin promoter activity.

Because of lack of specific JNK inhibitors, over-expression of JIP1 protein was tested as a JNK inhibitor to investigate the involvement of JNK in glucose-mediated repression of insulin promoter. JIP1 protein is a scaffolding protein that was first identified as a cytosolic anchor protein that binds specifically to JNK (248). In some cell systems over-expression of JIP1 protein retains JNK in the cytoplasm thereby inhibiting JNK regulation of gene expression (248). Over-expression of JIP1 in INS-1 cells failed to prevent the repression of insulin promoter activity by high levels of glucose. In fact, high concentrations of JIP1 led to further repression of insulin promoter activity. The inability of JIP1 protein to prevent the repression insulin promoter activity can be interpreted in three possible ways. First, the amounts of plasmid used to express JIP-1

protein (0.25 to 2.0 μg) may have been insufficient to retain JNK in the cytoplasm. Indeed, these concentrations of JIP1 may have actually enhanced JNK activity through its scaffolding activity and led to a further repression of insulin promoter activity. Second, over-expressed JIP1 protein may have had non-specific effects on the INS-1 cell and may have inhibited essential kinases or transcription factors required for insulin gene expression. Third and more likely is that JIP1 protein was unable to reverse glucose-induced repression of insulin promoter activity because other mechanisms may be involved in this repression.

Several investigators have demonstrated that high levels of glucose repress insulin expression by a variety of mechanisms. These various mechanisms include c-Myc, CCAAT/Enhancer-binding protein β (C/EBPβ), Pdx-1, and C1 activator (3, 85, 86, 121, 126, 274). c-Myc expression has been shown to increase in islets from diabetic 90 % pancreatectomized rats (4) and from rats made hyperglycemic with glucose clamps (275). It was demonstrated that over-expression of c-Myc suppresses insulin gene expression by inhibiting BETA2-mediated transcriptional activation (274). Investigators have also reported that supraphysiological glucose concentrations increase the expression of the transcription factor C/EBPβ in different pancreatic β-cell lines (86) as well as in Zucker diabetic fatty rats and 90 % pancreatectomized rats (85). In these diabetic models, repression of insulin mRNA was correlated with increased C/EBPβ mRNA (85). In addition, over-expression of C/EBPβ repressed insulin promoter activity by inhibiting binding activity of E47 transcription factor (86). Pdx-1 and C1 activator are also associated with the repression of insulin gene expression (2, 123). High levels of glucose reduce DNA binding activities of Pdx-1 and C1 activator to insulin promoter activity (2,

3, 121, 126). Overall, these results strongly suggest that repression of insulin gene expression involves various mechanisms, which might include JNK signaling pathway. Interestingly, Noguchi *et al.* (276) demonstrated that c-Myc is specifically phosphorylated by JNK in numerous cells types treated with UV radiation. One can postulate that hyperglycemia increases JNK activity that leads to phosphorylation and activation of c-Myc, which can repress insulin gene expression.

p38 MAP kinase is another stress-activated protein and that is involved in acute glucose regulation of Pdx-1 (272), suggesting a possible role of p38 MAP kinase in glucose-induced repression of insulin promoter activity. Inhibition of p38 MAPK, however, did not prevent glucose-induced insulin promoter repression. In contrast, inhibition of p38 MAPK lowered insulin promoter activity when cells were cultured in either 4.0 or 16.7 mM glucose. These results can be interpreted in three different ways. First, p38 MAP kinase may be required for insulin promoter activity, thus inhibition of p38 MAP kinase would reduce insulin promoter activity irrespective of glucose concentration. Such as interpretation is supported by previous reports that demonstrated exposure of β-cells to acute glucose induces Pdx-1 phosphorylation by p38 MAP kinase, resulting in activation and translocation of Pdx-1 to the nucleus where it stimulates insulin gene expression (272). Second, the concentration of p38 kinase inhibitor (SB203580) used may have had non-specific effects and may be inhibiting essential kinases that regulate insulin gene expression. Third, additional mechanisms besides JNK and p38 kinase may be involved in the repression of insulin promoter activity.

To investigate which elements are involved in JNK-induced repression of insulin promoter activity, a truncated insulin promoter containing sequences from –230 to + 30

(termed INS(-230)CAT) was analyzed. Over-expression of JNK repressed INS(-230)CAT activity in cells cultured in low glucose concentrations. Unexpectedly, over-expression of JNK also repressed the –230 insulin promoter activity containing mutations in the A1, A3, and C1 elements. These results can be explained if JNK affects an upstream target such as a co-activator that is necessary for Pdx-1 and C1 activator to bind DNA and transactivate insulin gene.

The lack of identifiable JNK target(s) on the insulin promoter suggests that JNK protein affects an upstream target(s) that regulates insulin gene transcription such as co-activators and co-repressors. Recently, Pessah, M *et al.* (277) demonstrated that JNK represses transcriptional activity by inhibiting the interactions among transcription factors and co-activators by inducing transcription factors to interact with co-repressors. Furthermore, Qiu *et al.* (96) demonstrated that the synergistic interactions between Pdx-1, Beta2, and E47 are mediated through the co-activator p300. They showed that the adenovirus E1A protein interacts with p300 and disrupts the interaction between p300 and Pdx-1, thus inhibiting insulin gene transcription (96). A possible mechanism by which JNK inhibits insulin promoter activity could be by inhibiting the ability of co-activators to interact with several transcription factors such as Pdx-1, Beta2, and E47. Over-expression of increasing concentrations of p300 and/ or cAMP-response element-binding protein (CBP), both well known co-activators, did not prevent JNK inhibitory effects on INS(-230)CAT activity (data not shown). These results, however, do not rule out the possibility that JNK can affect other co-activators.

It is noteworthy that INS(-230)CAT is repressed only 37.3 % (n=3) in INS-1 cells cultured in high levels of glucose (data shown in next chapter), while the INS(-327)CAT

is repressed 70 to 80 % (126). These results further suggest that high levels of glucose repress insulin promoter activity through several potential sites involving various mechanisms. In addition, characterization of the 5' region of the insulin promoter suggests that the repression of insulin promoter activity involves other mechanisms that affect the distal insulin promoter as will be discussed in the next chapter.

In conclusion, glucose-induced β-cell dysfunction likely involves the generation of reactive oxygen species that can activate stress-activated signaling pathways including JNK and this ultimately leads to detrimental changes in pancreatic β-cell gene expression.

V. CHRONIC EXPOSURE OF INS-1 CELLS TO HIGH GLUCOSE CONCENTRATIONS DECREASES INSULIN GENE REPORTER ACTIVITY THROUGH NOVEL REGULATORY ELEMENTS

1. Abstract

Chronic hyperglycemia induces β-cell dysfunction that is associated, in part, with reduced insulin gene promoter activity. We have reported that insulin promoter activity is markedly repressed in INS-1 cells cultured in 16.7 mM glucose compared to 4.0 mM glucose. We now report that insulin promoter activity is gradually increased when INS-1 cells are cultured for 48 hrs in 4 and 6 mM glucose compared to 2.0 mM glucose. In contrast, glucose concentrations above 8 mM cause a concentration-dependent reduction in promoter activity. In INS-1 cells, repression of insulin promoter activity by elevated glucose levels has been associated with reduced binding activity of Pdx-1 and the C1 activator (RIPE3b1). Studies designed to assess the role of Pdx-1 and C1 activator in glucose-induced insulin promoter repression led to the discovery that the majority of the repression mapped to the sequences upstream of the Pdx-1 and C1 activator binding elements. Truncation analysis indicated that a strong repression site is located between –327 and –261 nucleotides. This region includes the X minienhancer, which contains the A5/Core and E4 elements, and the Z minienhancer, which includes a palindrome sequence and the E3 element. As observed with insulin promoter activity, the activities of the X and Z minienhancers had a biphasic response to glucose. Thus, the X and Z minienhancer activity increased in cells cultured in media containing 6.0 mM glucose compared to 2.0 mM glucose, and decreased in cells cultured in glucose concentrations

above 8.0 mM. DNA binding activities to the A5/Core element, palindrome/E3a element, and palindrome/E3a-E3b elements indicated three different-sized complexes, which were markedly reduced in cells cultured in 16.7 mM glucose. Super-shift mobility assays demonstrated that Pdx-1, Nkx6.1, and Pax6 do not form part of the three different-sized complexes, and these complexes were not effectively competed by the rat I E2 element. These data demonstrate that high levels of glucose can functionally repress the X and Z minienhancers, and this repression is correlated with decreased binding activity of their binding complexes. Glucose-induced insulin promoter repression was partially prevented when all three A5/Core, palindrome, and E3 elements were mutated, suggesting that these elements mediate some of the glucose-induced insulin promoter repression. In conclusion, these data indicate the existence of additional transcriptional mechanisms by which elevated glucose concentrations can repress insulin promoter activity.

2. Introduction

Mammalian glucose homeostasis is tightly controlled by insulin production and release. Glucose, on the other hand, is one of the major physiological regulators of insulin gene expression and secretion (278). Acute changes in glucose concentrations increase insulin synthesis by controlling insulin transcription (80), insulin mRNA stability (193), and insulin gene translation (279). Glucose responsiveness of insulin gene transcription is associated with increased insulin promoter activity (95). Several glucose-responsive elements in the – 400 insulin promoter have been identified (91). These elements belong to three classes of sequences, A elements, E elements, and the C1 element (Fig. 16). The A elements, A1-A5, except for the A2, are characterized by AT rich sequences with a consensus sequence of TAAT (280), and bind several transcription factors including the pancreatic duodenal homeobox-1 (Pdx-1) transcription factor (281). Pdx-1 is essential for pancreas development and β-cell differentiation (282), and is a major transactivator of the human insulin gene (91, 283-285). The A3 element is highly regulated by glucose and its deletion has a detrimental effect on insulin transcription (281). The E elements have a consensus sequence of CANNTG. This sequence is recognized by a heterodimer of two helix-loop-helix factors, including the ubiquitously expressed proteins E12/E47 (286) and the β-cell specific factor, Beta2/Neuro D (66). There are two main E elements, E1 and E2, and both are regulated by glucose. Mutations within these elements decrease insulin promoter activity (92). Transcriptional activity of an isolated E1 element is very low, but is potentiated by the addition of adjacent regulatory elements (95, 287, 288). Therefore, E elements are required for insulin gene transcription, and their activity is dependent on other elements such as the A1 and the C1

element. In addition to the A and E elements, the C1 element is also regulated by glucose (289). The C1 element, with a C-rich sequence, binds the C1 activator (RIPE3b1) (74, 94, 290) that has only recently been cloned (5). Finally, an additional glucose-responsive element in the distal human insulin promoter, the Z minienhancer (-292/-243), was recently described using primary culture islets (89). The Z minienhancer contains the previously described negative regulatory element (NRE) (87, 88) and binds the ZaI complex that has not been well characterized.

The NRE is located between positions –279 and –261 on the human insulin promoter and deletion of sequences from –270 to –258 leads to a 25-fold increase in promoter activity in HIT-T15 cells (87). In this cell line, the NRE activity is modulated positively by sequences located from –279 to – 341 (88). The NRE resembles a silencer because its negative activity is partially independent of location and orientation, and can suppress a variety of promoters (88). The role of NRE has not been fully described but may be involved in restricting expression of the insulin gene to β-cells.

Chronic hyperglycemia, as observed in Type II diabetes mellitus, causes β-cell dysfunction including decrease glucose-stimulated insulin secretion and suppression of insulin gene expression. *In vivo* and *in vitro* studies have demonstrated that hyperglycemia suppresses insulin gene expression by decreasing insulin promoter activity. Decreased promoter activity is correlated with reduced binding activity of Pdx-1 and the C1 activator (RIPE-3b1) (2, 85, 121, 126, 291).

Our laboratory has shown that chronic exposure of INS-1 cells to high levels of glucose decreases insulin promoter activity and this is associated with reduced binding activity of Pdx-1 and the C1 activator (126). Decreased Pdx-1 binding activity is

associated with a post-translational decrease in Pdx-1 mRNA levels (126). The decrease in Pdx-1 and C1 activator binding activity is readily reversible by incubation of INS-1 cells in low glucose concentrations (126). Nevertheless, over-expression of Pdx-1 is not sufficient to prevent chronic hyperglycemia from inhibiting insulin promoter activity (L.K. Olson, unpublished data). Furthermore, other investigators have suggested that hyperglycemia can increase c-Myc and C/EBPβ levels, thus suggesting a more complicated mechanism (86, 274). Thus, the mechanism by which insulin gene expression is decreased in β-cells chronically exposed to supraphysiological glucose concentration remains to be completely elucidated. Studies described within this chapter were performed to investigate other possible promoter elements in the insulin gene that are involved in glucose-induced repression of insulin promoter activity in INS-1 cells.

3. Results

3.1 Glucose regulates insulin promoter activity in a biphasic manner in INS-1 cells.

Our laboratory has reported that exposure of INS-1 cells to 16.7 mM glucose for 24 to 48 hrs leads to marked suppression of insulin promoter activity (126). We have also shown that insulin mRNA levels start to diminish when cells are incubated in glucose concentrations at or above 8.0 mM glucose (126). We analyzed insulin promoter activity at different concentrations of glucose and tested whether promoter activity has the same pattern as insulin mRNA levels. To assess promoter activity, INS-1 cells were transiently transfected with an insulin promoter CAT reporter gene (INS(-327)CAT), in which chloramphenicol acetyl transferase (CAT) gene expression is regulated by sequences from –327 to +30 of the human insulin promoter (3). Cells were then treated

with 2.0, 4.0, 6.0, 8.0, 14.0 or 16.7 mM glucose for 48 hrs. Insulin promoter activity increased 2.2 ± 0.3 or 3.6 ± 0.6-fold in cells incubated in 4.0 or 6.0 mM glucose, respectively, compared to 2.0 mM glucose (Fig. 15). In contrast, incubation of cells in concentrations greater than 8.0 mM glucose led to a concentration-dependent reduction in insulin promoter activity. Thus, incubation of INS-1 cells in 16.7 mM glucose led to an 82.9 ± 1.4% reduction in insulin promoter activity, compared to cells incubated in 4.0 mM glucose.

3.2 The A1, A3, and C1 (RIPE3b1) regulatory elements have a minor role in mediating glucose-induced repression of insulin promoter activity in INS-1 cells.

The decrease in insulin mRNA and promoter activity observed in INS-1 cells incubated in high levels of glucose is correlated with a reduction in DNA binding activity of Pdx-1 and the C1 activator (126). To determine the relative roles of Pdx-1 and C1 activator binding elements in insulin promoter repression in cells treated with high levels of glucose, a simplified insulin promoter vector termed INS(-230)CAT was constructed. INS(-230)CAT is a CAT reporter gene regulated by insulin promoter sequences from –230 to + 30 (Fig. 16). This reporter gene contains the A1 (-79 to –84) and A3 (-210 to –216) elements where Pdx-1 binds, and the C1 element (–116 to -124) where the C1 activator (RIPE3b1) binds. Unexpectedly, INS-1 cells transfected with INS(-230)CAT and incubated in 16.7 mM glucose demonstrated only a small (~ 34 %) suppression of

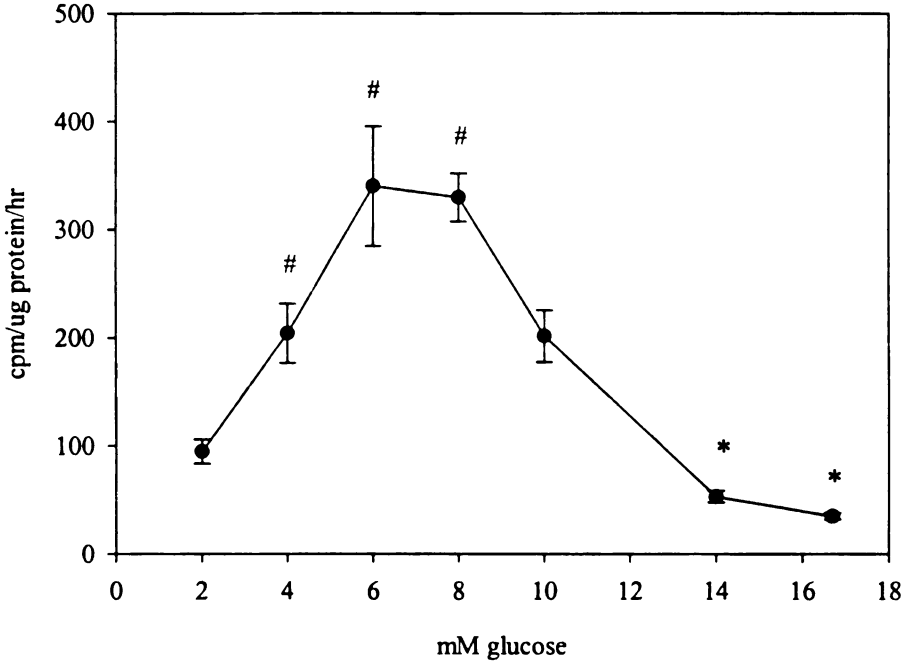


Figure 15. Glucose regulates insulin promoter activity in a biphasic manner in INS-1 cells. INS-1 cells were transiently transfected with INS(-327)CAT vector, and then were incubated in different concentrations of glucose for 48 hrs. Cells were then harvested and assayed for CAT activity. Values are the mean ± SE of four individual experiments done in duplicates (n=4). # indicates that the values are significantly increased compared to 2.0 mM glucose ($p < 0.01$). Asterisk indicates that the values are significantly reduced compared to 4.0 mM glucose ($p < 0.005$).

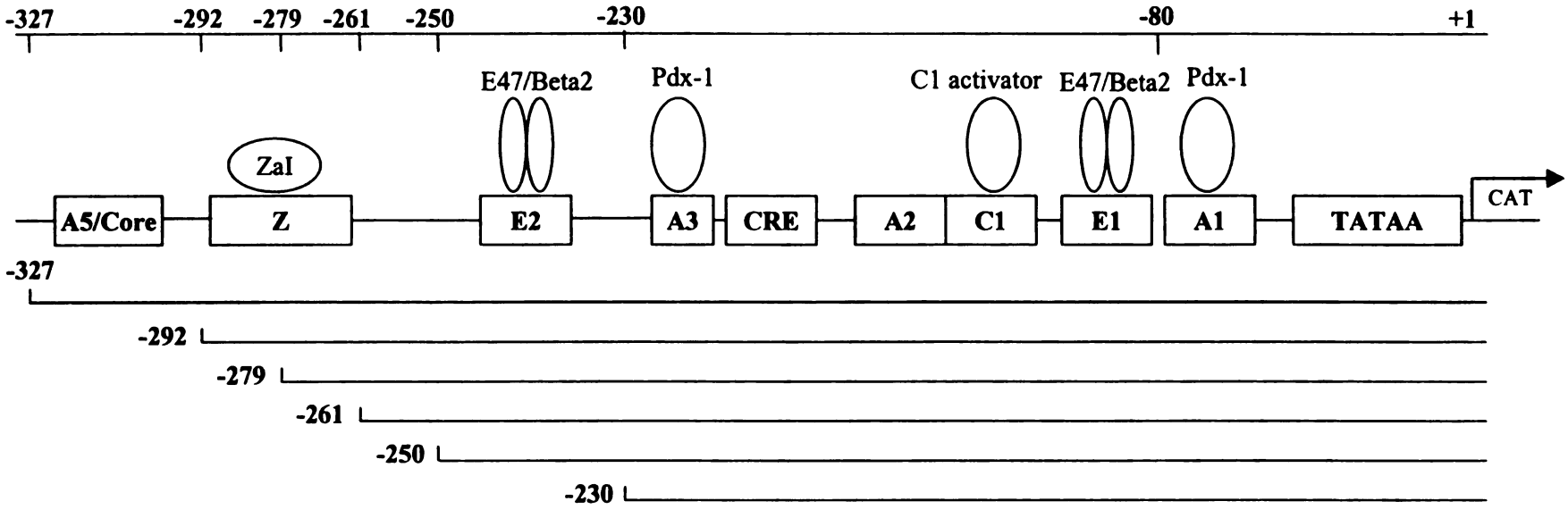


Figure 16. Schematic representation of human insulin promoter and truncated insulin promoter vectors. Regulatory promoter elements are boxed. Proteins binding to these elements are indicated above each box. Insulin promoter vectors are represented by lines.

promoter activity compared to cells incubated in 4.0 mM glucose (Fig. 17B). Consistent with our previous report (126), INS(-327)CAT expression was markedly suppressed (~ 80 %) in cells incubated in 16.7 mM glucose compared to 4.0 mM glucose (Fig. 17A). To analyze the role of Pdx-1 and the C1 activator in the repression of INS(-230)CAT, the A1, A3 and/ or C1 elements were mutated. As expected, mutation of both A1 and A3 elements led to a large (71.2 ± 3.3 %) decrease in basal INS(-230)CAT expression, and importantly, the repression of glucose-induced insulin promoter activity was prevented (Fig. 18). In a similar manner, mutation of the C1 element (mC1) led to a 74.5 ± 3.9 % decrease in basal INS(-230)CAT expression and also prevented glucose-induced insulin promoter repression (Fig. 18). In conclusion, these data indicate that the A1, A3, and C1 elements are involved in glucose-induced repression of INS(-230)CAT, however, most of the repression of the insulin promoter lies upstream of –230.

3.3 Upstream regulatory elements play a major role in glucose-mediated repression of insulin promoter activity.

To investigate the upstream elements involved in glucose-induced repression of insulin promoter activity, a series of truncated insulin reporter genes were constructed(Fig. 16). Consistent with previously reported results (126), the INS(-327)CAT expression was repressed 79.1 ± 1.6 % when INS-1 cells were incubated in 16.7 mM glucose compared to 4.0 mM glucose (Fig. 19). Deletion of the promoter region from – 327 to –292, INS(-292)CAT, which removes the A5 element (-292 to –

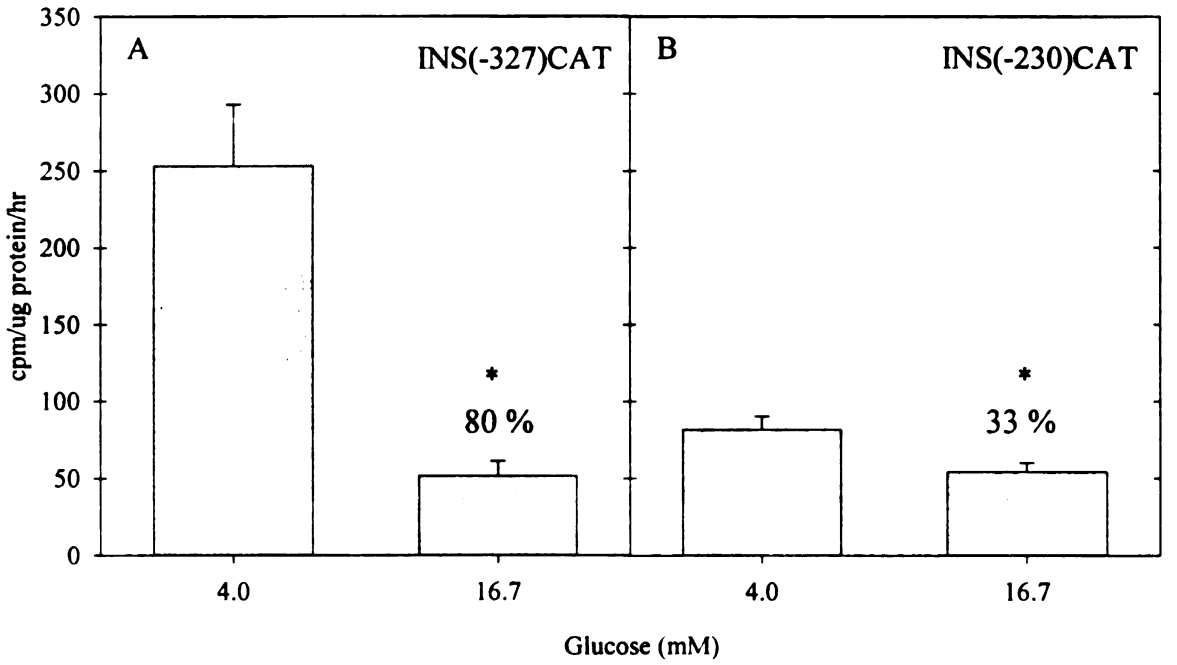


Figure 17. The –230 insulin promoter has a smaller repression than the –327 insulin promoter in INS-1 cells cultured in 16.7 mM glucose. A. INS-1 cells were transiently transfected with INS(-327)CAT vector (A) or INS(-230)CAT (B) and then cultured in 4.0 or 16.7 mM glucose for 48 hrs. Cells were then harvested and assayed for CAT activity. Values are the means ± SE of five independent experiments done in duplicates (n=5). Asterisk indicates that values were significantly different between 4.0 and 16.7 mM glucose ($p < 0.02$).

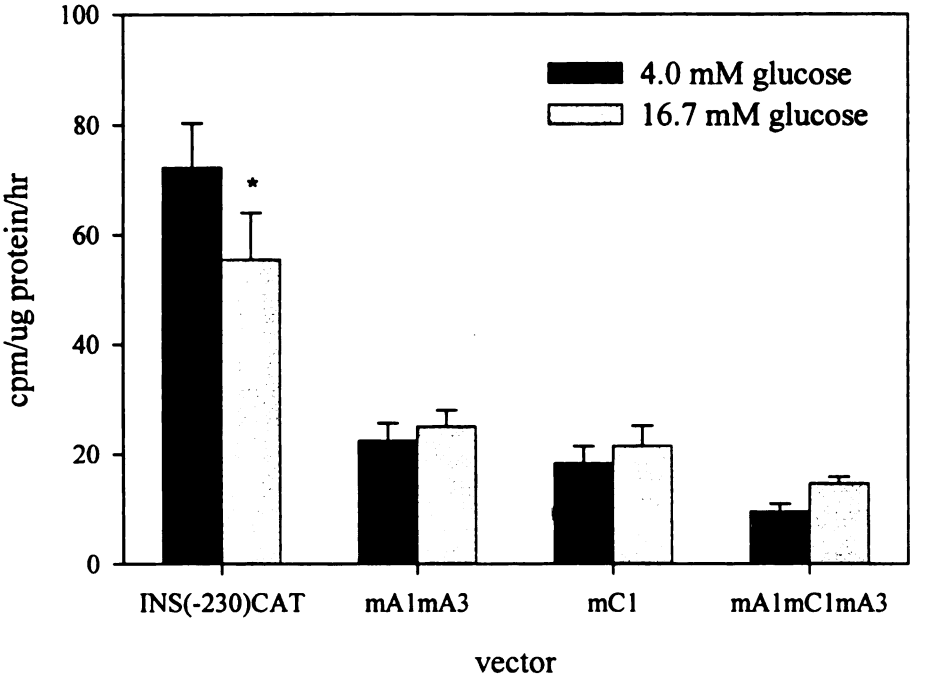
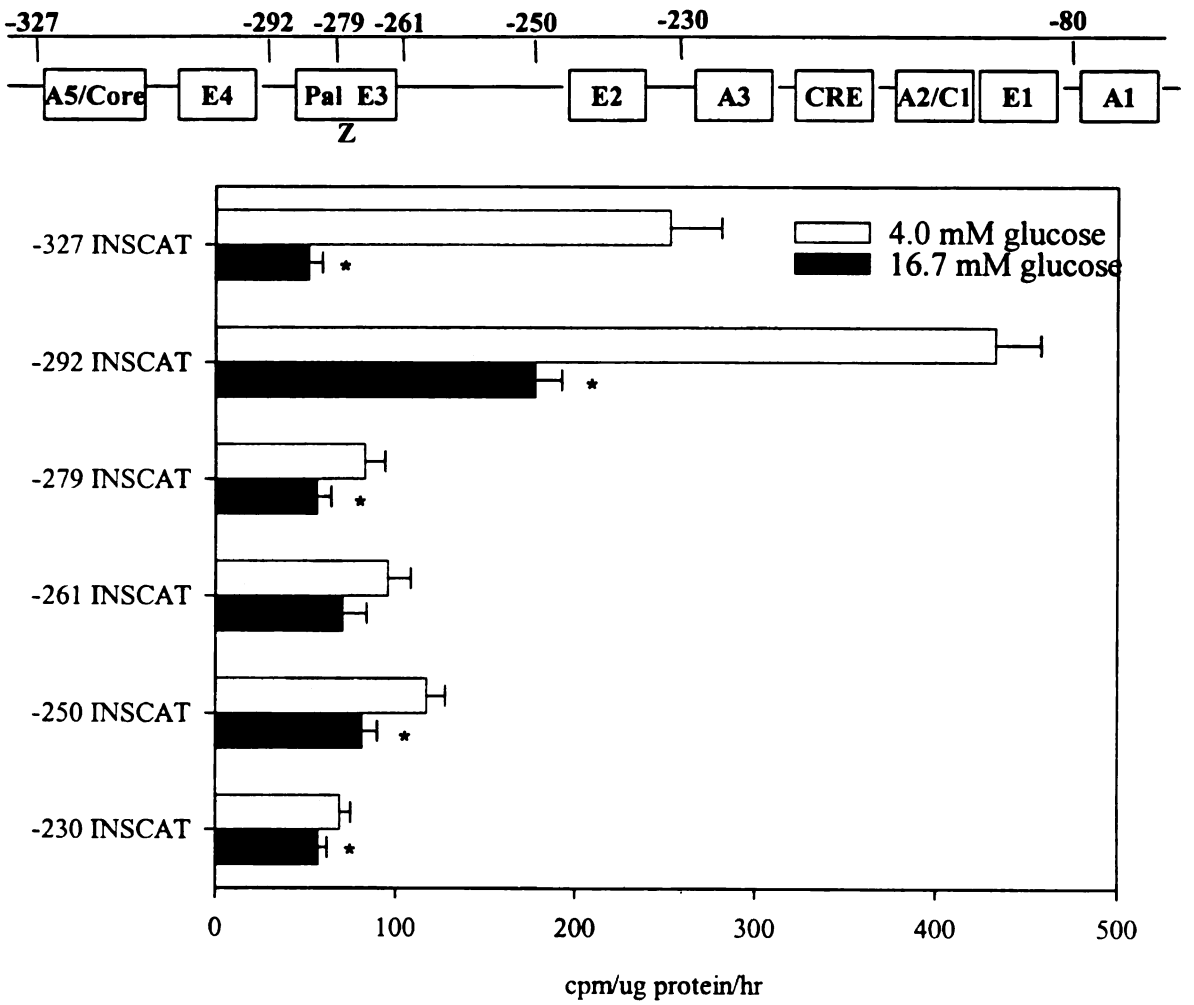


Figure 18. Mutations of both A1 and A3 or C1 prevent glucose-induced repression of the INS(-230)CAT promoter activity. INS-1 cells were transiently transfected with different vectors, shown in the figure, and were then cultured in 4.0 or 16.7 mM glucose for 48 hrs. Cells were then harvested and CAT activity was assayed. mA1mA3 represents mutated A1 and A3 elements. mC1 represents mutated C1 element and mA1mC1mA3 represents the mutations of the three elements. INS(-230)CAT and mC1 values are the mean ± SE of 5 independent experiments done in duplicates (n=10). mA1mA3 and mA1mC1mA3 values are the mean of ± SE of 4 independent experiments done in duplicates (n=8). Asterisk indicates that values were significantly different between 4.0 and 16.7 mM glucose ($p < 0.05$).

Figure 19. Truncation analysis of –327 insulin promoter activity in INS-1 cells cultured in 4.0 and 16.7 mM glucose. INS-1 cells were transiently transfected with different truncated insulin promoter vectors shown in Figure 16 and in the above schematic representation of insulin gene promoter (not to scale). The cells were then cultured in 4.0 or 16.7 mM glucose for 48 hrs. Values represent 5 experiments done in duplicates (n=10) with the means ± SE. Asterisk indicates that values were significantly different between 4.0 and 16.7 mM glucose ($p < 0.05$).


-327
-292
-279
-261
-250
-230
-80
A5/Core
E4
Pal E3
Z
E2
A3
CRE
A2/C1
E1
A1
4.0 mM glucose
16.7 mM glucose
-327 INSCAT
-292 INSCAT
-279 INSCAT
-261 INSCAT
-250 INSCAT
-230 INSCAT
0
100
200
300
400
500
cpm/ug protein/hr

314), the enhancer core element (Core) (-317 to –309), and an E-box like element named the E4 element (-300 to -294), caused an approximately two-fold increase in CAT expression in both 4.0 and 16.7 mM glucose compared to INS(-327)CAT (Fig. 19). INS(-292)CAT expression was repressed 59.1 ± 2.5 % in cells incubated in 16.7 mM glucose compared to 4.0 mM glucose. Deletion of the promoter region from –327 to –279, INS(-279)CAT, which removes the A5/Core, E4 and destroys a palindrome sequence (-284 to -273) contained in the Z minienhancer (89) and negative regulatory element (NRE) (88), led to only a 27.9 ± 5.4 % repression of CAT expression in cells cultured in 16.7 mM glucose compared to 4.0 mM glucose. Removal of promoter sequences from –327 to –261, INS(-261)CAT, which deletes the A5/Core, E4 element, the palindrome, and the E3 element (-273 to –257), did not further decrease the ability of 16.7 mM glucose to suppress insulin promoter activity. Further removal of promoter sequences up to –230 did not lead to any additional decrease of the high glucose-induced suppression of insulin promoter activity. These data suggest that regulatory elements within –327 and –279 promoter sequences are involved in glucose-induced repression of insulin promoter activity.

3.4 The Z, X, and Y minienhancers contain promoter sequences involved in glucose-induced biphasic regulation of insulin promoter activity.

Sander *et al.* (89) recently characterized glucose regulation of distal human insulin promoter sequences from –341 to –243 using multimerized (5 copies) regions of promoter (named minienhancers) linked to the –85 bp minimal promoter from the rat

insulin I gene promoter. They divided this region into X (-342 to –293), Y (-317 to –268) and Z (-292 to –243) minienhancers (Fig. 20). The X and Y minienhancers both contain the A5/Core element, while the Y minienhancer also contains a palindrome and the 5' region of the E3 element. The Z minienhancer contains a palindrome sequence and the E3 element. The E3 element contains two E-like sequences that we have termed E3a and E3b, separated by four base pairs (Fig. 20). The Z minienhancer was further divided into the Za (-292 to –263) and Zb (-274 to –246) minienhancers. The Za minienhancer (-292 to –261) contains a palindrome sequence and the E3a (-273 to –268), while the Zb minienhancer (-275 to –245) contains both the E3a and E3b (-263 to –257) and not the palindrome (Fig. 20). Sander *et al.* (89) demonstrated that the Z minienhancer had a very strong transcriptional activity and its activity was further increase in primary islets cultured in 16.0 mM glucose compared to 2.0 mM glucose. They concluded that the Z minienhancer is a glucose-responsive element only in primary cultured islets. In contrast, the X and Y minienhancers had very weak transcriptional activity when transfected in fetal islets and did not respond to glucose.

In order to investigate possible upstream regulatory elements involved in the glucose-induced repression of insulin promoter activity, INS-1 cells were transiently transfected with the multimerized (5 copies) Z, X, or Y minienhancers and were then incubated in 2.0, 4.0, 6.0, 8.0, or 16.7 mM glucose for 48 hrs. The –85 to +1 rat insulin promoter (–85 rINSCAT/pFOXCAT2) was found to be only slightly glucose-responsive in INS-1 cells, with a 1.8 ± 0.2 or 2.7 ± 0.1-fold increase in CAT expression in cells

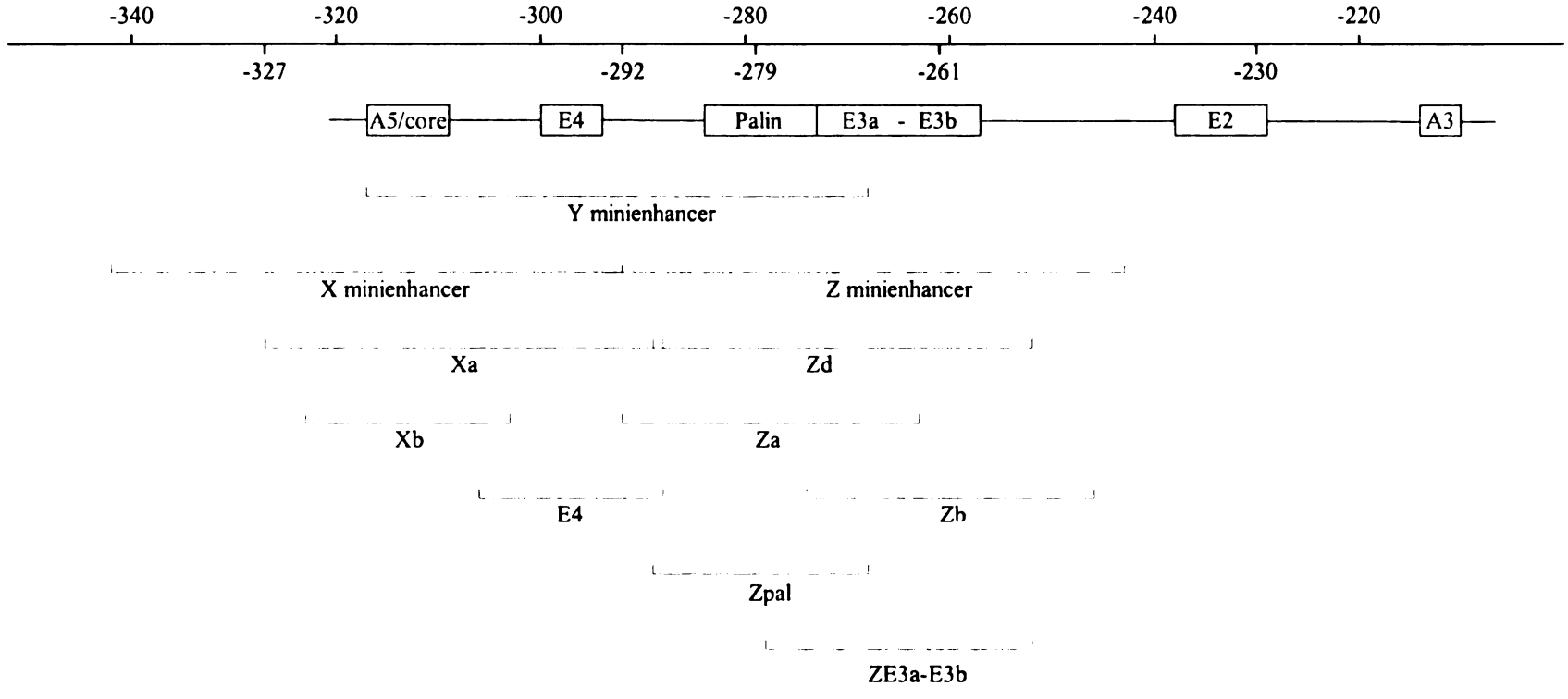


Figure 20. Schematic representation of distal insulin promoter sequences. Insulin promoter elements are boxed. Minienhancer elements used in multimerized reporter vectors are shown underneath of promoter elements, as well as probes used for mobility-shift assays.

incubated in 6.0 or 16.7 mM glucose, respectively, compared to 2.0 mM glucose (Fig. 21). We believe this small response is not insulin promoter specific because we have also found a small response with the Rous sarcoma virus minimal promoter (Fig. 14, chapter I). In contrast, the Z minienhancer had a large activity compared to the –85 rINSCAT. The Z minienhancer had a 3.1 ± 0.2-fold increase when cells were incubated in 6.0 mM glucose compared to incubation in 2.0 mM glucose (Fig. 21). As observed with INS(-327)CAT, glucose concentrations greater than 8.0 mM glucose led to a decrease in Z minienhancer activity compared to cells incubated in 4.0 or 8.0 mM glucose. Incubation of INS-1 cells in 16.7 mM glucose led to a 38.2 ± 5.1 % reduction in Z minienhancer activity compared to cells incubated in 4.0 mM glucose (Fig. 21). In order to determine whether the palindrome and/ or E3a-E3b elements were involved in the glucose-induced repression of the Z minienhancer, we analyzed the ability of glucose to repress the Za minienhancer, which contains the palindrome and the E3a element, and the Zb minienhancer, which contains only the E3a and E3b elements (Fig. 20). As observed with the Z minienhancer, both the Za and Zb minienhancers activity had a biphasic response to glucose with a ~2.7 and ~4.1-fold increase, respectively, when cells were incubated in 6.0 mM glucose compared to 2.0 mM glucose (Fig. 22). When cells were incubated in 16.7 mM glucose, Za and Zb minienhancers activity were also repressed by ~ 80.0 % and ~ 73.0 %, respectively, compared to 4.0 mM glucose. These data suggest that the E3a element, which is contained in both the Za and Zb minienhancers, may be involved in the repression of insulin promoter activity by high levels of glucose. Alternatively, independent elements within the Za and Zb minienhancers may be involved in glucose-induced insulin promoter repression.

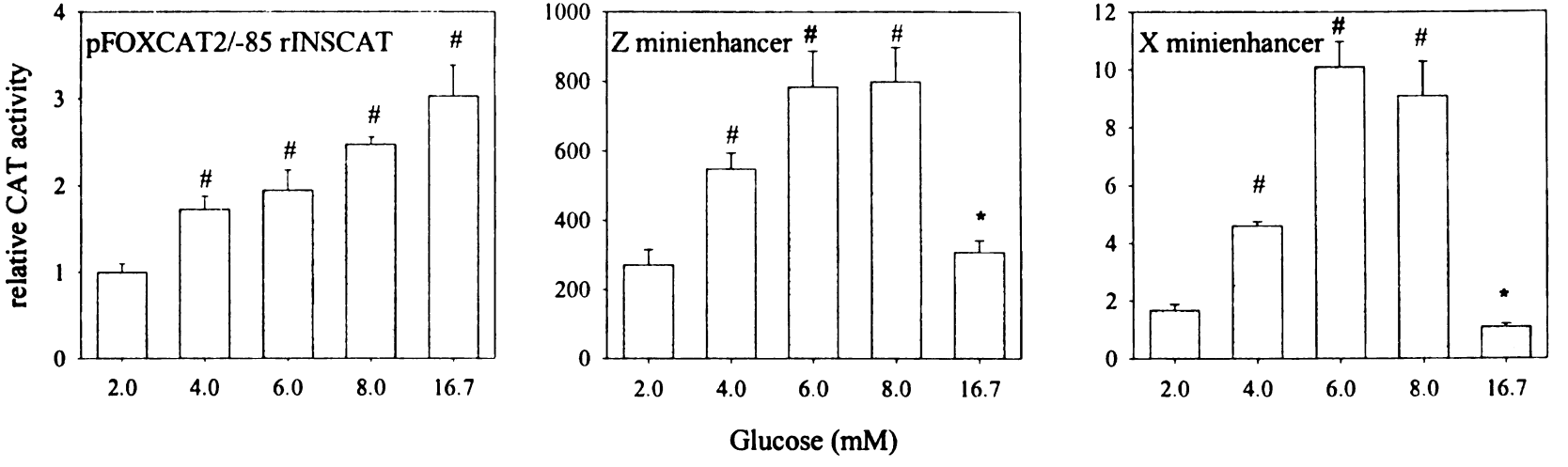


Figure 21. The Z and X minienhancers are repressed when INS-1 cells are cultured in 16.7 mM glucose. INS-1 cells were transiently transfected with plasmids containing five tandem copies of the minienhancers shown or a control vector containing –85 rat I promoter driving the CAT gene in pFOXCAT2. Cells were then cultured for 48 hrs in concentrations of glucose as shown in the figure. The values are relative to the pFOXCAT2 expression vector in cells cultured in 2.0 mM glucose. The values are the mean ± SE of two independent experiments done in duplicates (n=4). # indicates that the values were significantly different compared to 2.0 mM glucose ($p < 0.01$). Asterisk indicates that the values were significantly different between 4.0 and 16.7 mM glucose ($p< 0.01$).

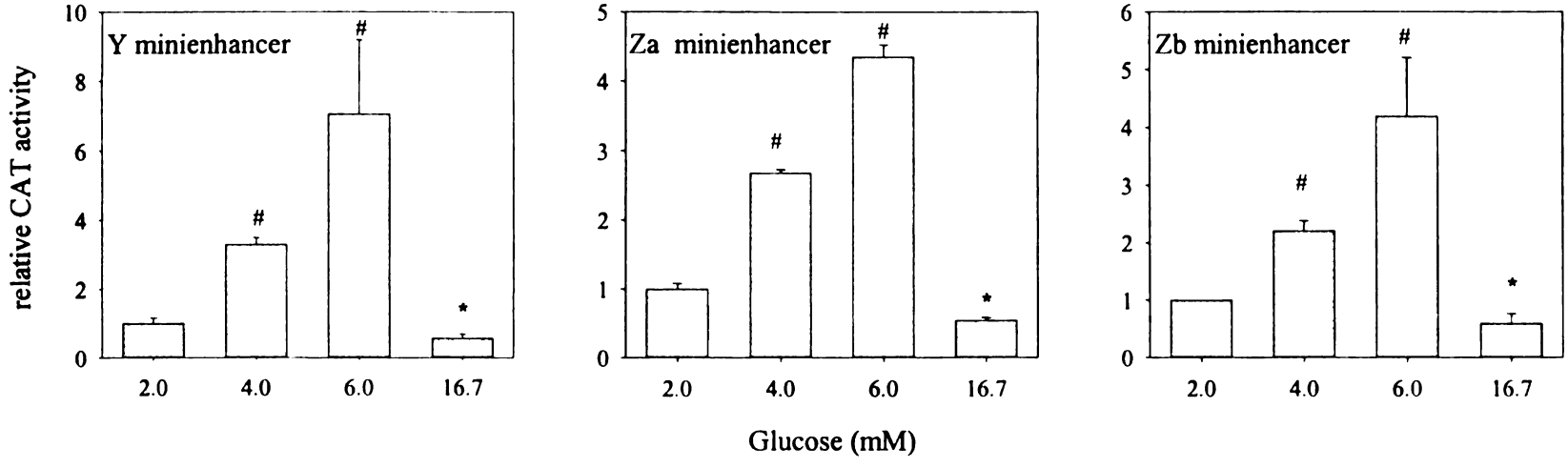


Figure 22. The Y, Za , and Zb minienhancers are repressed when INS-1 cells are cultured in 16.7 mM glucose. INS-1 cells were transiently transfected with plasmids containing five tandem copies of the minienhancers shown. Cells were then cultured for 48 hrs in different concentrations of glucose, as shown in the figure. The values are relative to the expression vectors in cells cultured in 2.0 mM glucose. The values are the mean ± SE of four independent experiments done in duplicates (n=4). # indicates that the values were significantly different compared to 2.0 mM glucose ($p < 0.03$). Asterisk indicates that the values were significantly different between 4.0 and 16.7 mM glucose ($p < 0.03$).

Deletion of insulin promoter sequences from –327 to –292, INS(-292)CAT, demonstrated that high levels of glucose mediated some promoter repression through this region. This region contains the A5/Core and the E4 elements. To determine the effect of glucose on this general region, INS-1 cells were transiently transfected with the X minienhancer reporter gene and incubated in 2.0, 4.0, 6.0, 8.0, or 16.7 mM glucose for 48 hrs. The X minienhancer activity was markedly lower than the Z minienhancer activity (Fig. 21), as previously observed in fetal rat islets (89). Interestingly, the X minienhancer also had a biphasic response to glucose, with a 5.3 ± 0.7-fold increase when cells were incubated in 6.0 mM glucose compared to 2.0 mM glucose (Fig. 21). The X minienhancer activity was also markedly reduced when cells were incubated in 8.0 or 16.7 mM glucose (Fig. 21). The X minienhancer activity was decreased by 74.4 ± 0.2 % in cells incubated in 16.7 mM glucose compared to 4.0 mM glucose. These data suggest that high glucose concentrations repress the insulin promoter activity, in part, through the promoter region from -327 to –292.

We also analyzed the ability of glucose to repress the Y minienhancer activity, which contains the A5/Core, the E4, the palindrome, and the E3a element (Fig. 20). INS-1 cells were transiently transfected with Y minienhancer reporter vector and cells were then incubated in media containing 2.0, 4.0, 6.0, or 16.7 mM glucose for 48 hrs. As expected, the Y minienhancer also had a biphasic glucose response with a 7.1 ± 2.1-fold increase in cells incubated in 6.0 mM glucose compare to 4.0 mM glucose (Fig. 22). The Y minienhancer was also repressed by ~ 82.0 % in cells incubated in 16.7 mM glucose compared to 4.0 mM glucose. Overall, these data suggest that the A5/Core, the E4

element, the palindrome, and the E3 element all have possible roles in mediating glucose-induced repression of insulin promoter activity.

3.5 High levels of glucose repress DNA binding activity to the A5/Core and E3 regulatory elements.

INS-1 cells incubated in 16.7 mM glucose significantly repressed the Z, X, and Y minienhancer activities. To analyze whether glucose-induced repression of Z, X, and Y minienhancer promoter activities correlate with a reduction in DNA binding activity, we tested nuclear extracts of INS-1 cells incubated in 4.0 or 16.7 mM glucose for the ability to bind labeled double-stranded oligonucleotides within the Z and X minienhancers. Because the palindrome (-284 to –279) and the E3a-E3b elements (-273 to –258) are potential regulatory elements within the Z minienhancer, we analyzed DNA/nuclear extract binding activity with a probe termed Zd (-289 to –252) (Fig. 20). As shown in Figure 23, four specific DNA/protein complexes (Zd1-4) were observed in nuclear extracts of INS-1 cells incubated in either 4.0 or 16.7 mM glucose for 48 hrs. The fastest migrating complex (Zd1) consistently showed reduced binding activity in cells cultured in 16.7 mM glucose (Fig. 23, compare lanes 2 and 3, and Fig. 24A). Binding activities of all four complexes were specific to the Zd probe because they were completely competed with 100-fold molar excess of the unlabeled Zd probe (Fig. 23, compare lane 2 and 3 with 4 and 5). The palindrome sequence alone was not responsible for Zd1-4 complexes because competition with the excess unlabeled palindrome probe (Z_{pal}) did not affect binding (Fig. 23A, lanes 6 and 7). Competition analysis showed that the slowest

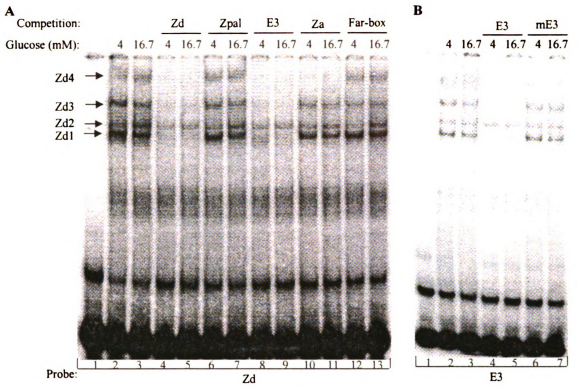


Figure 23. Glucose-sensitive complex binds to the palindrome sequence and the E3 element. Equal amounts of nuclear extracts from INS-1 cells incubated in media containing 4.0 or 16.7 mM glucose were analyzed for binding to a ^{32}P-labeled Zd probe (A) or E3 probe (B) (see Table 3). (A) Competition with 100-fold molar excess of unlabeled Zd probe (wt) (lanes 4 and 5), palindrome probe (lanes 6 and 7), E3 probe (lanes 8 and 9), Za probe (lanes 10 and 11) and Far-box probe (lanes 12 and 13). (B) Competition with 100-fold molar excess of unlabeled E3 probe (wt) (lanes 4 and 5) and mE3 probe (lanes 6 and 7). Shown is a representative experiment of 3 three independent experiments.

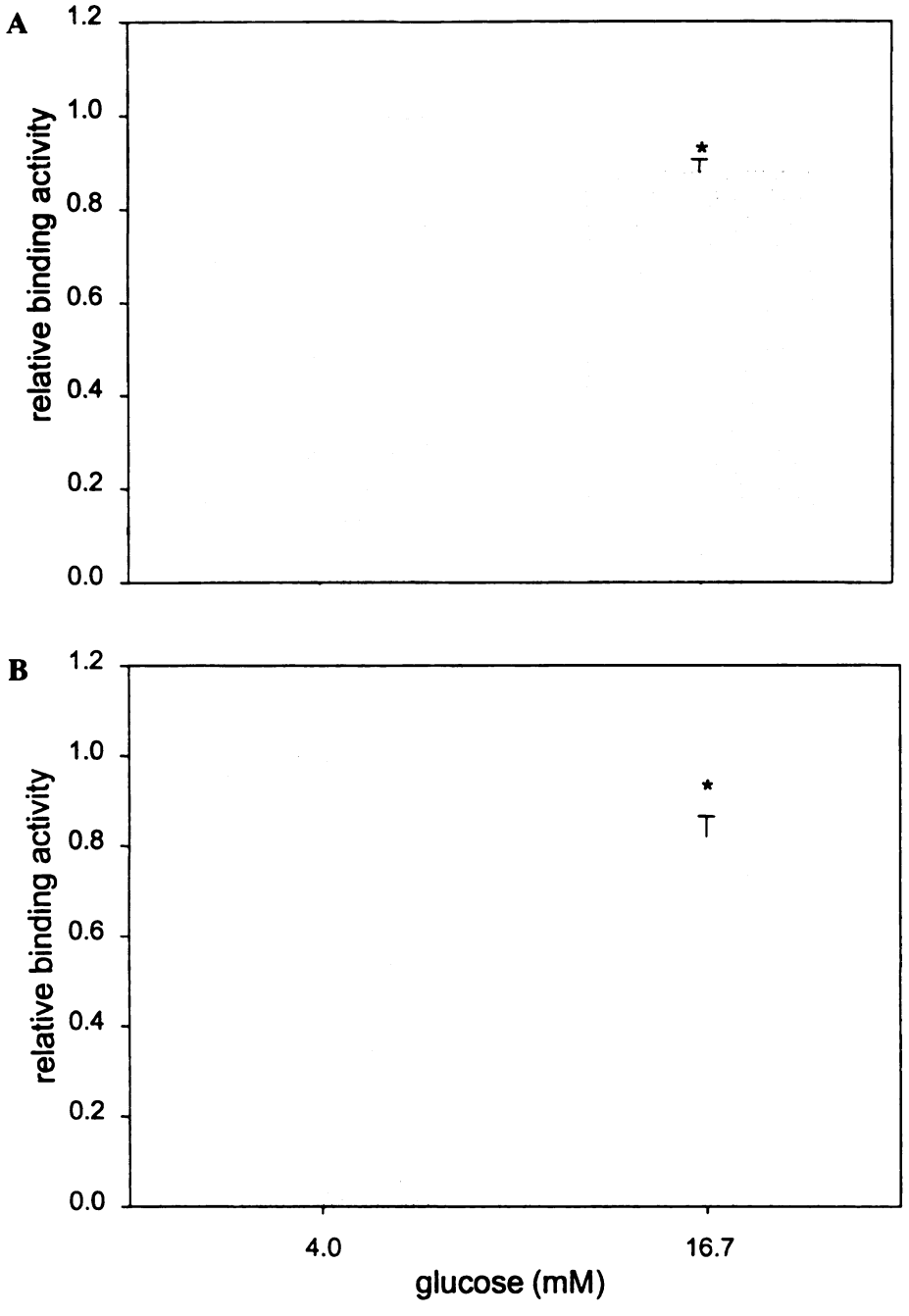


Figure 24. High levels of glucose reduces binding activity of the Zd1 and E3 probes. Fig. 24A and 24B show the binding activity of the slowest migrating complex that bind to the Zd and E3 probes, respectively. Values represent mean ± SE of three independent experiments (n=3). Asterisk indicates that 16.7 mM glucose significantly decreases binding activity compared to 4.0 mM glucose ($p < 0.03$).

migrating complex (Zd4) was effectively competed for by the Za probe, which contains the palindrome and the E3a elements (Fig. 23A, lanes 10 and 11). These data suggest that either the E3a element alone or the E3a element and palindrome sequence are required for Zd4 binding. Competition analysis also showed that the E3 probe, which contains the E3a and E3b elements effectively competed for Zd1-4 binding complexes (Fig. 23A, lanes 8 and 9). To test whether the E12/E47 and Beta2 transcription factors are contained in the Zd1-4 complexes, the Zd probe was competed for binding with an E-like element from the rat I promoter, the rat FAR-box. The Zd1-4 complexes were not competed for by the rat I FAR-box probe (Fig. 23A, lanes 12 and 13), indicating that the E12/E47 and Beta2 transcription factors do not bind to these sequences. Interestingly, protein binding activity to a labeled E3 element (E3a and E3b elements), termed Z_{E3aE3b} probe, showed similar pattern of DNA/protein complexes to those observed for the Zd probe (Fig. 23B, lanes 2 and 3 compared to Fig. 23A, lanes 2 and 3). High levels of glucose reduced binding activity of the fastest migrating complex (Fig. 23B, lanes 2 and 3 and Fig. 24B). The DNA/protein binding activities were specific to the Z_{E3aE3b} probe because the 100-fold molar excess of unlabeled Z_{E3aE3b} competed efficiently with all complexes (Fig 23B, lanes 4 and 5). When the Z_{E3aE3b} probe was competed with a probe containing mutations on E3a and E3b elements, the Zd1-4 complexes were not competed (Fig. 23B, lanes 6 and 7). These data strongly suggest that the DNA binding complexes of the Zd probe require the E3a and E3b elements, and there is a trend that high levels of glucose decrease Zd1 binding activity.

High concentrations of glucose also reduced the Za minienhancer activity (Fig. 22B). Since the Za probe lacks the E3b element, it was important to determine

whether DNA binding activity to the Za probe was regulated by glucose. As shown in Fig. 25A, three complexes bind to the Za probe (Za1-3). The Za2 complex binding activity was markedly decreased in nuclear extracts from INS-1 cells incubated in 16.7 mM glucose compared to 4.0 mM glucose (Fig 25A, compare lanes 2 and 3 and Fig. 26). These complexes were specific to Za probe since addition of excess unlabeled Za probe prevented formation of Za1-3 complexes (Fig. 25A, lanes 4 and 5). Mutation of –271 (T to G), which changed the third base-pair of the E3a element, did not affect binding of the three Za complexes (Fig. 25B, lanes 4 and 5). In contrast, a mutation at –273 (C to G), which changed the first base-pair of the E3a element, led to loss of both Za1 and Za2 complexes (Fig 25B, lanes 6 and 7). More over, a mutation at both –283 (C to A) and -282 (T to G), both contained in the palindrome, led to a complete loss in binding activity for all three Za complexes (Fig. 25B, lanes 8 and 9). Overall, these data suggest that the Za2 complex, which is a glucose-sensitive complex, requires both the palindrome and the E3a element for binding. In addition, these data demonstrated that separation of the palindrome sequence and the E3a element from the E3b element led to different DNA/protein complexes than the complex bound to the three elements together. These data suggest that there could be some synergic interactions among the palindrome, E3a, and E3b elements for binding transcription factors. Similar observations have been made by Sander *et al.* (89).

Experiments performed in this study showed that high levels of glucose repressed the X minienhancer activity (Fig. 21). Therefore DNA/binding activity to elements within the X minienhancer was examined. The Xa probe (-323 to –288), which

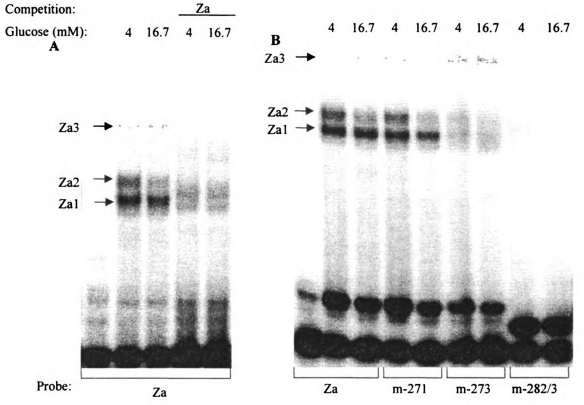


Figure 25. Glucose-sensitive complex binds to the Za element region. Equal amounts of nuclear extracts from INS-1 cells incubated in media containing 4.0 or 16.7 mM glucose were analyzed for binding to a ^{32}P-labeled Za probe (A), and to a ^{32}P-labeled m-271, m-273 or m-282/3 (B). See Table 3 for sequences of the probes. Panel A, lanes 4 and 5 contained 100 X-fold unlabeled Za probe. Shown is a representative experiment of 4 independent experiments.

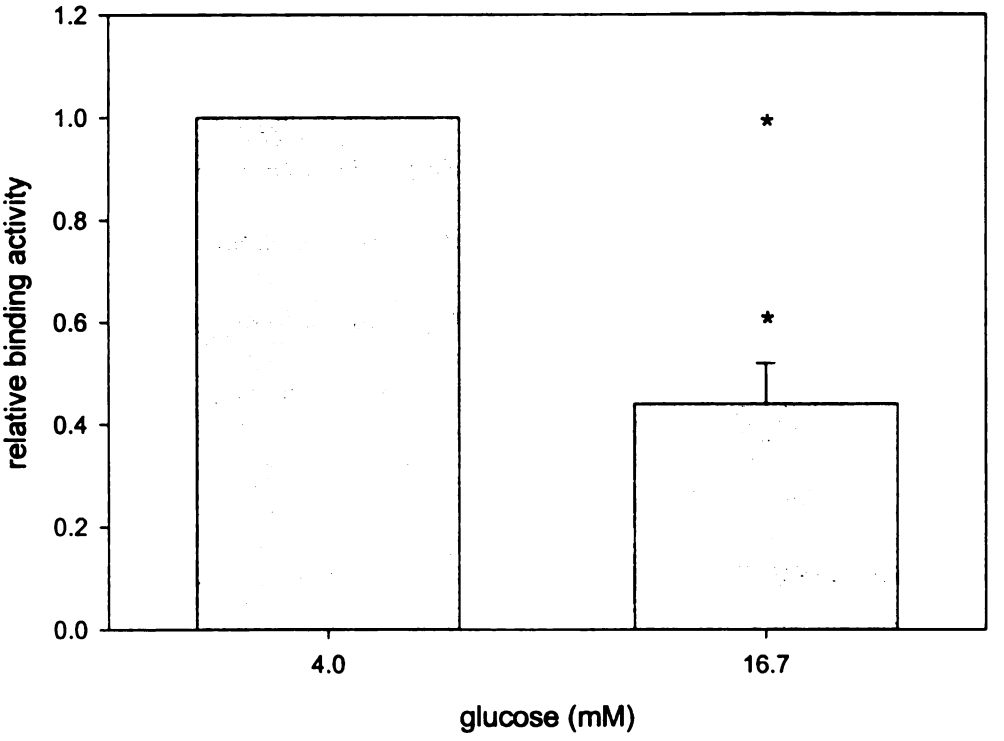


Figure 26. High levels of glucose reduce binding activity of the Za1 complex. High levels of glucose reduce binding activity of the slowest migrating complex of the Za probe. Data shown are the mean ± SE of four independent experiments (n=4). Asterisk indicates that 16.7 mM glucose significantly decreases binding activity compared to 4.0 mM glucose ($p < 0.0002$).

contains the A5/Core (-317 to –309) and the E4 element (-300 to –294) (Fig. 20), was labeled and analyzed for DNA/protein binding activity with nuclear extracts from INS-1 cells incubated in 4.0 or 16.7 mM glucose for 48 hrs. As shown in Figure 27, multiple DNA/protein complexes bound to the Xa probe. Importantly, a slow migrating complex, Xa1, was markedly decreased in nuclear extracts derived from cells cultured in 16.7 mM glucose (Fig. 27, compared lanes 2 and 3). Addition of 100-fold molar excess of unlabeled Xa probe competed with Xa1 binding, demonstrating that the binding of this complex was specific to the Xa probe.

To determine whether the glucose-sensitive complex, Xa1, requires the A5/Core and/ or E4 element contained in the Xa probe, nuclear factor binding activity to a probe that contained only the A5/Core sequence, termed the Xb probe (-323 to –303), was examined (Fig. 20). As shown in Figure 28, only a single slow-migrating complex formed with the Xb probe. Importantly, this complex was markedly lower in nuclear extracts from cells incubated in 16.7 mM glucose (Fig. 28, lanes 2 and 3). This complex was readily competed by excess unlabeled Xb probe (Fig. 28, lanes 4 and 5), indicating the specificity of this complex. A mutant A5/Core probe, where the third base-pair of A5 and two overlapping sequences of A5/Core were mutated (ATG to CGT), did not compete for binding (Fig. 28, lanes 6 and 7), suggesting that this glucose-sensitive complex binds directly to A5 and Core elements. Since the A5 element is similar in sequence to a Pdx-1 binding site (TAAT), and Pdx-1 binding activity is regulated by glucose, it was necessary to examine whether Pdx-1 was part of this complex. Competition analysis with 100-fold molar excess of unlabeled A1 and A3 probes, which

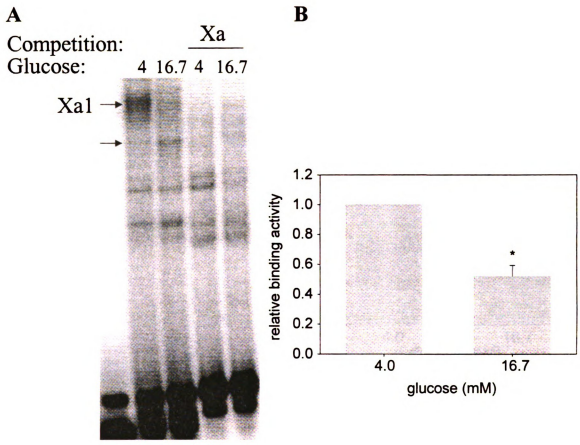


Figure 27. Glucose-sensitive complex binds to the Xa element region. A. Equal amounts of nuclear extracts from INS-1 cells incubated in media containing 4.0 or 16.7 mM glucose were analyzed for binding to a ^{32}P-labeled Xa probe. Lanes 4 and 5 contain 100-fold molar excess unlabeled Xa probe. Shown is a representative experiment of 4 independent experiments. B. Summary of the Xa1 binding activity of 4 independent experiments with values of the mean ± SE (n=4). Asterisk indicates that 16.7 mM glucose significantly decreases binding activity compared to 4.0 mM glucose ($p < 0.004$).

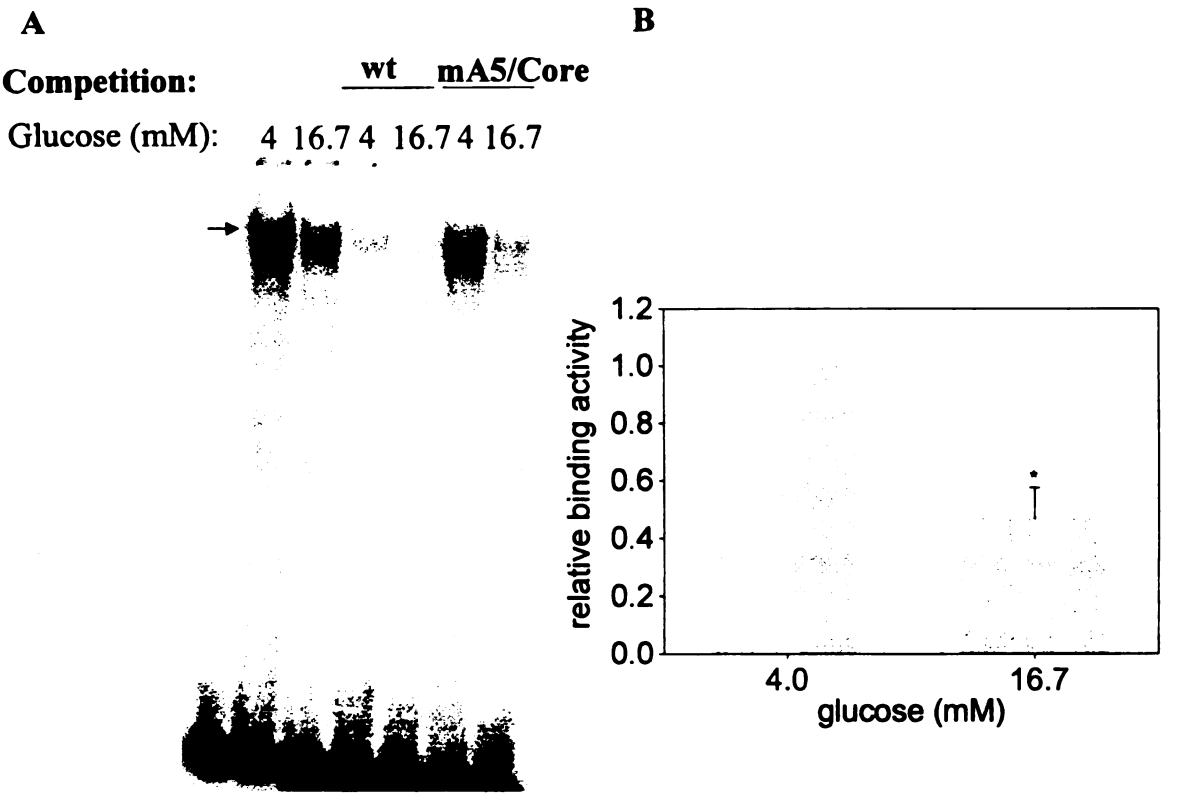


Figure 28. Glucose-sensitive complex binds to the A5/Core sequence (Xb probe). A. Equal amounts of nuclear extracts from INS-1 cells incubated in media containing 4.0 or 16.7 mM glucose were analyzed for binding to a ^{32}P-labeled Xb probe (A5/Core). Lanes 4 and 5 contain 100 -fold unlabeled Xb probe. Lanes 6 and 7 contain 100 -fold molar excess unlabeled mutated Xb probe (mA5/CORE). B. Summary of binding activity of three independent experiments with values of the mean ± SE (n=3). Asterisk indicates that 16.7 mM glucose significantly decreases binding activity compared to 4.0 mM glucose ($p < 0.002$).

have Pdx-1 binding sites, did not diminish binding of the glucose-sensitive complex (Fig. 29, lanes 6, 7 and 8, 9, respectively). Furthermore, addition of a Pdx-1 antibody did not supershift or disrupt the glucose-sensitive complex (Fig. 29, lanes 10 and 11). It was also necessary to determine whether Nkx6.1 or Pax6 formed part of the glucose-sensitive complex, since both transcription factors bind to A-like elements (76, 292). Addition of a Nkx6.1 or Pax-6 antibody did not supershift or disrupt binding of the glucose-sensitive complex (Fig. 29, lanes 12 and 13, data not shown for Pax-6). A positive control that demonstrates that high levels of glucose reduce binding activity of Pdx-1 to the A1 element, and that an antibody that recognizes Pdx-1 supershift Pdx-1 are shown in Figure 30. High levels of glucose reduce A1 binding activity (Fig. 30 comparing lanes 2 and 3). Pdx-1 antibody recognizes Pdx-1 within this complex (Fig. 30 comparing lanes 10 and 11).

Because the Xa element contains the A5/Core and the E4 element, it was necessary to determine whether there was any glucose-regulated binding activity to the E4 element (-300 to –294). Nuclear extracts from INS-1 cells incubated in 4.0 or 16.7 mM glucose were analyzed for the ability to bind to the E4 probe (-306 to –288) (Fig. 20). A single-complex bound the E4 probe and its binding activity was not decreased in nuclear extracts from cells incubated in 16.7 mM glucose (Fig. 31, lanes 2 and 3). This complex was specific for the E4 probe because, a 100-fold molar excess of unlabeled E4 probe effectively competed for the complex (Fig. 31, lanes 4 and 5).

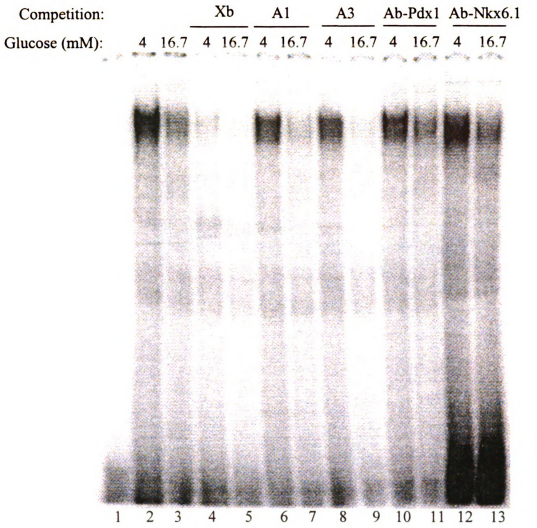


Figure 29. Neither Pdx1 nor Nkx6.1 form part of the complex that binds to the A5/Core element. Equal amounts of nuclear extracts from INS-1 cells incubated in media containing 4.0 or 16.7 mM glucose were analyzed for binding to a ^{32}P-labeled Xb probe (A5/Core). Competition with 100-fold molar excess of unlabeled Xb probe (wt) (lanes 4 and 5), A1 probe (lanes 6 and 7), A2 probe (lanes 8 and 9), and the addition of 1µl anti-Pdx-1 antibody (lanes 10 and 11) or the 1 µl anti-Nkx6.1 antibody (lanes 12 and 13). Shown is a representative experiment of three independent experiments where as the super-shift experiments were only performed once.

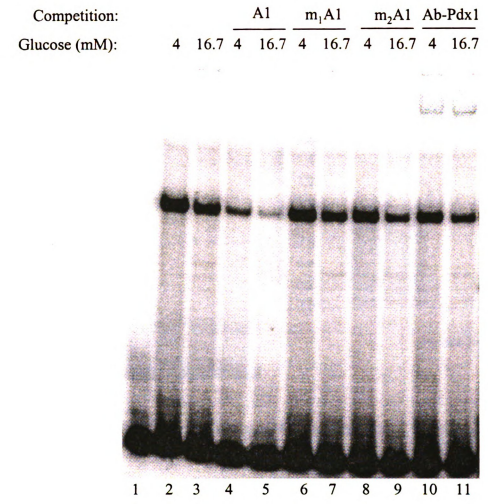


Figure 30. High levels of glucose reduce binding activity to the A1 element. Equal amounts of nuclear extracts from INS-1 cells cultured in 4.0 or 16.7 mM glucose were analyzed for binding to a ^{32}P-labeled A1 probe. Lanes 4 and 5 contain 100-fold molar unlabeled A1 probe. Lanes 6-7, and 8-9 contain 100-fold molar excess unlabeled mutated A1 probe, m1A1 and m2A1, respectively. Shown is a representative experiment of 2 independent experiments.

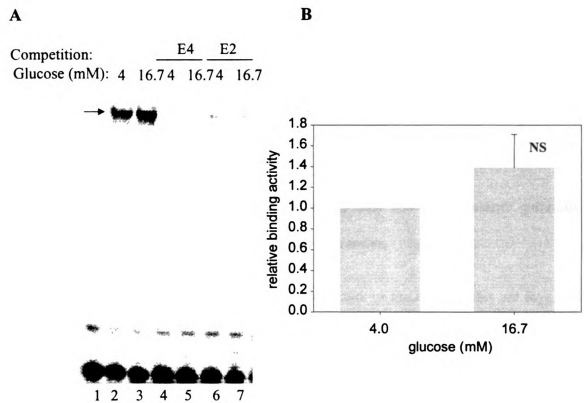


Figure 31. Glucose does not decrease binding activity of the complex that binds to the E4 element. A. Equal amounts of nuclear extracts from INS-1 cells incubated in media containing 4.0 or 16.7 mM glucose were analyzed for binding to a ^{32}P-labeled E4 probe. Competition analysis with 100-fold molar excess of unlabeled E4 probe (wt) (lanes 4 and 5) or FAR-box (rat E2 element) (lanes 6 and 7). B. Summary of binding activity of three independent experiments with values of the mean ± SE ($p < 0.17$). NS, not significantly different.

An unlabeled Far-box probe also competed for this complex (Fig. 31, lanes 6 and 7), indicating that the E12/E47/Beta2 heterodimer possibly binds to this site. Overall, these data suggest that there are three insulin promoter elements, A5/Core, palindrome, and E3 element, through which high levels of glucose might mediate insulin promoter repression.

3.6 Mutation of the A5/Core, the palindrome, and the E3 elements partially diminished glucose-induced insulin promoter repression

To determine whether the A5/Core, the palindrome and/ or the E3 elements are involved in mediating glucose-induced insulin promoter repression, site-specific mutations of all these elements were made within the full length of insulin promoter, INS(-327)CAT. The INS(-327)CAT vector containing mutations in the palindrome, in both the palindrome and the A5/Core, or in both the palindrome and the E3 element were termed mPalINS(-327)CAT, mPal/A5/CoreINS(-327)CAT, and mPal/E3INS(-327)CAT, respectively. Mutations of all three elements, A5/Core, palindrome, and E3 within the INS(-327)CAT vector was termed mA5/Core/Pal/E3INS(-327)CAT. All mutated sequences of these elements are shown in Table 3. As expected, INS(-327)CAT expression was repressed 86.6 % in INS-1 cells cultured in 16.7 mM glucose compared to 4.0 mM glucose (Table 4). Mutations of the palindrome sequence as well as mutations of both the palindrome and the A5/Core element did not prevent glucose-induced repression of the insulin promoter activity. The mPalINS(-327)CAT and the mA5/Core/Pal INS(-327)CAT expressions were repressed 80.0 % and 74.8 %, respectively. The repression of

Name	4.0 mM glucose CAT activity	16.7 mM glucose CAT activity	% repression CAT activity
INS(-327)CAT	127.2 ± 6.2	17.0 ± 2.5	86.6 *
mPal INS(-327)CAT	210.8 ± 9.5	42.8 ± 4.2	80.0 *
mA5/Core/Pal INS(-327)CAT	182.2 ± 27.3	45.9 ± 9.4	74.8 *
mPal/E3 INS(-327)CAT	96.2 ± 7.4	34.5 ± 2.7	64.2 *
mA5/Core/Pal/E3 INS(-327)CAT	37.7 ± 6.8	20.1 ± 4.0	46.6 *

Table 4. Mutations of all three A5/Core, palindrome, and E3 elements partially diminished glucose-induced repression of the INS(-327)CAT. INS-1 cells were transfected with insulin promoter vectors. The cells were then cultured in 4.0 or 16.7 mM glucose for 48 hrs. mPal represents mutated palindrome sequence. mA5/Core/Pal represents mutated A5/Core and palindrome sequences. mPal/E3 represents mutated palindrome and E3 sequences. mA5/Core/Pal/E3 represents mutated A5/Core, palindrome, and E3 sequences. Values are the means ± SE of three individual experiments (n=3). Asterisk indicates that values at 16.7 mM glucose were significantly lower than at 4.0 mM glucose of given vector ($p < 0.04$). Two experiments were performed by Diana Ye, Department of Pharmacology, Michigan State University.

the insulin promoter activity by high glucose concentrations was slightly prevented when both the palindrome and E3 elements were mutated. The mPal/E3INS(-327)CAT expression was repressed by 64.2 % in cells cultured in 16.7 mM glucose compared to 4.0 mM glucose. Importantly, mutation of all three elements, mA5/Core/Pal/E3 INS(-327)CAT expression was only 46.6 % repressed in cells cultured in 16.7 mM, compared to 4.0 mM glucose. These results strongly suggest that the palindrome, A5/Core, and E3 elements mediate part of glucose-induced insulin promoter suppression, and that A1, A3, and C1 elements might mediate the rest of the suppression.

4. Discussion

In vitro and *in vivo* models of hyperglycemia have shown that insulin gene expression is suppressed when β-cells are chronically exposed to high glucose concentrations (110, 118). Reduced expression and/ or binding activity of transcription factors including Pdx-1 and C1 activator (2, 3, 113, 121, 126) and increased expression of C/EBPβ and c-Myc (85, 274) have been suggested to mediate some of the glucose-induced changes in promoter activity. To gain a more in-depth understanding of the role of Pdx-1 and C1 activator on these events we examined a more simplified insulin promoter vector that contains sequences from –230 to + 30, termed INS(-230)CAT. High levels of glucose led to only a minor repression of INS(-230)CAT expression compared to the large repression observed on the full length promoter INS(-327)CAT. Mutations of the Pdx-1 and/ or C1 activator binding sites abolished the suppression found in the INS(-230)CAT expression. These results indicate that Pdx-1 and C1 activator are involved,

but have a minor role in glucose-induced repression of the INS(-327)CAT expression, and suggest that additional mechanisms mediate insulin promoter repression. The participation of additional mechanisms are also illustrated in INS-1 cells transfected with a Pdx-1 expression vector, because Pdx-1does not prevent decreased insulin promoter activity in cells cultured in high levels of glucose (L.K. Olson, unpublished data). Studies in HIT-T15 cells, a Syrian hamster insulinoma, also demonstrated that over-expression of Pdx-1 alone is not sufficient for full recover of promoter activity (122). Although loss of C1 activator binding likely plays an important role in the downregulation of insulin promoter activity, its exact role has not been determined since it has only recently been cloned. Overall, these studies suggest that there are other mechanisms by which exposure of β-cells to high levels of glucose represses insulin promoter activity and that these mechanisms may mediate repression through sequences upstream of the –230 insulin promoter.

Deletion of human insulin promoter sequences from –327 to –279 reduced insulin promoter repression by glucose to similar levels observed by deleting sequences from –327 to –230. Deleting sequences upstream of –279 removes the A5/Core element, the E4 element, and disrupts the palindrome sequence by dividing it in half. It has been shown that palindrome sequences can bind transcription factors such as glucocorticoid receptor (GC) and estrogen receptor (293), suggesting that similar transcription factors can bind to the insulin palindrome sequence. In fact, Goodman *et al.* (90) demonstrated that two complexes from nuclear extracts of two insulinoma cells lines, HIT-T15 and RIN-m5F, bind to the human insulin NRE and that a consensus sequence GC oligonucleotide efficiently competes for binding of these complexes. Interestingly, the insulin

palindrome sequence is adjacent to the E3 element, and Sander *et al.* (89) have proposed that transcription factors that bind to these adjacent sites might interact and that their binding activity might be dependent upon one another. Therefore, disruption of the palindrome may disrupt binding to the E3 element. If this is true, deletion of sequences upstream of –279, which disrupts the palindrome, could be predicted to have similar effects as deleting sequences upstream –261 that removes the E3 element. This hypothesis is in agreement with our deletion studies that showed that deleting upstream sequences from –279 had similar glucose-induced promoter repression as the reporter gene with upstream sequences of –261 deleted. Overall, these results demonstrate that high levels of glucose suppress insulin promoter through sequences from –327 to –261. The A5/Core, E4, palindrome, and E3 elements are the most likely sites to mediate glucose-induced repression of the insulin promoter.

The Z minienhancer, which contains the palindrome sequence and the E3 element, was markedly repressed in INS-1 cells cultured in 16.7 mM glucose, compared to 4.0 mM glucose. In contrast to our results, Sander *et al.* (89) demonstrated that the Z minienhancer was a potent transcriptional enhancer in primary fetal islet cells cultured in 16.0 mM glucose compared to 2.0 mM glucose. The inconsistency with Sander *et al.* (89) results of the Z minienhancer in fetal islets and our results in INS-1 cells might be explained by the biphasic glucose regulation of this minienhancer. Sander *et al.* (89) only cultured fetal islet cells in 2.0 or 16.0 mM glucose, potentially missing the large glucose induction of the Z minienhancer activity that occurred in INS-1 cells cultured in 2.0 to 6.0 mM glucose. Therefore, the Z minienhancer could have shown repression in fetal islet cells incubated in 16.7 mM glucose compared to 4.0 or 6.0 mM glucose as was

observed in INS-1 cells. In fact, if we just compare the Z minienhancer activity in INS-1 cells incubated in 2 mM vs. 16.7 mM glucose we could have concluded that the Z element was induced by glucose as observed in fetal islets. Our studies showed that most of the suppression of the Z minienhancer activity occurred at glucose concentrations between 6.0 to 16.7 mM glucose and these concentrations were not examined in fetal islet cells.

Sander *et al.* (89) reported that the Z minienhancer activity is repressed compared to controls (pFOXCAT) in two β-cell lines transformed by Simian virus large T-antigen. The authors suggested that the Z minienhancer activity is repressed in these transformed cell lines because they have a high proliferation rate, in contrast to differentiated fetal islets, which do not divide rapidly in culture. In contrast, we found that INS-1 cells, which are transformed by gamma irradiation, express the Z minienhancer at high levels compared to the pFOXCAT control. Our results suggest that rapid proliferation does not block the Z minienhancer activity, but that T-antigen transformation may block Z minienhancer activity. Nevertheless, INS-1 cells do show enhanced proliferation when cultured at high levels of glucose concentrations, but this is unlikely to account for changes in promoter activity because G1 arrested INS-1 cells still had decreased insulin mRNA expression when cultured in elevated glucose (126). Consistently with this idea, Clark *et al.* (88) reported binding of a possible repressor, Oct-1, to the NRE that is within the Z minienhancer in HIT-T15 cells. Further experiments need to be performed to elucidate differences between Z minienhancer activity in different tumor β-cell lines.

Mobility-shift assays performed with nuclear extracts from INS-1 cells demonstrated a binding complex to the palindrome and E3a element, termed the Za2 complex, and its binding activity is decreased from 4.0 to 16.7 mM glucose. In contrast, nuclear extracts from fetal islet cells revealed a binding complex to the same region that its binding activity is increased from 2.0 to 16.7 mM glucose (89). The difference between the binding activities of these complexes could be explained by the biphasic glucose regulation of the Z minienhancer activity. In INS-1 cells, Z minienhancer activity markedly increases from 2.0 to 6.0 mM glucose, and slightly increases from 2.0 to 16.7 mM glucose, suggesting that binding activity to the palindrome and E3a element most likely increases as observed in nuclear extracts from fetal islet. Additional experiments need to be performed to elucidate Z element binding activity of nuclear extracts from INS-1 cells and fetal islets.

Mobility-shift assays performed with nuclear extracts from INS-1 cells also demonstrated that there are two different glucose-sensitive complexes that bind to the Z element: one complex binds to the palindrome and E3a-E3b elements, and a different complex binds to the palindrome and only the E3a element. These results indicate that removing the E3b element from the palindrome-E3a-E3b sequences shows different complexes, suggesting that the palindrome and the E3 element work in a synergistic manner, as suggested by Sander *et al.* (89). As discuss above, these results also agree with our truncation analysis that showed that deleting sequences upstream from –279, which disrupts the palindrome, had the same glucose-induced repression as deletion of sequences upstream from –269. Overall, these results highly suggest that transcription factors that bind to the palindrome, E3a, and E3b elements interact as has been shown for

other transcription factors that regulate insulin promoter activity. For example, E47/E12, which binds to the E1 element, interacts with Pdx-1 that binds to A1 element (64), and C1 activator that binds to the C1 element (289).

Activity of the X minienhancer, which contains the A5/Core and the E4 element, also showed a biphasic glucose regulation. Mobility-shift assays demonstrated a glucose-sensitive complex that binds to the A5/Core element, we termed Xa1. Interestingly, this complex appears to be distinct from other known transcription factors that bind to A-like elements within insulin promoter. These results suggest a novel transcription factor that binds to the A5 element and whose binding activity is diminished by high levels of glucose. In contrast to the A5/Core, palindrome, and E3 elements, binding activity to the E4 element showed a glucose-stimulated binding complex. Interestingly, this E4 element behaves like the E2 element in that glucose enhances its binding activity (88, 123). In addition, the E2 element (Far-box element) from the rat I insulin promoter competed for the complex bound to E4 element. E12/E47 and Beta2 transcription factors bind to E elements, but super-shift mobility assays demonstrated that Beta2 did not bind to E4 element (data not shown). These results indicate that transcription factors that recognize E2 rat element may bind and regulate the E4 element.

DNA binding activity analyses of sequences from –327 to –242 of insulin promoter demonstrated three major glucose-sensitive elements, the A5/Core, the palindrome, and the E3. Site-specific mutations of all these three elements in the –327 insulin promoter markedly diminished the ability of glucose to repress the insulin promoter activity. These results demonstrate that, in fact, glucose mediates insulin promoter repression, in part, through the A5/Core, palindrome, and E3 elements. These

results indicate that additional mechanism besides changes in Pdx-1, C1 activator, c-Myc, and C/EBPβ mediate glucose repression of insulin promoter.

Indeed, recently experiments have demonstrated that hyperglycemia is causing a more global change in gene expression and phenotype. For example, it has been demonstrated that hyperglycemia impairs glucose-induced insulin secretion by altering gene expression involved in glucose metabolism and insulin secretion. In Zucker diabetic fatty rats and 90 % pancreatectomized rats, genes involved in glucose metabolism including glucose transporter Glut2, glucokinase, mitocondrial glycerol-3-phosphate dehydrogenase, and pyruvate carboxylase decreased progressively as hyperglycemia increased (100, 101). The same phenomenon was observed in genes involved in insulin secretion such as potassium channel Kir6.2 and voltage dependent Ca^{2+} channel (4, 101, 113). In 90 % pancreatectomized rats there is also an increase in glucose-6-phosphatase, hexokinase I, and lactate dehydrogenase A (LDH) gene expression, which normally are expressed at low levels in islets (4). The implications of the changes of these genes lead to reduced glucose-induce insulin secretion. The changes in other genes are not clearly understood, but one can postulate that increased LDH expression, which produces lactate from pyruvate by using NADH, depletes NADH levels, which are required for mitochondrial ATP production.

Expression of important genes involved in β-cell differentiation is also altered by chronic hyperglycemia. In the 90 % pancreatectomy rat diabetic model, gene expression of hepatic nuclear factor (HNF) -3β, 4α, 1α, paired-box homeodomain-6 (Pax-6), NK-homeodomain factor Nkx6.1, Beta2, and Pdx-1 were gradually decreased with increasing

levels of hyperglycemia (4). One can postulate that these global alterations of gene expression may contribute not only to decrease glucose-stimulated insulin secretion but also to the degeneration of β-cells that eventually leads to β-cell apoptosis.

Chronic hyperglycemia induces β-cell dysfunction by altering a wide-ranging of gene expression involved in glucose metabolism, glucose-induced insulin secretion, and β-cell differentiation. Because of this broad effect of hyperglycemia in β-cell function, it is not surprising that hyperglycemia mediates insulin promoter repression through several promoter elements. The mechanisms by which high levels of glucose induce such diverse changes in β-cell function are poorly understood.

VI. CONCLUSIONS AND FUTURE STUDIES

Pancreatic β-cells control blood glucose levels in a narrow range by responding to even small changes of blood glucose, which is the major secretagogue, and secrete the right amount of insulin into circulation. Failure of β-cells to secrete insulin leads to the development of diabetes. Type II diabetes is characterized by a combination of insulin resistance and altered glucose-induced insulin secretion (294). As a compensatory mechanism for insulin resistance, β-cell hypertrophy and changes in expression of key glucose metabolism enzymes are observed that lead to an increase in insulin secretion. Hypertrophy is found in 90 % pancreatectomized rats (4), in Zucker diabetic fatty rats (188), and after 96 hr of glucose infusion (295). Hypertrophy and hyperinsulinimea have been associated with increased c-Myc and hexokinase I expression, respectively (4).

Failure of the β-cell to compensate for insulin resistance because of inability to further increase β-cell mass leads to increased blood glucose levels and this has been associated with β-cell dysfunction. An important characteristic of β-cell dysfunction is decreased glucose-induced insulin secretion, which further exacerbates the diabetic condition. Decreased glucose stimulation of insulin secretion has been extensively studied in the last decade and is correlated with alterations in genes involved in glucose metabolism and insulin secretion. Recent studies in the 90 % pancreatectomized rat model demonstrated downregulation of a variety of genes involved in glucose metabolism such as insulin, Glut2, gluocokinase, mitochondrial glycerol phosphate dehydrogenase, and pyruvate carboxylase (4). Genes involved in insulin release are also

downregulated such as the potassium channel Kir6.2, the voltage-dependent calcium channel α 1D, and calcium ATPase channel (SERCA3) (4).

The pathogenesis of glucose-induced insulin secretion during diabetes involves a variety of genes that lead to downregulation of insulin gene expression. Decreased binding activity and/ or expression of Pdx-1 and C1 activator (3, 126), (113) are associated with downregulation of insulin gene expression. This thesis demonstrated that additional mechanisms are involved in glucose-induced repression of insulin promoter activity. Functional analysis and mobility-shift assays showed that the A5/Core, palindrome, and E3 elements are also involved in the insulin promoter repression. Some of the mechanisms involved in the repression of insulin promoter activity are correlated with increased c-Myc and C/EBPβ expression (86, 274). In addition, we demonstrated that hyperglycemia might repress insulin promoter activity through the JNK pathway. In line with our results, Kaneto *et al.* (8) showed that inhibition of JNK by over-expressing a dominant negative form of JNK in H_2O_2 treated isolated rat islets prevented repression of insulin mRNA and recovered Pdx-1 binding activity. Since high levels of glucose increase ROS, it is likely that elevated ROS levels by hyperglycemia activates JNK and subsequently represses insulin promoter activity.

Another potential mechanism by which high levels of glucose might repress insulin promoter activity is through chronic exposure of β-cell to insulin. As mentioned above, during the development of diabetes high levels of insulin are secreted to compensate for insulin resistance. Insulin is known to induce insulin gene expression (197) and one can postulate that chronic exposure of β-cells to insulin might repress insulin gene expression. In our experiments, INS-1 cells are cultured in 11.1 mM glucose

prior to treatment with 16.7 mM glucose for 48 hrs, which induces insulin secretion. Therefore, in addition to chronic exposure of cells to high levels of glucose, cells are also chronically exposed to insulin, which might contribute to the reduction of insulin promoter activity.

Repression of glucose-induced insulin secretion by hyperglycemia is also accompanied by changes of important genes involved in β-cell differentiation. In the 90 % pancreatectomized rat model a decrease in Pdx-1, Nkx6.1, Pax6, Beta2, HNF1α, HNF4α, and HNF3β gene expression is observed (4). Recently, it was demonstrated that expression of genes that are normally suppressed in the β-cell are increased in correlation with increasing glucose levels (131). Increased expression of lactate dehydrogenase-1 (LDHA), glucose-6-phosphatase, fructose-1,6-bisphosphatase, peroxisome proliferator-activated receptor (PPAR γ), and uncoupling protein 2 (UCP-2) were observed in the 90 % pancreatectomized rat model (131). The role of the alteration of all these gene's expressions is not understood yet and it is not known if changes in mRNA levels are correlated with changes in protein activities. The important point is that there is a global change in gene expression that can be involved in β-cell adaptation to hyperglycemia or β-cell decompensation due to hyperglycemia.

Hyperlipidemia has been also associated with detrimental effects on β-cell function (296). Chronic exposure of β-cell to free fatty acids (FA) increases basal insulin release but decreases glucose-induced insulin secretion (reviewed in Ref. 294). Hyperlipidemia also alters glucose-induced insulin secretion in the presence of high levels of glucose, which has been correlated with decreased insulin gene expression and furthermore with decreased Pdx-1 expression (133, 134, 295). Recently, *in vivo* studies

demonstrated that hyperglycemia is required for hyperlipidemia to alter insulin gene expression (139). This was demonstrated in the Zucker diabetic fatty rat, which contains high content of triglycerides (TG) in both blood and islets. When the rats were treated with a reagent, phlorizin, that lowers blood glucose levels and islet TG content without affecting blood TG content, clearly shows that hyperglycemia is associated with increased TG islet content and decreased insulin mRNA levels (139). The role of hyperlipidemia in the repression of glucose-induced insulin secretion is not clear since the 90 % pancreatectomized rat model does not have high levels of TG and there is repression of insulin secretion. Nevertheless, the possibility can not be ruled out that chronic exposure of β-cells to TG contributes to decrease glucose-induced insulin secretion when the diabetic state is more severe.

The biochemical mechanisms by which hyperglycemia alters β-cell function are not well understood. One potential hypothesis is that there is a generalized mechanism, which activates other pathways that lead to the global changes in gene expression. A likely mechanism is reactive oxygen species since hyperglycemia can generate reactive oxygen species by several mechanisms including glucose autoxidation, glycation, advanced glycation end-products, and induction of the polyol pathway (reviewed in Ref. 8). In a non-hyperglycemic state, superoxide molecules are produced by mitochondria during the process of oxidative phosphorylation. During hyperglycemia there is an increased in superoxide levels, which can inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (299) that subsequently leads to accumulation of glycolytic metabolites such as glyceraldehyde-3-phosphate (GAP) and fructose-6-phosphate (F6P). GAPDH can lead to de novo synthesis of diacylglycerol (DG), which leads to activation

of protein kinase C that is known to be activated in diabetic conditions (177). Accumulation of F6P leads to the activation of the hexosamine pathway, which increases modification of proteins by *O*-linked *N*-acetylglucosamine (300). The AGE pathway can also be increased by GAP (299). One can hypothesize that inactivation of superoxide production by the michondria could inhibit activation of PKC, hexosamine, polyol, and AGE pathways, that subsequently meliorate detrimental effects in diabetes. In fact, Nishikawa *et al.* (301) demonstrated that inhibition of electron transport in the mitochondria prevents activation of PKC, formation of AGE, and sorbitol accumulation in vascular endothial cells exposed to 30 mM glucose. These results indicate that there is a generalize mechanism, production of ROS, that leads to the activation of several pathways involved in the pathogenesis of diabetes.

The biochemical mechanisms by which hyperglycemia alters glucose-induced insulin secretion involves, at least in part, generation of chronic oxidative stress (7, 157, 158, 170). Interestingly, it has been proposed that hyperlipidemia induces β-cell dysfunction by inducing oxidative stress (141). One can postulate that hyperglycemia and hyperlipidemia intercommunicate to induce β-cell dysfunction mediated by generation of oxidative stress. Recently, it was shown that in the 90 % pancreatectomized rat model there is an increase of antioxidant gene expression such as heme oxygenase-1 and glutathione peroxide (173). These results indicate that pancreatic islets induce a protective mechanism against the production of ROS during hyperglycemia.

Future studies

We demonstrated that JNK activity increased in INS-1 cells cultured in high levels of glucose and that over-expression of JNK repressed insulin promoter activity in cells cultured in low levels of glucose. These results led to the correlation that glucose-induced insulin promoter repression is mediated by activation of JNK. However, it was not possible to directly demonstrate that JNK mediated the insulin promoter repression. Recently, Kaneto *et al.* (8) demonstrated that isolated islets treated with H_2O_2 repress insulin expression through activation of JNK. Prevention of H_2O_2 –induced activation of JNK and repression of insulin gene expression was demonstrated by infecting isolated rat islets with an adenovirus expressing a dominant negative JNK (DN-JNK). The DN-JNK is a kinase-inactive JNK where the ATP-binding site is mutated. DN-JNK is phosphorylated, but it is not able to phosphorylate downstream targets. Therefore, it may be feasible to prevent glucose-induced activation of JNK in INS-1 cells over-expressing this DN-JNK. I hypothesize that inhibition of endogenous JNK by over-expressing DN-JNK may partially prevent repression of insulin gene promoter activity in INS-1 cells cultured in high levels of glucose.

Recent experiments demonstrated that a generalize pathway, production of superoxide through the mitochondrial electron transport chain, may be involved in the complications of diabetes (296, 298). Superoxide production induces activation of PKC, polyol, hexosamine, and AGE pathways. It would be interesting to test whether inhibition of the production of superoxide by the mitochondrial electron chain pathway prevents hyperglycemia-induced insulin promoter activity in INS-1 cells. These experiments could be achieved by over-expressing a manganese superoxide dismutase

gene, which is specific for the superoxides produced by mitochondria. I hypothesize that over-expression of Mn-superoxide dismutase would prevent repression of insulin promoter activity in INS-1 cells cultured in high levels of glucose.

Studies have demonstrated that reactive oxygen species are involved in glucose-induced repression of insulin secretion (7, 158). Tanaka *et al.* (7) demonstrated that treatment of HIT-T15 cells with NAC and AG, two antioxidants, prevent repression of insulin promoter and Pdx-1 binding activity by hyperglycemia. Previous experiments performed in our laboratory demonstrated that INS-1 cells cultured with these two antioxidants did not prevent insulin promoter repression, in contrast promoter activity was further repressed. In these experiments, INS-1 cells were treated with higher concentrations of NAC and AG than used by Tanaka *et al.* (7). The high concentrations of NAC and AG could have had non-specific effects and therefore hindered the effects of ROS on insulin promoter activity. Future experiments investigating the role of ROS in insulin promoter repression should be designed with a dose concentration response of NAC and AG on promoter activity. I hypothesize that ROS is involved in glucose-induced repression of insulin promoter activity, thus NAC and AG should partially prevent some of this insulin promoter repression.

It was very interesting to discover that high levels of glucose regulated the distal insulin promoter region. Deletion of sequences from –327 to –292 increased basal insulin promoter activity, suggesting that there is some basal repression within this small region. This area contains the A5/Core and E4 element. To our knowledge no one has demonstrated any transcription factor binding activity in this area. Our studies demonstrated that proteins that bind to the rat I E2 element recognize the human E4

element. Beta2 and E12/47 transcription factors heterodimerize and bind to the E2 elements. Mobility-shift assays demonstrated that Beta2 does not form part of the complex that binds to the E4 element. I hypothesize that E12/E47 transcription factors might bind to the E4 element and possibly other helix-loop-helix transcription factors as well. Electrophoretic mobility shift assays with an antibody specific for Pdx-1 or Pax6 demonstrated that the complex that binds to the A5/Core element does not contain Pdx-1 and Pax6, and the human A1 element does not compete for binding with this complex. Overall, future experiments can be design to identify transcription factors that bind to the A5/Core and E4 element. It will be interesting to investigate whether the new transcription factors that bind to A5/Core element also bind to the other A-elements, and whether these transcription factors are regulated by glucose.

Hyperglycemia represses insulin promoter activity through several elements including the palindrome. It has been demonstrated that estrogen and glucocorticoid receptors can bind to palindromic sequences (293). In fact, glucocorticoid receptors can bind to the human palindrome sequence (90). Interestingly, glucocorticoids are known to induce insulin resistance that is usually accompanied with β-cell dysfunction (302, 303). HIT-T15 cells and isolated β-cells treated with dexamethasone, a synthetic glucocorticoid, induces a decrease in insulin secretion and mRNA levels (304). Treatment of HIT-T15 cells with dexamethasone for 48 to 72 hrs leads repression of a reporter vector driven by the human palindrome and E3 element, known as the NRE element (90). It is also interesting that β-cells are the only islet cells that contain glucocorticoid receptors (305). I hypothesize that the repression of insulin promoter activity observed during hyperglycemia might be mediated, in part, by glucocorticoids.

To test whether glucocorticoids mediate repression of insulin gene expression, diabetic animal models such as the Zucker diabetic fatty rat can be treated with a glucocorticoid antagonist like RU-486 from 6 through 12 weeks of age when the animal becomes diabetic. Then insulin secretion and insulin mRNA can be measured.

Sander *et al.* (89) showed that high levels of glucose increase binding activity to the Za element from extract of fetal and adult pancreatic islets. In contrast, nuclear extracts from INS-1 cells showed that glucose reduced binding activity to the Za element. The difference between these experiments is the basal glucose concentration. It is important to clarify these results by comparing binding activity to the Z element from extracts of fetal islet and INS-1 cultured at the same glucose concentrations. I hypothesize that Z element-binding activity is repressed when both INS-cells and fetal islets are incubated in 16.7 mM glucose compared to 4.0 or 6.0 mM glucose.

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