EFFECTS OF GLUCOSE AND FREE FATTY ACIDS ON ERK1/2 IN PANCREATIC β-CELLS

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**Dedicated to**
**my parents and the love of my life, Kim Jones**

# EFFECTS OF GLUCOSE AND FREE FATTY ACIDS ON ERK1/2 IN PANCREATIC β-CELLS

**by**

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**DISSERTATION**

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Donald Ervin Arnette, Jr., Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2005

Supervising Professor: Melanie Cobb, Ph.D.

Diabetes is a growing problem in the United States. There is a growing occurrence of obesity in the United States, which directly adds to the incidence and occurrence of diabetes. Our lab has previously showed that the extracellular signal-regulated protein kinases (ERKs) 1 and 2 are activated by elevated concentrations of glucose and are involved in insulin transcription. In this study we examine the mechanism by which glucose induces ERK1/2 activity. The effects of free fatty acids on ERK1/2 activity were also examined. In this study a rat insulinoma cell line was used.

The rat insulinoma cell line INS-1 is a model system which is commonly used to represent pancreatic β-cells.

Elevated glucose activates ERK1/2 in INS-1 cells. With the use of several pharmacological agents which interrupt calcium signaling, it was concluded that calcium signaling is involved in glucose-induced ERK1/2 activation. Calmodulin and the calmodulin-dependent phosphatase calcineurin were determined to be required for glucose-induced ERK1/2 activity as well as glucagons-like peptide induced ERK1/2 activity. This activation was also shown to require the release of calcium from intracellular stores.

It has been well documented that free fatty acids (FFAs) have negative effects on pancreatic β-cells. FFAs have been shown to decrease insulin secretion. The effects of FFAs on ERK1/2 activity were also examined. Chronic exposure of FFAs causes constitutive activation of ERK1/2 in INS-1 cells. This constitutive activity of ERK1/2 was determined to be protein kinase C (PKC) independent. The FFA-induced ERK1/2 activity resulted in a ERK1/2 nuclear localization pattern that is dramatically different from the usual pattern of ERK1/2 nuclear localization. It has been previously shown that ERK1/2 phosphorylate insulin transcription factors that are required for maximal glucose-induced insulin transcription. This altered ERK1/2 nuclear localization may affect the phosphorylation of these transcription factors, which might explain how chronic exposure to FFAs inhibits insulin transcription.

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**Publications presented in this dissertation**

1. Arnette D, Gibson TB, Lawrence MC, January B, Khoo S, McGlynn K, Vanderbilt CA, Cobb MH. Regulation of ERK1 and ERK2 by glucose and peptide hormones in pancreatic beta cells. J Biol Chem. 2003 Aug 29;278(35):32517-25.

**List of Figures and Tables**

**List of Abbreviations**

| | |
|---|---|
| ACC | acetyl coA carboxylase |
| ADP | adenosine 5’ diphosphate |
| AMPK | AMP-activated protein kinase |
| ATP | adenosine 5’ triphosphate |
| bHLH | basic helix-loop-helix |
| BSA | bovine serum albumin |
| P1P2 | phosphatidylinositol-4,5-bisphosphate |
| cAMP | cyclic AMP |
| bZIP | basic region leucine zipper |
| CaMK | Calmodulin-dependent-protein-kinase |
| CBP | CREB binding protein |
| CPT1 | carnitine-palmitoyl-transferase-1 |
| CRE | cAMP response element |
| CSA | cyclosporin |
| DAG | diacylglycerol |
| DM | diabetes mellitus |
| ERK | extracellular singal-regulated protein kinase |
| FFA | free fatty acid |
| G6P | glucose-6-phosphate |
| GLP-1 | glucagon like peptide |
| GLUT | glucose transporter |
| GSIS | glucose-stimulated insulin secretion |

| | |
|---|---|
| IDDM | insulin-dependent diabetes mellitus |
| IRS | insulin receptor substrate |
| JNK | c-Jun amino-terminal kinase |
| KRBH | Krebs-Ringer-bicarbonate-HEPES |
| MAP | mitogen-activated protein |
| MEK | MAPK/ERK kinase |
| NIDDM | noninsulin-dependent diabetes mellitus |
| PAA | phenylacetic acid |
| PAGE | polyacrylamide gel electrophoresis |
| PI3K | phosphatidylinositol-3'-kinase |
| PKA | protein kinase A |
| PMA | phorbol 12-myristrate 13-acetate |
| SDS | sodium dodecyl sulfate |
| ZDF | Zucker diabetic fatty |

## Chapter 1: General Overview

### DIABETES

The control of blood glucose levels, glucose homeostasis, requires the function of many organs, which include the liver, muscle, adipose tissue, the pancreas and specific regions of the brain (1). Dysfunction in any of these organs can lead to diabetes and/or lipid disorders. Diabetes mellitus (DM) comprises a group of common metabolic disorders that share the phenotype of hyperglycemia. There are several types of diabetes which are caused by an interaction of genetics, environmental factors, and lifestyle (2). Type 2 diabetes is characterized by a defect in the secretion of insulin by the pancreatic islet β cells in response to elevated blood glucose concentrations, and a decrease in the action of insulin on its target tissues (1). The metabolic dysregulation associated with diabetes causes secondary pathophysiologic changes in multiple organ systems (2). These defects aggravate each other so that the secretion of insulin is not adequate to override the insulin resistance in target tissues; at this point hyperglycemia is constant even in a fasted state (1).

### Classification and Pathogenesis

Diabetes mellitus is classified based on the pathogenic process which leads to hyperglycemia, instead of earlier criteria such as age of onset or type of therapy. There are two broad categories of DM, type 1 and type 2.

Autoimmune β-cell destruction which leads to insulin deficiency is characteristic of type 1. Type 2 is a group of disorders which are characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production (2). A period of abnormal glucose homeostasis, known as impaired fasting glucose, precedes type 2 DM (2). There are two features which are currently used to classify DM that are different from the previous criteria. First, the terms insulin-dependent diabetes mellitus (IDDM) and noninsulin-dependent diabetes mellitus (NIDDM) are no longer used. Because many individuals with type 2 DM eventually require insulin treatment to maintain euglycemia, the term NIDDM was confusing. Secondly, age is no longer considered for classification. In most cases type 1 DM occurs prior to the age of 30; however, an autoimmune, β-cell destruction can occur at any age. The risk for type 2 DM increase with age, but it also occurs in children, especially in obese adolescents (2).

The prevalence of DM is increasing rapidly. Although the incidence of both type 1 and type 2 is increasing, type 2, which is often triggered by obesity, is expected to rise more rapidly in the future due to increased obesity and reduced activity levels in the population. DM occurs in approximately 5% of the worldwide population and it is estimated that within the next ten years it will have increased by greater than 40% to afflict 220 million people (3).

## GLUCOSE

Blood glucose concentration is tightly regulated by a homeostatic mechanism that involves several different tissues. In the absorptive state, these tissues increase their rate of glucose utilization; while during the fasting state, they release glucose into the blood from glycogen stores or newly synthesized glucose from gluconeogenic precursors (4). These mechanisms of glucose utilization and production are regulated by hormones, mostly insulin and glucagon, as well glucocorticoids, growth hormone, epinephrine, glucagon like peptde (GLP-1) and gut hormones. Pancreatic β-cells can sense variations in blood glucose concentration and secrete insulin to prevent hyperglycemia. The autonomic nervous system impacts insulin secretion, innervating pancreatic islets. The autonomic nervous system is also involved in the control of glucose utilization by muscle, glucose production by the liver, and food intake or energy storage and expenditure (4). Glucose is also the key regulator of insulin secretion by the pancreatic β-cell.

### Glucose Transporters

Glucose is a six-carbon sugar, which requires the presence of specific transporter proteins for transport through biological membranes since it is a polar molecule. These transporter proteins, glucose transporters (GLUTg), catalyze the facilitative transport of glucose across the plasma membrane. Five transporters, GLUT1 to GLUT5, were initially identified and extensively characterized (5-7).

Glucose transporters have twelve transmembrane domains. Their functions are distinguished by hexose specificity, affinity for substrate, and differential regulation of their expression by metabolic conditions, hormones and glucose. The number of glucose transporters recognized increased through bioinformatic searches of gene databases for sequences related to GLUT1 to GLUT5 (8). The most recently discovered sequences are referred to as GLUT6 to GLUT12.

Glucose transporters are divided into three classes. Class I transporters consists of GLUT1 to GLUT4; class II consists of GLUT5, GLUT7, GLUT9, and GLUT11; class III contains GLUT6, GLUT8, GLUT10, and GLUT12. GLUT1, GLUT3, and GLUT4 are high affinity glucose transporters (Km for glucose <1mM). GLUT2 has a low affinity for glucose ( Km for glucose is ~17 mM) (7). The GLUT2 transporter is found in hepatocytes, pancreatic β-cells, small intestine, and the kidney (9). Because normal circulating concentrations of glucose range between 3.9 and 5.6 mM, the rate of transport through this transporter will be directly proportional to the glucose concentration. Therefore, when circulating levels of glucose are high (postprandial state), there is a net flux of glucose into the hepatocytes of the liver and pancreatic β-cells. In contrast, intracellular concentrations of free glucose increase in hepatocytes, not through uptake, but as a result of glycogenesis and glucogenesis during periods of low circulating glucose (fasting) (10). Pancreatic β-cells must be highly sensitive to changes in plasma glucose concentrations to appropriately regulate the amount of insulin secreted.

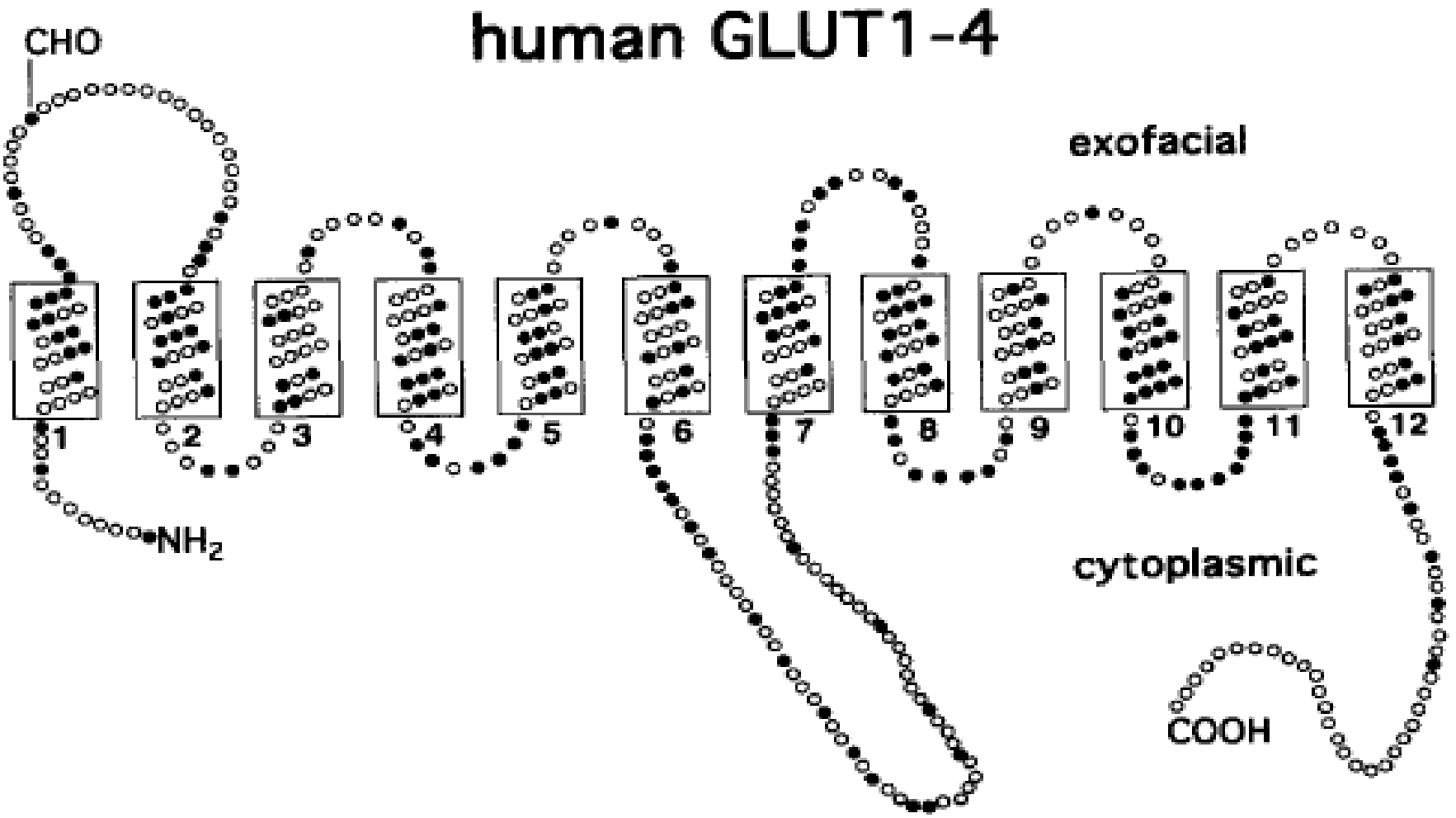



Figure 1.1 Predicted secondary structure of GLUT1. Olson and Pessin 1996 (Ref 10)

Therefore, the high Km of the Glut2 glucose transporter guarantees that the transporter is not saturated at physiologic levels of glucose, and glucose flux will remain proportional to the plasma glucose concentration.

**Glucose Sensing**

Metabolic signals mediate glucose regulation of insulin secretion (11-14). As mentioned previously, pancreatic β-cells express GLUT2 glucose transporters, which permit rapid glucose uptake, regardless of the extracellular sugar concentrations (15). At low glucose levels (<2.5 mM), little substrate is phosphorylated in β-cells, probably because of low expression in these cells of the high affinity hexokinase isoforms (hexokinase I, hexokinase II or hexokinase III) (16). It is likely that nonendocrine cells contaminate preparations of isolated islets and cause relatively high measurements of hexokinase(17). Unlike β-cells, most other cell types express hexokinase at sufficiently high levels to keep intracellular glucose-6-phosphate (G6P) concentrations constant, and maintain the basal ATP production necessary for maintaining cellular functions even at low extracellular glucose concentrations (18). In these cells, the main forces for metabolic flux are the rate of glucose transport and/or G6P consumption, keeping the energy charge of the cells constant regardless of the extracellular glucose levels (18;19). In pancreatic β-cells, however, the absence or very low expression of hexokinase results in a glycolytic flux and low ATP/ADP levels at glucose levels < 2.5mM, levels essential for maintaining a low basal rate of insulin

secretion. The low affinity glucokinase (hexokinase IV) phosphorylates glucose in β-cells when plasma glucose levels > 2.5mM (16).

**Glucose-Stimulated Insulin Secretion**

The secretion of insulin by pancreatic β-cells is modulated by various nutrients, neurotransmitters, and peptide hormones. Glucose is the only nutrient secretagogue which alone is capable of promoting the release of insulin at concentrations within its physiological range. However, many additional nutrients, including fatty acids, and amino acids also influence insulin secretion. Therefore, the islets of Langerhans can be viewed as a fuel sensor, which simultaneously integrates the signals of many nutrients and modulators to secrete insulin according to the need of an organism (20). Glucose-stimulated insulin secretion (GSIS) is initiated by glucose uptake through GLUT2 and its phosphorylation by glucokinase yielding G6P. G6P is then metabolized and leads to the activation of mitochondrial metabolism to generate ATP (4). A rise in the cytoplasmic ATP/ADP ratio causes closure of an ATP-sensitive $K^+$ channel. This channel consists of two subunits. One is Sur1, the receptor for certain oral hypoglycemics (sulfonylureas, meglitinides), which are used therapeutically to induce insulin secretion by causing closure of the $K^+$ channel. The other protein is Kir6.2, the channel itself and a target of therapeutic $K^+$ channel openers like diazoxide, which inhibits insulin secretion from insulinomas.

Closure of this $K^+$ channel induces β-cell membrane depolarization, which opens voltage-dependent calcium channels, primarily L-type, which leads to an influx of calcium. The influx of $Ca^+$ then triggers exocytosis of the insulin granules.

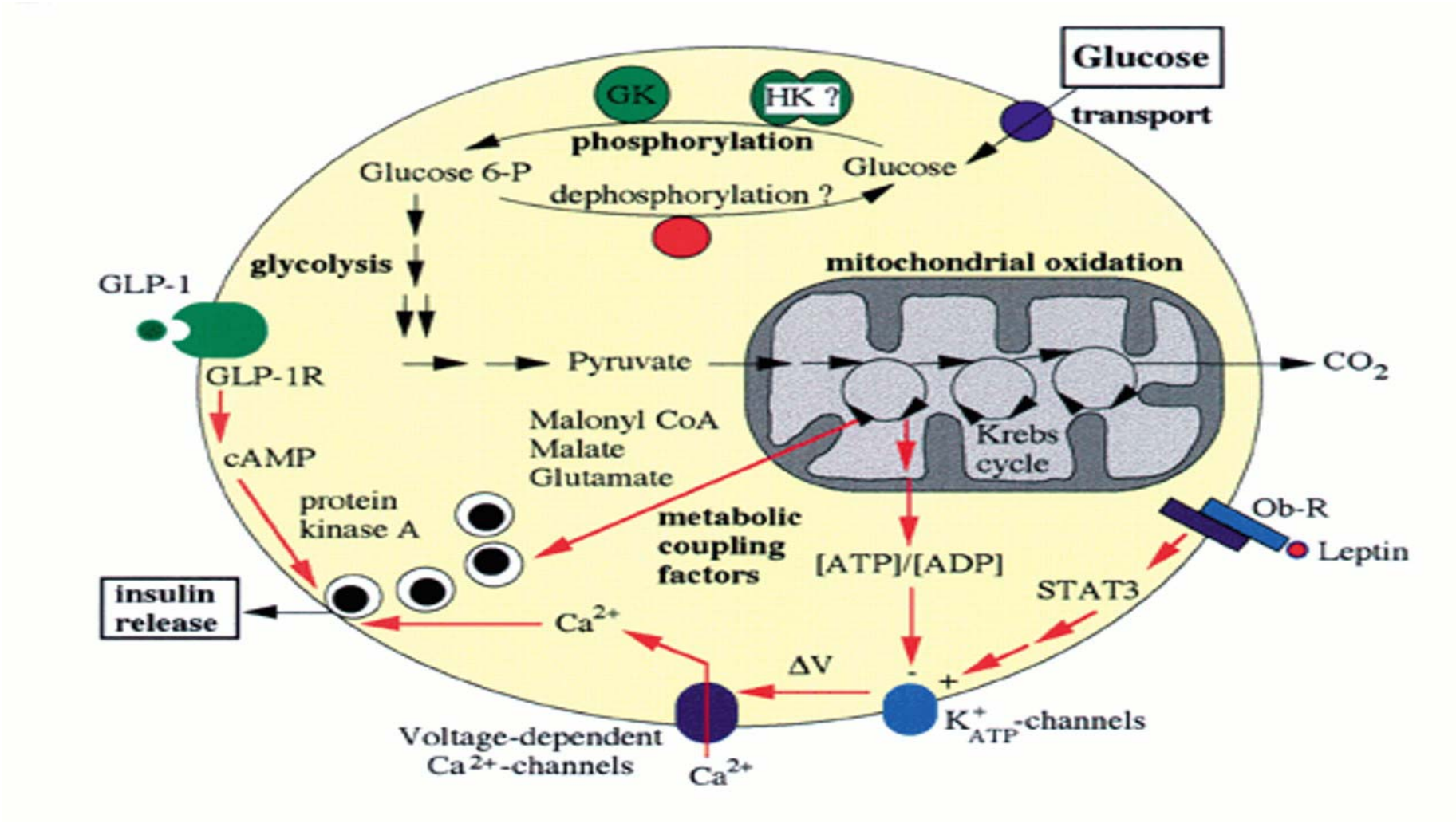



Figure 1.2 Metabolic hypothesis of glucose stimulated insulin release from β-cell. Schuit *et al* 2001 (Ref 16)

When plasma glucose rises, nuclear glucokinase is released into the cytosol (21;22). The crucial role of glucokinase is to modulate metabolic signaling in β-cells. This has been concluded from three observations. First, β-cells with lower glucokinase expression have lower GSIS than those with a higher expression (23). Second, reduced expression of glucokinase or functional enzyme activity is associated with an inadequate insulin secretory response to glucose in individuals with maturity-onset diabetes of the young (24). Third, the disruption of the glucokinase gene in β-cells causes altered GSIS in mice (25;26). Glucose phosphorylation represents the rate-controlling step in GSIS as indicated by similar dose-dependent increases in glucose phosphorylation, utilization, oxidation and GSIS (27).

**INSULIN**

The mammalian insulin gene is expressed exclusively in the β-cell of the pancreas. Insulin regulates energy metabolism after there is a rise in plasma glucose levels. Insulin signaling initiates multiple events. Insulin can increase the storage of glucose, amino acids, and fat. These are related by promoting the synthesis of glycogen, protein, and lipids (28). Insulin can also inhibit the amount of glucose that is produced by the liver.

**Action**

After insulin is secreted into the circulation, it binds to its receptor in target sites. This binding of insulin to its receptor stimulates intrinsic tyrosine kinase activity, which leads to receptor autophosphorylation and the recruitment of intracellular signaling molecules, known as insulin receptor substrates (IRSs). These and other adaptor proteins initiate a complex cascade of phosphorylation and dephosphorylation reactions which result in the activation of multiple insulin signaling pathways.

One of the key pathways that insulin activates is the phosphatidylinositol-3'-kinase (PI3K) pathway. The autophosphorylation of the insulin receptor leads to the translocation and activation of PI3K. This translocation and activation is through an interaction of the PI3K SH2 domain with phosphotyrosine residues of the receptor and IRS1. PI3K then phosphorylates its substrate, PtdIns(4,5)$P_2$. This phosphorylation results in the production of the second messenger PtdIns(3,4,5)$P_3$ which recruits PDK-1, Akt/PKB, and some atypical PKC isoforms through their PH domains. Phosphorylation by PDK-1 then activates Akt and the previously mentioned PKC isoforms (29). The activation of this pathway is one of the essential insulin signaling mechanisms, which

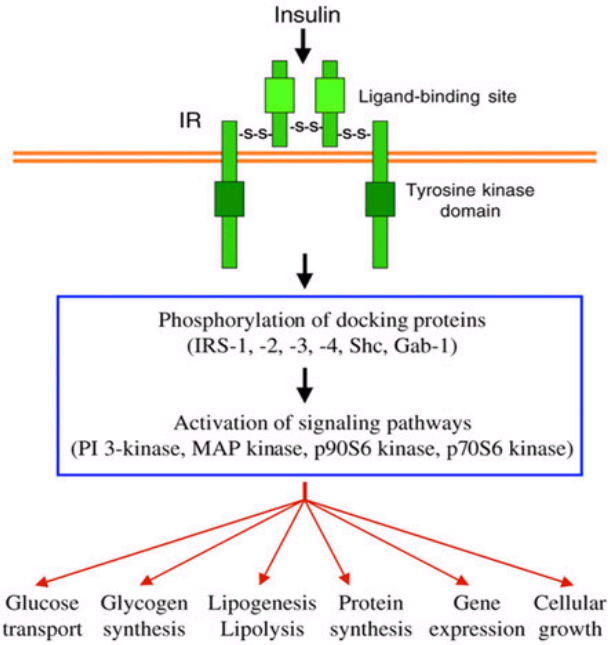



Fig 1.3: Insulin signaling through the insulin receptor leads to multiple effects in target tissues. (Baudry EMBO Rep. 2002 Apr;3(4):323-8)

stimulates the translocation of GLUT4 to the cell surface. This translocation event is crucial for glucose uptake by skeletal muscle and fat. Glycogen synthesis, protein synthesis, and lipogenesis are all enhanced by insulin signaling pathways(2).

**Insulin gene transcription**

The insulin gene is located on chromosome 11p15.5 in humans and consists of three exons and two introns (30). The insulin promoter is defined as the sequences immediately upstream of the transcription start site. The insulin gene promoter and the transcription factors binding to this region largely determine cell-specific expression of the insulin gene in the β-cell. Mutagenesis studies have revealed multiple sequences along the promoter that contribute to its overall activity, by functioning as binding sites for sequence specific DNA-binding proteins found in the nucleus of the β-cell.

The insulin promoter is a large complex formed by many different proteins (31). Insulin genes in different species share a number of conserved DNA motifs in their 5' flanking region, implying that they may be regulated by similar trans-acting factors. E, A, and C1/RIPE3b elements seem to play major roles in the expression of the insulin gene (32). Neither the E element nor the A element by themselves have any significant transcriptional activity in β-cells; but together, they can increase activity of a linked promoter in a β-cell specific fashion (33).

The E elements are binding sites for protein dimers formed by heterodimerization between two members of the family of basic helix-loop-helix (bHLH) proteins: a ubiquitous class A bHLH protein (products of the E2A gene, E12, E47, and E2/5) and a cell type-specific bHLH protein (neuroD1/BETA2) (34-37). The A elements can bind many homeodomain family proteins; the most common in β-cells is PDX1 also known as IPF-1,STF-1 and IUF-1 (38-41). PDX1 and the bHLH proteins E47 and BETA2 physically interact through their DNA binding domains. In vivo, there are many other proteins in the nucleus that can impact the bHLH-PDX1 interaction. DNA binding by the PDX1/bHLH complex may increase the occupancy of the E-A sites on the insulin promoter. However, this is not sufficient. Maximum transcriptional activation requires interaction with the basal transcriptional machinery (31). The grouping of transcription factors on DNA creates complexes of protein interaction sites that recruit or stabilize binding of the RNA polymerase II transcription initiation complex (42). The DNA-bound transcription factors are linked with the basal transcription machinery; this process is enhanced by non-DNA binding coactivators (31). The increased stability provided by co-activators plays an essential role in the formation and the function of the insulin promoter transcription activation complex.

PDX1 is not the only protein that can bind to the A elements and cooperate with the bHLH dimer to activate insulin transcription. There are several homeodomain transcription factors that can bind to the A elements. Most

of these cannot synergize with the bHLH dimer, but two can. These are LIM-homeodomain proteins Lmx1.1 and Lmx1.2 and their interactions with the bHLH dimer lead to increased transcriptional activity relative to the PDX1-bHLH combination (31).

Because Lmx1 proteins can synergize with the bHLH dimer, this suggests that other proteins in the β-cell nucleus may be able to substitute for PDX1 in the transcription activation complex on the insulin promoter (31). A study suggested that PDX1, E2A and BETA2 genes are not required for insulin gene transcription. Mice homozygous null for E2A transcribe the insulin gene at normal rates (43). Mice with BETA2 mutations show that this bHLH protein is important for the maintenance of β-cells, but is not necessary for insulin gene transcription (44). These results suggest that other bHLH proteins expressed in the pancreas can substitute for BETA2. Although PDX1 is not necessary for insulin transcription, PDX1 can activate the transcription of many genes involved in glucose sensing and metabolism like GLUT2 and glucokinase (45). It has also been shown that humans heterozygous for inactivating mutations of PDX1 are predisposed to diabetes, and other mutations in human PDX1 are associated with type 2 diabetes and MODY(46;47).

**Glucose and Insulin Transcription**

The formation of an effective transcription complex depends on all of the interacting proteins; therefore, the net activity of the promoter can be regulated by altering the concentration or function of any one protein in the complex. Glucose

appears to regulate insulin gene transcription through its effects on many of the proteins in the transactivating complex (48-50). Glucose causes an increase in PDX1 binding to the A element (40;41) and it increases the binding of the bHLH dimer to the E elements. This increased binding to the E element cooperates with the activated PDX1 bound at the A site to increase insulin gene transcription (31).

Elevated glucose has multiple effects on the β-cell, one of which is an increase in β-cell cyclic AMP (cAMP) levels (51). cAMP can induce insulin gene transcription through the cAMP response elements (CREs) (52), the human insulin gene has four CREs sites (53). This induction of transcription occurs mainly through binding of the CRE binding protein (CREB), a transcription factor and member of the basic region leucine zipper (bZIP) family, to CREs(52).

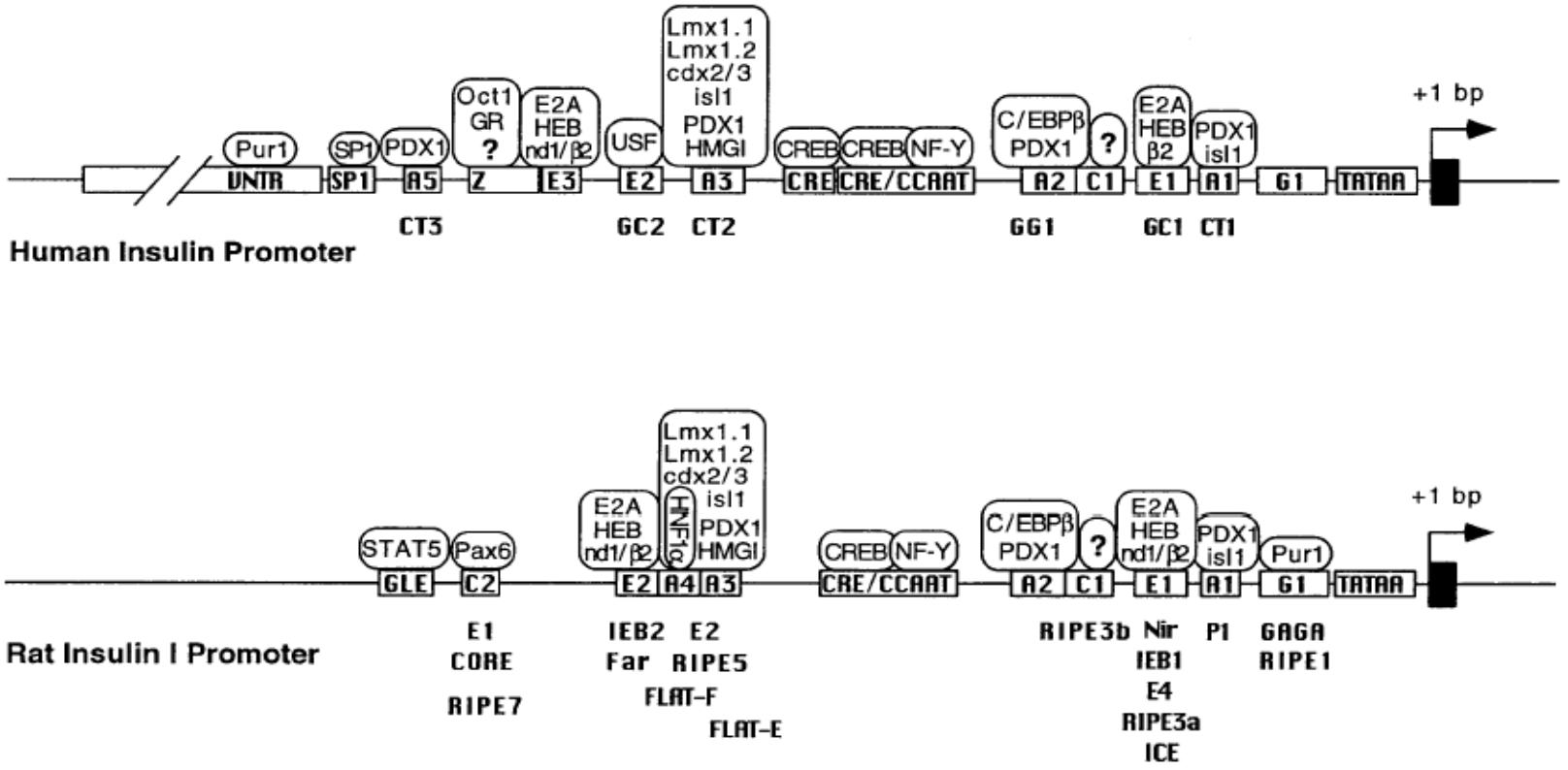



Figure 1.4 Human and rat insulin I gene promoters with known sequence elements and binding factors. K. Ohneda *et al* 2000 (Ref 31)

This mechanism involves the activation of protein kinase A (PKA). The phosphorylated CREB interacts with the basal transcriptional machinery by binding the co-activator CREB binding protein (CBP) which activates transcription (54). The hormones glucagon and GLP-1 can also cause an intracellular rise in cAMP levels (55;56) which can stimulate insulin gene transcription

The nuclei of β-cells contain many distinct protein complexes that bind specific sequences within the insulin promoter. Accumulating evidence suggests that no single protein complex alone accounts for cell-specific expression. It is more likely that the combination of transcription factors in the β-cell is unique, and that their interactions are needed to activate transcription of the insulin gene.

**β-CELL DYSFUNCTION**

Chronic hyperglycemia and hyperlipidemia can have negative effects on β-cell function. In the extreme these become glucotoxicity and lipotoxicity. Over time glucotoxicity and lipotoxicity contribute to the progressive deterioration of glucose homeostasis characteristic of type 2 diabetes. The mechanisms of glucotoxicity involve several transcription factors and are in part mediated by the generation of chronic oxidative stress. Lipotoxicity is probably mediated by the accumulation of a cytosolic signals derived from the fatty acid esterification pathway (57).

**Glucotoxicity**

There are many reports that suggest that chronic hyperglycemia impairs GSIS and insulin gene expression (58). Negative effects of chronic hyperglycemia on β-cell function include three distinct events: glucose desensitization, β-cell exhaustion, and glucose toxicity. Glucose desensitization is impairment of the insulin secretory response to further glucose stimulation of β-cells following chronic exposure to high concentrations of glucose (59;60). Exhaustion of the β-cell is the depletion of releasable intracellular insulin after prolonged exposure to a secretagogue. The term glucotoxicity describes the slow and progressively irreversible effects of chronic hyperglycemia on β-cell function, which occurs after prolonged exposure to elevated glucose. In addition to causing functional changes, chronic hyperglycemia can also decrease β-cell mass by inducing apoptosis (61;62).

The decreased activity of two β-cell transcription factors, PDX1 (63) and the activator of RIPE3b (MafA) is associated with the impairment of insulin gene expression after prolonged exposure to elevated glucose levels (63). Increased expression of the insulin gene transcriptional repressor CCAAT/enhancer binding protein β has also been reported (64). The generation of chronic oxidative stress is thought to play a role in the biochemical mechanism of glucotoxicity (65-67). In the insulin secreting cell, HIT-T15, the generation of reactive oxygen species in the presence of a reducing sugar or chronic exposure to elevated glucose leads to decreased transcription of the insulin gene (66;67). Chronic exposure of isolated

islets to elevated glucose levels leads to impaired β-cell function and apoptosis (68). In some instances this impairment can be prevented with the administration of antioxidants. Antioxidants have also been shown to normalize plasma glucose levels and restore insulin secretion, insulin content, and insulin mRNA levels in Zucker diabetic fatty (ZDF) rats (67). These findings support the hypothesis that glucotoxicity is at least partially mediated by chronic oxidative stress. Another proposed mechanism includes decreases in β-cell signaling molecules such as intracellular malonyl-CoA, which causes an increase in fatty acid oxidation (69). Glucotoxicity is also responsible for increasing insulin resistance, in part through the inhibition of the glucose transporter system (70).

**Lipotoxicity**

Lipotoxicity has many similarities to glucotoxicity. Lipotoxicity serves as a link between obesity and insulin resistance. Fatty acids, which are essential fuels for β-cells in the normal state, become toxic when chronically present in excessive levels. Prolonged exposure of β-cells to fatty acids increases basal insulin release but inhibits GSIS (71). The elevation of fatty acids has also been shown to cause insulin resistance in individuals with and without type 2 diabetes, mostly through the inhibition of insulin- stimulated glucose uptake in muscle cells (72).

There are several views on the exact mechanism of lipotoxicity in the pancreatic β-cell. One is based on the idea that prolonged exposure to fatty acids

is associated with alterations in lipid metabolism and changes in glucose metabolism (73). This model indicates that the simultaneous presence of elevated glucose and fatty acids results in accumulation of cytosolic citrate, the precursor of malonyl-CoA. Malonyl-CoA inhibits carnitine-palmitoyl-transferase-1 (CPT1), the enzyme that transports fatty acids into the mitochondrion (57). Long term inhibition of CPT1 results in cytosolic accumulation long chain fatty acyl CoAs, which are thought to mediate the negative effects of chronically elevated fatty acids (73). This model suggests that glucose concentration plays a critical role in the effects of fatty acids.

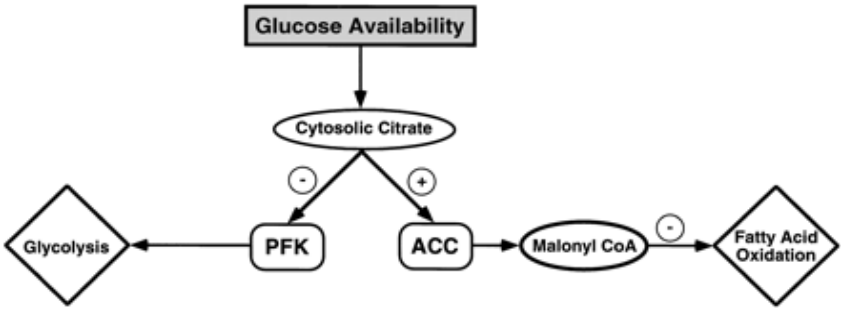



Figure 1.5 Regulation of glycolysis and fatty acid oxidation by cytosolic citrate and phosphofructokinase (PFK). Ruderman *Am J Physiol. 1999 Jan;276(1 Pt 1):E1-E18*

Another study has focused on the same pathway. This study also showed that chronically elevated fatty acids blunt the responsiveness of β-cells to glucose stimulation, but suggest that this disturbance begins with the downregulation of the expression of acetyl-CoA carboxylase, an enzyme involved in the formation of malonyl-CoA (74). Both studies agree that chronically elevated fatty acids

alter the normal functions of malonyl-CoA, which disrupts the rate of fatty acid oxidation in the β-cell.

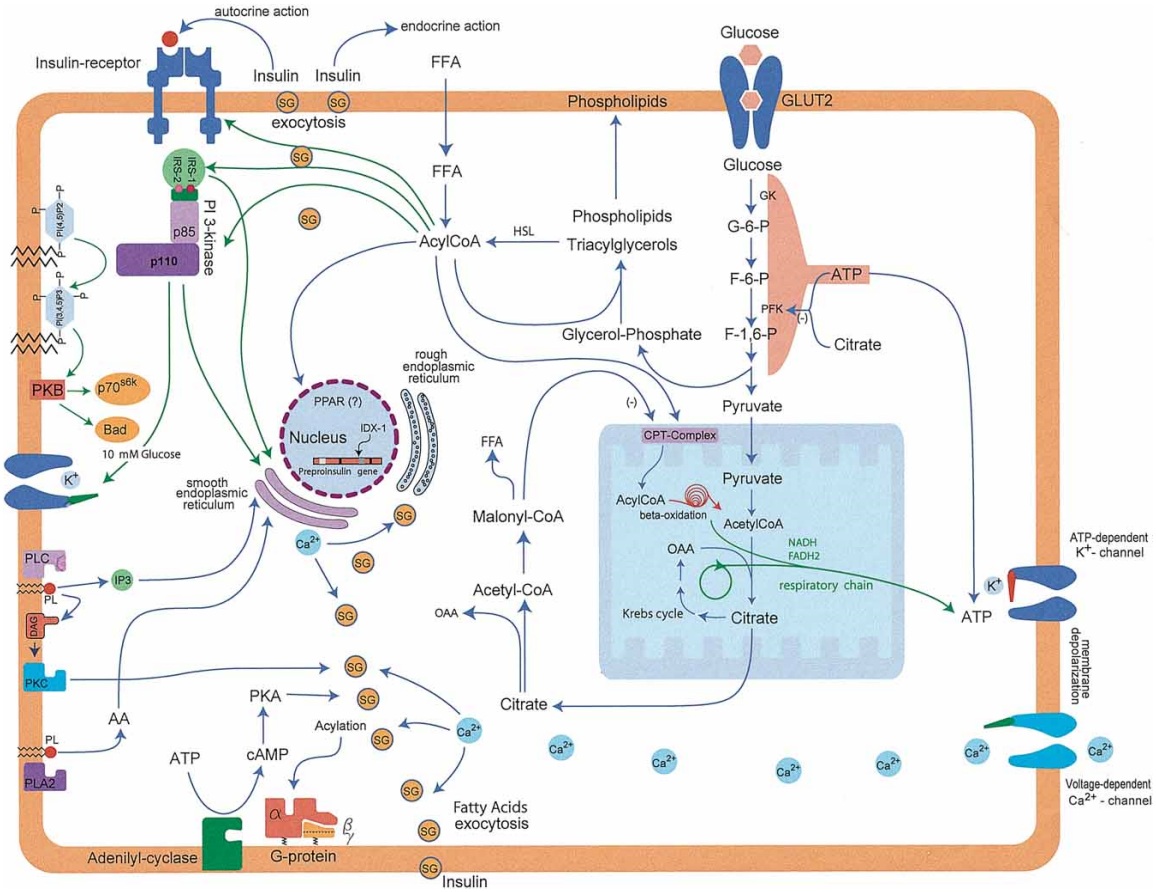



Figure 1.6 A summary of the possible effects of fatty acids on insulin secretion in pancreatic β-cells. Blue lines indicate established effects or interactions, whereas green lines indicate events, where mechanisms are not fully established.

Haber *J Cell Physiol. 2003 Jan;194(1):1-12.*

**MAP kinases and insulin gene transcription**

Glucose activates a wide variety of signaling pathways and second messengers to regulate β-cell function. Among these are the mitogen-activated protein (MAP) kinases ERK1 and ERK2 (75;76). MAP kinases are components of highly conserved kinase cascades important for transmitting extracellular information to coordinate cellular responses. ERK1 and ERK2 have been studied more than any other MAP kinases, and they have been implicated in many signal transduction pathways. ERK1 and ERK2 have a nearly universal involvement in responses to ligands and cooperate with other signal transducers to change cellular functions (77-79). ERK1 and ERK2 are generally associated with cell proliferation; however, they modulate many other responses including activities of membrane enzymes such as phospholipase A2 (80), cell attachment and motility (77;78), and gene transcription, (79). ERK1 and ERK2 are also highly expressed in the nervous system in post mitotic-neurons. A number of studies suggest that they make significant contributions to long term potentiation (81-83). Some targets of ERK1/2 in pancreatic β-cells have been identified and are discussed below.

**Glucose-induced insulin transcription**

Glucose activates the MAP kinases ERK1 and ERK2 in islet-derived cells (75;76). ERK1/2 are activated over the same glucose concentrations, from 2 to 10

mM, as those that elicit insulin secretion. Potentiators of insulin secretion potentiate ERK1/2 activation. ERK1/2 are not required for GSIS, but glucose increases the amount and activity of ERK1/2 in the nucleus of β-cells, suggesting a role for ERK1/2 in insulin gene transcription. Similar to GSIS, glucose-induced ERK1/2 activation requires glucose metabolism. Our lab previously showed that ERK1/2 regulates proinsulin mRNA levels in INS-1 pancreatic β-cells as well as transcription from the rat insulin I promoter in β-cells. We also showed that ERK1/2 regulate insulin gene transcription by a glucose-responsive element. ERK sensitive motifs are contained within the glucose responsive element of the insulin promoter, E2A3/4(-247/-198bp) (84). When ERK1/2 is blocked with either K52R ERK2 (kinase inactive) or PD98059, a selective, cell-permeable inhibitor of MAP kinase kinase (MEK 1/2), glucose dependent transcriptional activity of E2A3/4 in INS-1 cells is suppressed.

**ERK1/2 and factors regulating gene transcription**

We have begun to elucidate the signaling mechanism of glucose induction of the glucose responsive element. Our studies examined the ability of MAP kinase family members to phosphorylate several factors known to bind to the A and E boxes. The proteins E47, Beta2, PDX1, and LMX1.1 were used as in vitro substrates for activated MAP kinase. ERK2 phosphorylates Beta2, E47/E12 and

PDX1 but not LMX1.1 in vitro. Phosphorylation of BETA2 was also inferred in cells expressing a Beta2 mutant (Beta2 S274A). This mutation eliminated the major ERK2 phosphorylation site. The mutated BETA2 did not exhibit the decreased electrophoretic mobility that is observed in response to the phosphorylation of wild type Beta2 by ERK2.

The ERK2 phosphorylation sites in both E47 and Beta2 are located in their respective activation domains. To determine if the phosphorylation of the ERK2 sites was required for the transactivating activity of E47, Beta2, and PDX1, we used multiple serine mutations in these proteins. We were able to conclude that ERK2 does phosphorylate PDX1 in response to glucose to increase its transactivating activity and that Beta2 transactivation is also dependent on the activation of ERK1/2 (84).

I focused my studies on the mechanism of glucose induced ERK1/2 activation in β-cells. The majority of the studies were carried out in the rat insulinoma INS-1 cell line. Initially I investigated the effects of calcium signaling on glucose-induced ERK1/2 activity. I also observed the effects that β-cell lipotoxicity had on MAP kinase activity and insulin transcription. Initially we had made some observations about the glucose induced ERK1/2 pathway that followed logically from findings that had been previously collected. First, we believed that calcium was an important player in this pathway. This was based on

the fact that the chelation of extracellular calcium, with EGTA, inhibited glucose-induced ERK1/2 activation. Second, it was known that glucose metabolism was required for the activation of ERK1/2. Third, we thought that the traditional Ras/Raf/Mek pathway was involved in the activation of ERK1/2. This idea was based on the fact that the Mek inhibitor, PD98059, could block the activation of ERK1/2 and that dominant inhibitory mutants of Ras and Raf also blocked ERK1/2 activation. Finally, we were able to mimic ERK1/2 activation by exposing INS-1 cells to high concentrations, 25 mM, of KCl. This suggested that membrane depolarization is also involved in the activation of ERK1/2. With this knowledge, I developed hypotheses as to how ERK1/2 are activated in INS-1 cells in response to changes in extracellular glucose concentrations.

## Chapter 2: Regulation of ERK1/2 by glucose and Peptide Hormones in Pancreatic β-Cells

### Abstract

We showed previously that ERK1/2 were activated by glucose and amino acids in the pancreatic beta cells. Here we examine and compare signaling events that are necessary for ERK1/2 activation by glucose and other stimuli in beta cells. We find that agents that interrupt $Ca^{2+}$ signaling by a variety of mechanisms interfere with glucose- and glucagon-like peptide (GLP-1)-stimulated ERK1/2 activity. In particular, calmodulin antagonists and FK506 and cyclosporin, immunosuppressants that inhibit the calcium-dependent phosphatase calcineurin, suppress ERK1/2 activation by both glucose and GLP-1. $Ca^{2+}$ signaling from intracellular stores is also essential for ERK1/2 activation, as thapsigargin blocks ERK activation by glucose or GLP-1. The glucose-sensitive mechanism is distinct from that used by phorbol ester or insulin to stimulate ERK1/2, but shares common features with that used by GLP-1.

### Introduction

Insulin is produced by beta cells in the pancreatic islets of Langerhans in mammals. It is the key hormone that promotes the utilization and storage of glucose. Glucose, on the other hand, is the most important regulator of the secretion and biosynthesis of insulin by beta cells, creating a deceptively simple

primary loop controlling sugar metabolism. Signals from $Ca^{2+}$, inositol phospholipids, and cAMP are believed to mediate glucose effects on beta cells, but detailed knowledge of the pathways that control beta cell function is limited (1-6). Van Obberghen and colleagues were the first to show that glucose activates the mitogen-activated protein (MAP) kinases ERK1 and ERK2 in islet-derived cells (7). We and others have confirmed this finding (8-12). MAP kinases, also known as extracellular-signal regulated protein kinases (ERKs), are components of kinase cascades important for transmitting extracellular information to coordinate cellular responses. MAP kinases have been implicated in many physiological events ranging from cellular proliferation and differentiation to cell survival (13;14).

Glucose over its normal physiological concentration range increases the activity of ERK1/2 in pancreatic beta cell lines and intact islets (7;8;10). Glucose metabolism is required for ERK1/2 activation (8), as it is for insulin secretion. Glucose regulation of ERK1/2 has been reported in adipocytes, for example, which also have some capacity for glucose-sensing (15), but in few other cell types. Potentiators of insulin secretion, including forskolin, glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP) which promote cAMP synthesis, potentiate ERK activation, leading to the view that ERK1/2 perform functions that depend on the glucose-sensing machinery of beta cells (8;12;16).

Exposure of INS-1 cells to KCl induces $Ca^{2+}$ uptake and ERK activation (8). Inclusion of EDTA or EGTA in the medium blocks activation of ERKs by glucose in INS-1 cells and in islets. Experiments with chelators and with artificially low glucose suggest that ERKs are activated to a small but significant extent even at sub-threshold glucose concentrations, because the activity in the presence of chelators or at 0-1 mM glucose is lower than activity at 2.8-3 mM glucose in the absence of chelators (8). As confirmation that agents that stimulate insulin secretion via an effect on $Ca^{2+}$ influx also activate ERKs, effects of the oral hypoglycemic drugs glyburide and tolbutamide, which cause closure of the ATP-dependent potassium channel in the beta cell plasma membrane, were also examined (8;10). Treatment of INS-1 cells with 10 μM glyburide or 100 μM tolbutamide for 2 h increased immunoreactive insulin in the medium by 2-fold in the absence of glucose and also caused a discernible increase in ERK activity. Finally, blockers of L-type $Ca^{2+}$ channels interfere with glucose-induced ERK1/2 activation, also suggesting the importance of $Ca^{2+}$ influx on this kinase pathway (10).

Efforts to elucidate the mechanism of ERK1/2 activation have suggested a role for several signaling molecules as intermediates in the pathway. Notable among these is protein kinase C (PKC), which is implicated in one report but discounted by others (11;17). PKC causes activation of ERK1/2 downstream of G protein-coupled receptors that act through Gq and is also involved in prolonged activation caused by some other ligands (18;19). Likewise, glucose induces

tyrosine phosphorylation, but its role in ERK1/2 activation by glucose is controversial (11;17;20).

In this study we have examined regulation of ERK1/2 by several extracellular cues, including glucose, GLP-1, and insulin. Our goals were to determine to what extent these agents shared common mechanisms for ERK1/2 activation and to begin to define essential components of the pathways. Our studies reveal that glucose and GLP-1 converge on a common mechanism for ERK1/2 regulation that is distinct from that used by insulin. The mechanism we propose has significant differences from those previously suggested to control ERK1/2 in beta cells; we find a dependence on the natural release of $Ca^{2+}$ from intracellular stores and a sensitivity to inhibition by immunosuppressants that block the calmodulin-regulated phosphatase calcineurin.

**Methods**

*Materials* – Recombinant adenoviruses were prepared in this laboratory (K52R ERK2) as described (21) or were kind gifts of L. Klesse (MEK1 S217A, MEK1 S317E, S221E, Raf C4B, Raf BXB, G15A H-Ras) (22) and B. Rothermel (myocyte-enriched calcineurin interacting protein (MCIP1)) (23;24). Forskolin, cyclosporin A, and GLP-1 were purchased from Sigma. KN62, KN93, GF109203X, pituitary adenylyl cyclase-activating peptide (PACAP), the calmodulin antagonist W7, thapsigargin, dantrolene, 2-aminoethoxy-diphenyl borate (2APB), nifedipine, diazoxide, rapamycin, the Src inhibitor PP2, and wortmannin were purchased from Calbiochem.

*Cells* - INS-1 cells, either from early passages or subclones selected for increased glucose-stimulated insulin secretion kindly provided by Chris Newgard (6), were grown in RPMI 1640 medium containing 10% fetal bovine serum, 0.5 mM Hepes, pH 7.4, 10.2 mM L-glutamine, 50 mM sodium pyruvate, and 2.5 mM β-mercaptoethanol (25). Cells that were 60-80% confluent were pre-incubated for 1-2 h in Krebs-Ringer-bicarbonate-Hepes (KRBH) with 0 or 2 mM glucose prior to treatment. In the indicated experiments cells were infected with recombinant adenoviruses at multiplicities of infection of 10-100 for 1 h, 24 or 48 h prior to cell treatment. After treatment with the agents indicated in figure legends, the medium was removed and cells were washed with cold phosphate-buffered saline and harvested in 0.2 ml cold lysis buffer (50 mM Hepes, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.2 μg/ml phenylmethylsulfonyl fluoride, 0.1 M

NaF, 2 mM $Na_3VO_4$, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin (7)). After 20 min on ice, supernatants were collected following centrifugation for 10-30 min at 14,000 rpm in an Eppendorf microfuge and were stored at -80$^o$ C.

*Measurement of ERK1/2 Activity* - Equal amounts of lysate proteins (20-40 μg) were resolved in 10% polyacrylamide gels in sodium dodecyl sulfate and subjected to electrophoresis. Proteins were transferred to nitrocellulose at 700 mA for 1.5 h at 4$^o$C. Membranes were incubated in 5% nonfat milk/0.05% Tween-Tris-buffered saline (TBS) for 1 h, then in 1:3000 anti-phosphoERK1/2 antibody (BioSource or Sigma) in 1% nonfat milk/1% bovine serum albumin/0.05% Tween-TBS for 2 h, and finally in 1:5000 anti-rabbit IgG in 1% nonfat milk/1% BSA/0.05% Tween-TBS for 1 h at room temperature. Membranes were washed twice in 0.05% Tween-TBS and twice in TBS. After detection of phosphorylated ERK1/2 bands (43 kD and 41 kD, respectively) by enhanced chemiluminescence and autoradiography, membranes were stripped in 62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS for 30 min at 60$^o$C, rinsed with 0.05% Tween-TBS, and immunoblotted as above with 1:5000 Y691 anti-ERK1/2 rabbit polyclonal antibody (26).

**Results**

*Upstream components of the glucose-stimulated ERK1/2 pathway in INS-1 cells*-- To examine upstream components of the glucose-dependent MAP kinase cascade, we infected INS-1 cells with adenoviruses expressing kinase-defective or interfering mutants of proteins viewed as core components of the ERK1/2 pathway in other cell types (Ras, Raf, MEK1, ERK2) (14;22). Expression of mutants of the core components that have been shown to inhibit ERK1/2 activation in other cell types (13;14), K52R ERK2, S217A MEK1, Raf C4B, and G15A H-Ras, blocked glucose-stimulated ERK1/2 activity (Fig. 1A-C). In these experiments ERK1/2 activity was monitored in cell lysates with antibodies that selectively recognize the doubly phosphorylated, active forms of the kinases. Total ERK1/2 immunoreactivity demonstrated that equal amounts of the two proteins were present in each lane. Consistent with its inhibitory action, the kinase-defective mutant K52R ERK2 reduced phosphorylation of endogenous ERK1/2. We previously showed that MEK1 but not MEK2 was activated by glucose in INS-1 cells (9), consistent with the finding that the phosphorylation-defective mutant S217A MEK1 blocked ERK2 activation by glucose (Fig. 1B). The effects of the inhibitory Ras mutant and the Raf N-terminal fragment support the conclusion that a small GTP binding protein, either a Ras isoform or another GTPase that uses a common guanine nucleotide exchange factor, are required for ERK1/2 activation in INS-1 cells.

Tyrosine kinases often participate in ERK1/2 activation, and glucose has been reported to induce tyrosine phosphorylation in beta cells (15;20). We examined the potential roles of two tyrosine kinases, Src and PYK2, in inducing ERK1/2 activation. We found no evidence of glucose-induced changes in tyrosine phosphorylation of PYK2 in INS-1 cells (data not shown), suggesting that it is not involved. Src inhibitors including PP2 (27) caused a partial but significant reduction in ERK1/2 phosphorylation in response to glucose (Fig. 1D), suggesting a role for a Src-like kinase.

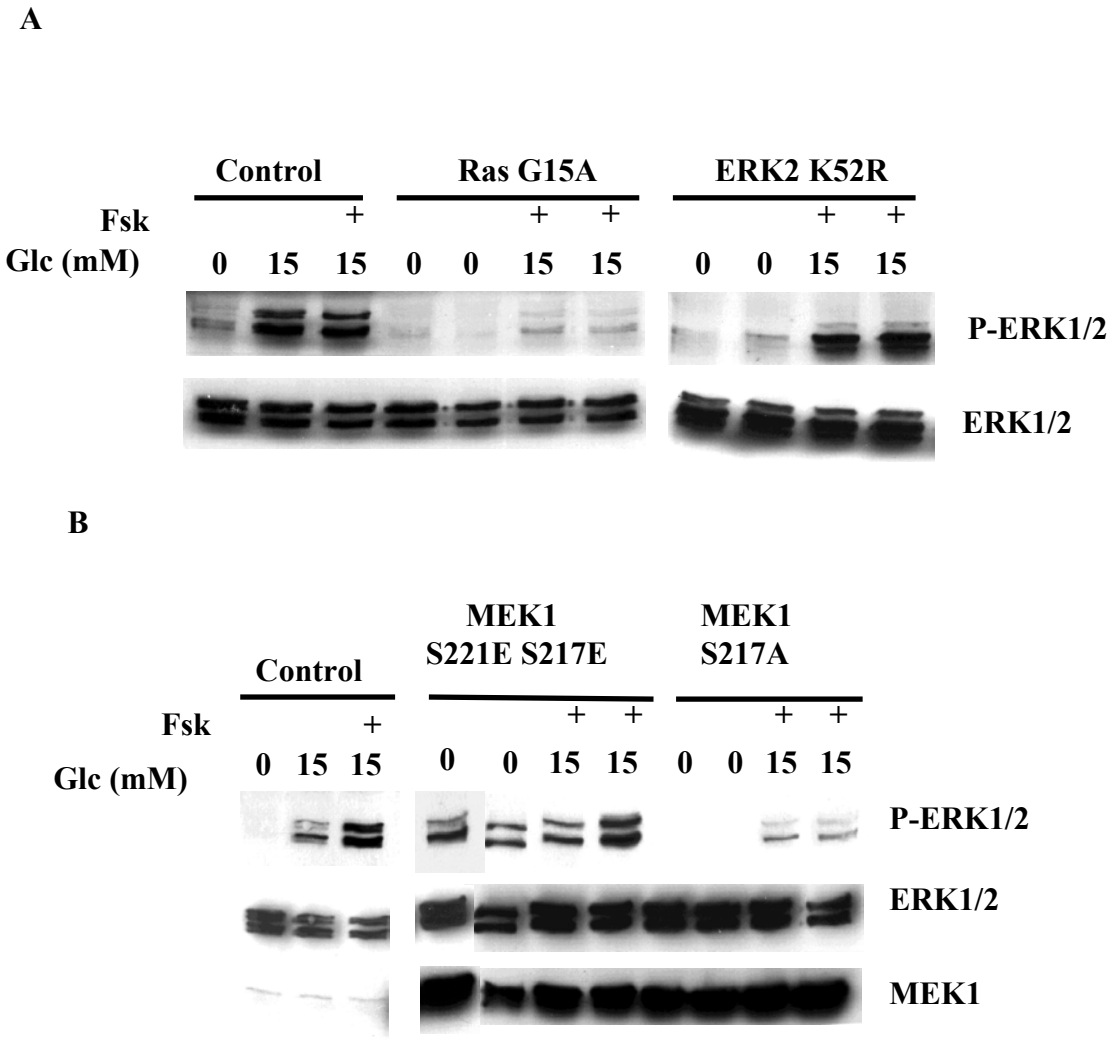
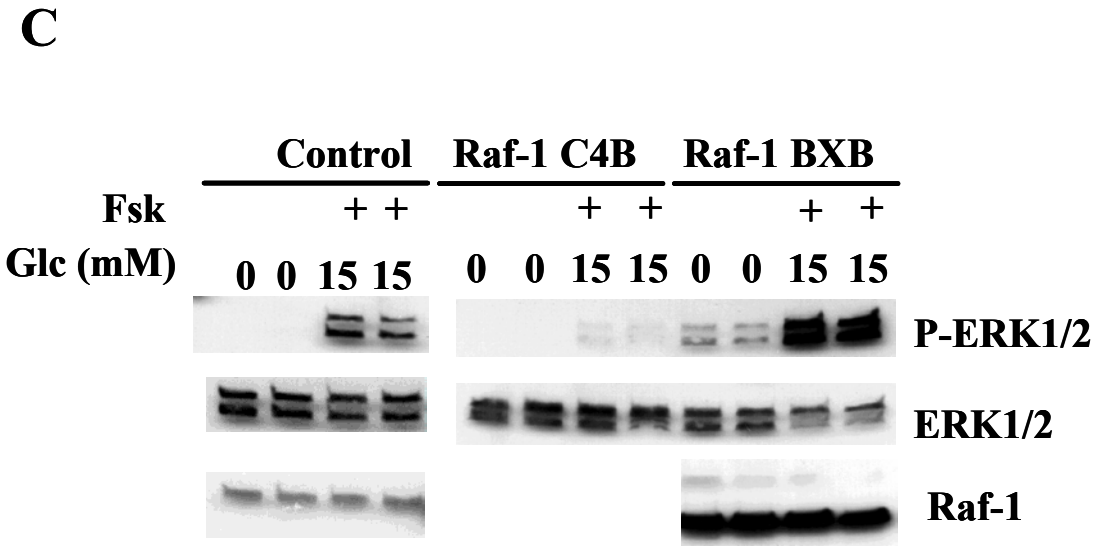



**Figure 2.1**. **Dominant negative mutants of Ras and Raf inhibit ERK1/2 activation by glucose.** In panels A-C, INS-1 cells were infected with recombinant adenoviruses expressing the indicated proteins. After 48 h, cells were preincubated in KRBH for 2h and either untreated or exposed to 15 mM glucose (Glc) or Glc plus 10 μM Fsk for 30 min. Lysate proteins were immunoblotted as described in Methods. **A.** Control, G15A Ras (inhibitory), K52R ERK2 (inhibitory). The phosphorylated ERK2 band visible in the lanes from samples expressing K52R ERK2 comes from the overexpressed ERK2 mutant not the endogenous protein. **B.** Control, MEK1 S217E, S221E (constitutively active), MEK1 S217A (inhibitory). (Contributed by Khoo Shih)



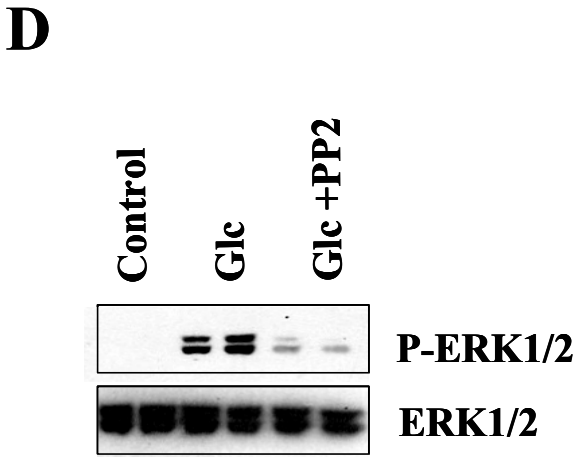




**Figure 2.1**. **Dominant negative mutants of Ras and Raf inhibit ERK1/2 activation by glucose.** (cont.) **C**. Control, Raf-1 C4B (N-terminal fragment lacking the kinase domain), Raf-1 BXB (constitutively active) **D.** Cells were preincubated in KRBH for 2 h, then 1 μM PP2 was added for 15 min before the addition of Glc for 30 min. Lysate proteins were immunoblotted as in Fig. 1. Representative data are shown. Experiments were repeated a minimum of 2-3 times. In all panels, duplicate lanes show samples from independent replicates. (Panel C contributed by Khoo Shih)

*Calmodulin is the calcium-dependent effector required for stimulation of ERK1/2 by glucose in INS-1 cells*-- We previously showed extracellular $Ca^{2+}$ was required for glucose-dependent ERK1/2 activation (8). Thus, we evaluated the effects of inhibitors that block calcium-dependent signaling molecules. First, we tested protein kinase C (PKC) inhibitors (27) because PKC is required in several systems for ERK1/2 activation and it has been suggested that this is also the case in beta cells (11;28). None of the inhibitors tested, including bis-indoylmaleimide (GF109203X), blocked glucose-induced ERK1/2 activity (Fig. 2A), although phorbol ester-stimulated activity was blocked (not shown). On the other hand, W7, a calmodulin antagonist, was a very effective inhibitor of ERK1/2 activation by glucose (Fig. 2B).

To probe the site of action of calmodulin, we tested the involvement of two important calmodulin targets, $Ca^{2+}$ and calmodulin-dependent protein kinases (CaMKs) and the calmodulin-dependent phosphatase calcineurin (27;29;30). CaMKII and calcineurin have both been linked to insulin secretion from beta cells (31-38). Not only did the CaMK inhibitors KN62 and KN93, which inhibit interaction of the kinases with calmodulin, both suppress glucose-dependent ERK1/2 activation (Fig. 2C), the immunosuppressant FK506, a calcineurin inhibitor, also prevented ERK1/2 activation by glucose (Fig. 2D).

To determine the specificity of action of FK506, we examined the effects of other immunosuppressants. Supporting an involvement of calcineurin,

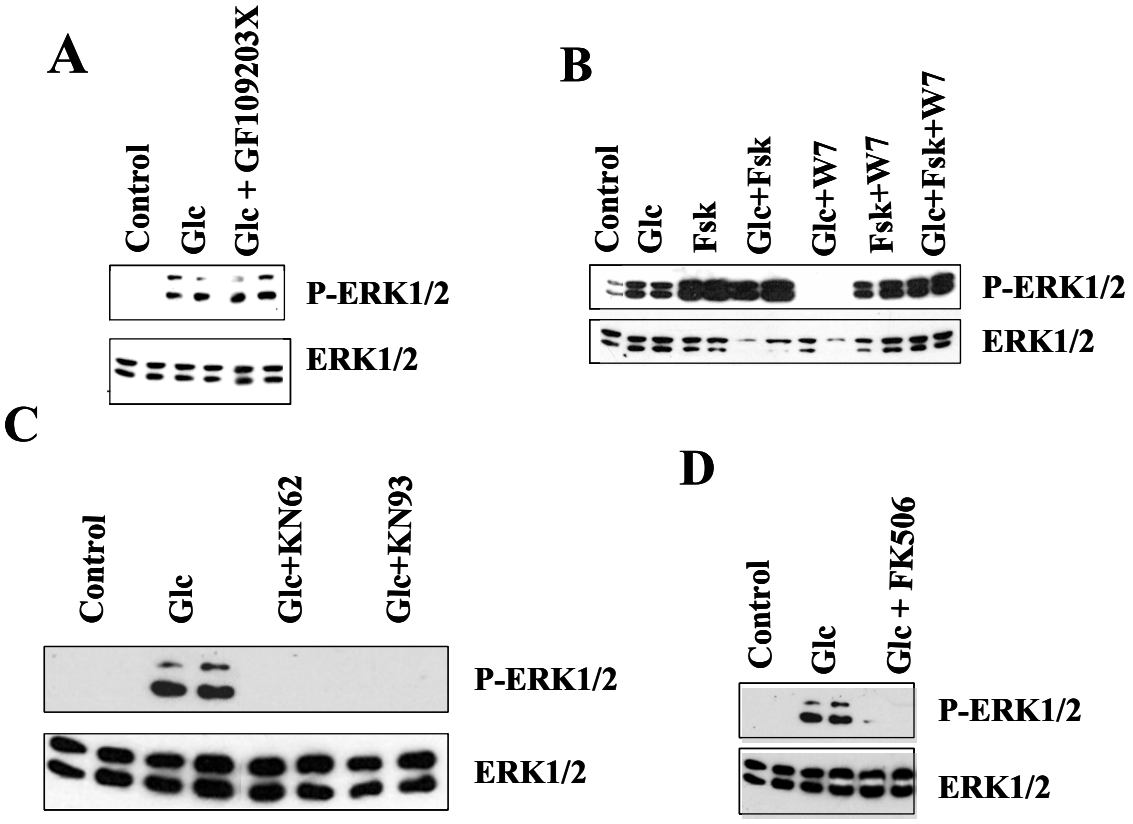



**Figure 2.2. Calcium targets required for activation of ERK1/2.** INS-1 cells were preincubated as in Fig. 1. The inhibitors were added as indicated 15 min prior to stimulation with 15 mM Glc or as indicated. ERK1/2 activities were detected by immunoblotting as in Fig. 1. **A.** 2 μM GF109203X **B.** 50 μM W7 followed by Glc, 10 μM Fsk, or Glc plus Fsk. One of 4 similar experiments **C.** 10 μM KN62 or KN93; **D.** 100 nM FK506. One of 10 similar experiments. In all panels, duplicate lanes show samples from independent replicates. Panels A and C show one of 2 comparable experiments.

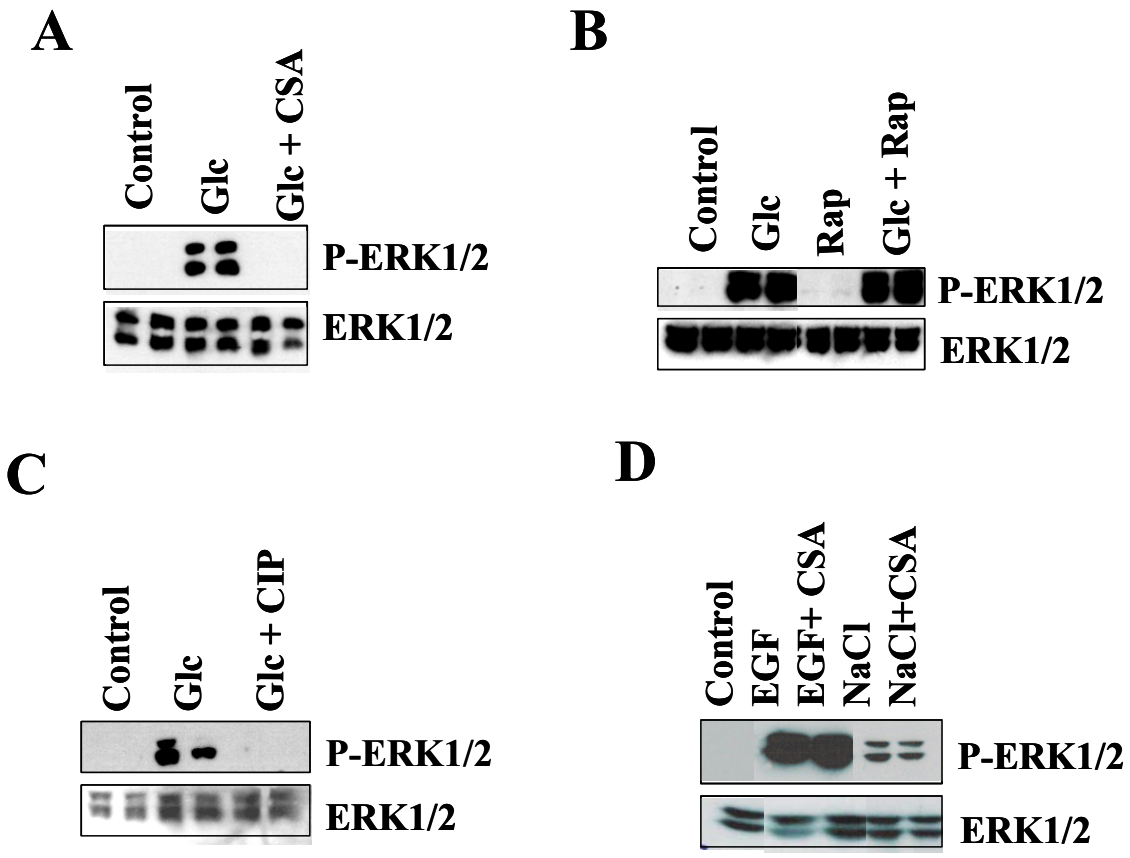



**Figure 2.3. Calcineurin inhibitors selectively prevent ERK1/2 activation by glucose in beta cells**. In **A-C** INS-1 cells were pre-incubated as in Fig. 1 and duplicate lanes show samples from independent replicates. Cells were untreated or pretreated with: **A.** 10 μM cyclosporin A (CSA); or **B.** 1 μM rapamycin (Rap) for 15 min, followed by the addition of 15 mM Glc for 30 min. **C.** Cells were infected with an adenovirus expressing mCIP or empty virus. After 24 h the cells were preincubated in KRBH for 2 h and then treated with 15 mM Glc for 30 min. **D.** 293 cells were pre-incubated in serum-free medium for 24 h, treated with 10 μM CSA for 15 min, and then stimulated with either 10 ng/ml EGF for 5 min or 0.5 M NaCl for 15 min. Experiments in **A** and **B** were repeated a minimum of 5 times and in **C** and **D** twice.

cyclosporin A and FK520, both of which are also calcineurin inhibitors, prevented ERK1/2 activation by glucose (Fig. 3A and not shown). In contrast, the immunosuppressant rapamycin, which targets mTOR not calcineurin, had no effect on glucose-induced ERK1/2 activity (Fig. 3B). Expression of myocyte-enriched calcineurin interacting protein, a calcineurin inhibitory protein (39), also prevented ERK1/2 activation by glucose (Fig. 3C), providing an independent line of evidence implicating calcineurin in ERK1/2 activation by glucose. To determine if calcineurin inhibitors influence activation of ERK1/2 in other systems, HEK293 cells were treated with EGF or NaCl to stimulate ERK1/2 activity in the presence or absence of cyclosporin A (Fig. 3D). The calcineurin inhibitor had no effect on ERK1/2 activity induced by either stimulus in 293 cells.

*The calmodulin/calcineurin-dependent mechanism is not common to all agents that stimulate ERK1/2 in INS-1 cells*-- Because we had clear evidence that calmodulin/calcineurin were required for ERK1/2 activation by glucose, we wished to determine if other agents that activate ERK1/2 also employ a mechanism dependent on these factors. We examined the potential involvement of calmodulin/calcineurin in ERK1/2 activation by phorbol ester which has not generally been found to use these signal transducers (Fig. 4A). Consistent with an independent mechanism of action, phorbol ester-stimulated activity was not affected by calcineurin inhibitors.

Hormones that stimulate adenylyl cyclase including GLP-1 (Fig. 4B,C) and PACAP (not shown) activate ERK1/2 ((12;16;40), this study). Although the extent of activation was usually less than with either glucose or forskolin, increased ERK1/2 phosphorylation was detected within 2-5 min and persisted for at least 10 min with either GLP-1 or PACAP. The effect on kinase activity was greater if 2 mM glucose was present in the medium. Activation of ERK1/2 by GLP-1 was sensitive to inhibition by W7, but like glucose largely insensitive to wortmannin (Fig. 4B). Although activity stimulated by forskolin alone was apparently less sensitive to the phosphatase inhibitors (Fig. 2B and not shown), activity stimulated by glucose plus GLP-1 or glucose plus forskolin was blocked by FK506 (Fig. 4C). In each of these cases, rapamycin did not reduce ERK1/2 activity.

Because insulin stimulates ERK1/2 in many tissues, and the agents above that trigger ERK1/2 also enhance insulin secretion, we wished to determine if induction of ERK1/2 activity by insulin in beta cells uses a calmodulin/calcineurin-dependent mechanism and is involved in their activation by glucose. Insulin and IGF-1 (not shown) stimulated ERK1/2 in INS-1 cells,

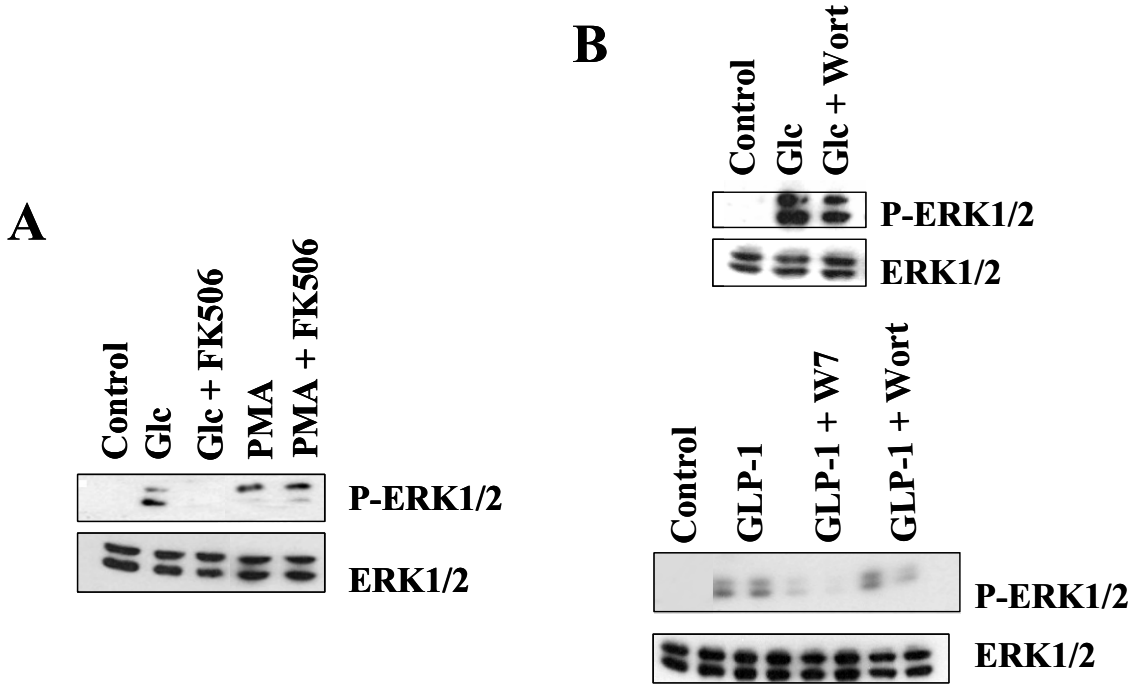



**Figure 2.4. Effects of inhibitors on ERK1/2 activation by hormones in INS-1 cells.** ERK1/2 activities were measured as in Fig. 1. **A.** Cells were exposed to 1 μM FK506 and then stimulated with either Glc for 30 min or 100 nM phorbol ester (PMA) for 15 min. **B.** (Upper panel) Cells were pretreated with 50 nM wortmannin (wort) for 15 min followed by 15 mM Glc for 30 min. (Lower panel) Cells were pretreated with wortmannin or W7 for 15 min and stimulated with Glp-1 for 5 min.

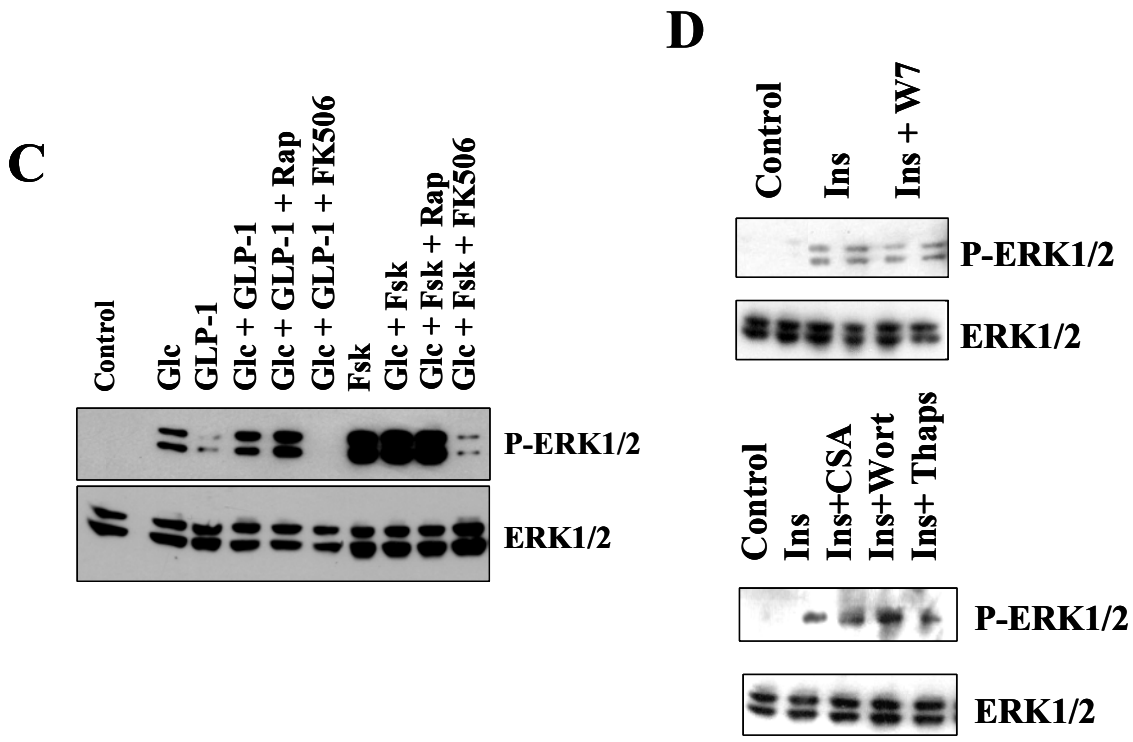



**Figure 2.4. Effects of inhibitors on ERK1/2 activation by hormones in INS-1 cells.(cont.)**

**C.** Cells in RPMI with serum and 2 mM Glc for 4 h were exposed to 100 nM Rap or 100 nM FK506 for 15 min, and then stimulated with 11 mM Glc plus 30 nM GLP-1 or Glc plus 10 mM Fsk for 15 min. **D.** (Upper panel) Cells were treated with W7 and stimulated with insulin for 30 min. (Lower panel) Cells were treated with 10 μM CSA, 1 μM wortmannin, or 1 μM thapsigargin (thaps) for 15 min, and then stimulated with insulin for 30 min. Some panels show duplicate lanes, which represent samples from independent replicates. Data shown are representative of 3 or more similar experiments.

but to a much reduced extent compared to glucose (Fig. 4D). Interestingly, activation of ERK1/2 by insulin was not blocked by wortmannin (lower panel), which inhibits phosphatidylinositol-3 kinase (PI-3 kinase) a major mediator of insulin action (41), although activation of Akt was inhibited (not shown). Wortmannin also had little effect on glucose-stimulated ERK1/2 activity (Fig. 4B). Stimulation of ERK1/2 by insulin, in contrast to glucose, was not reduced by W7 or cyclosporin A (Fig. 4D). These findings suggest that glucose and insulin use distinct mechanisms to trigger ERK1/2.

*Source of $Ca^{2+}$ required for glucose-dependent ERK1/2 activation*-- $Ca^{2+}$ was shown to be required in the earliest studies of this response (7;8). To explore this requirement further, we first depolarized cells by exposing them to 25 mM KCl (Fig. 5A). KCl caused a rapid but transient increase in ERK1/2 activity which had returned to control values by 10 min; glucose activated ERK1/2 more following a 30 min incubation in the presence of KCl than in its absence (not shown). Like activation by glucose, activation of ERK1/2 by KCl was blocked by calcineurin inhibitors (Fig. 5A).

We next examined the source of calcium required for ERK1/2 activation. Nifedipine, a blocker of L-type voltage-gated $Ca^{2+}$ channels (42), partially blocked glucose stimulation of ERK1/2 (Fig. 5B), as was reported (11;12). Similar results were observed with the related blockers, nisoldipine and nitrendipine (not shown). Under no condition was a complete blockade produced

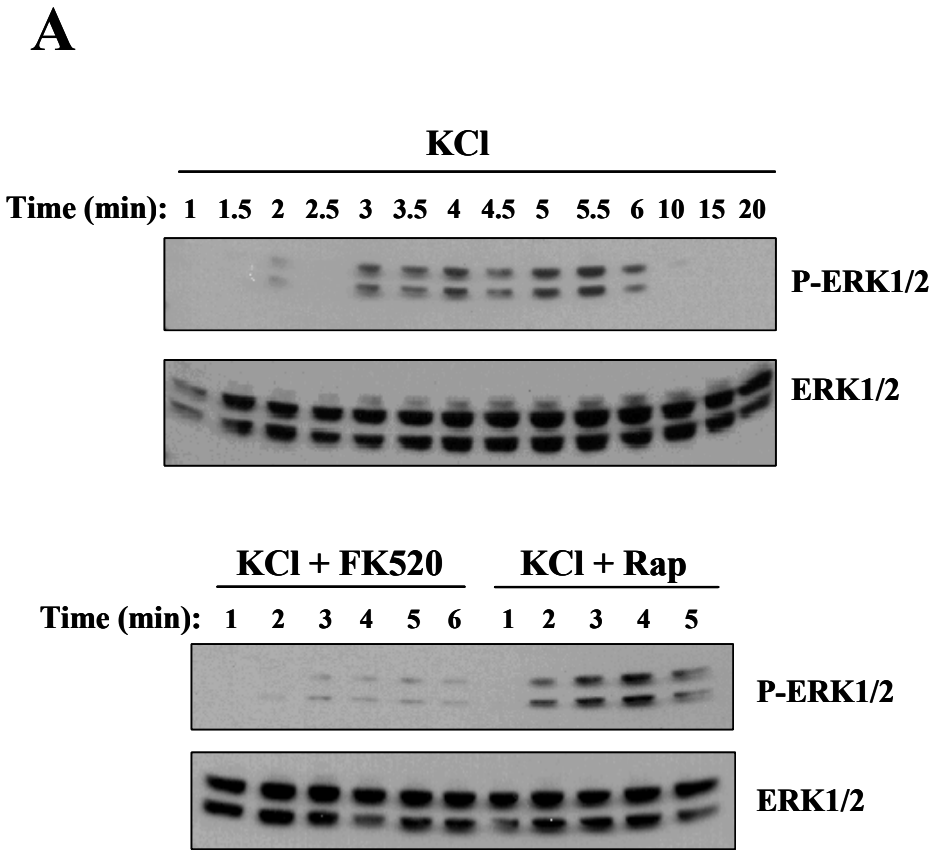



**Figure 2.5. Role of calcium influx in ERK1/2 activation. A.** INS-1 cells pre-incubated for 4 h in KRBH plus 2 mM Glc were exposed to 30 mM KCl for the indicated times either without or with a 15-min pre-incubation in 100 nM FK520 or rapamycin. (Contributed by Michael Lawrence)

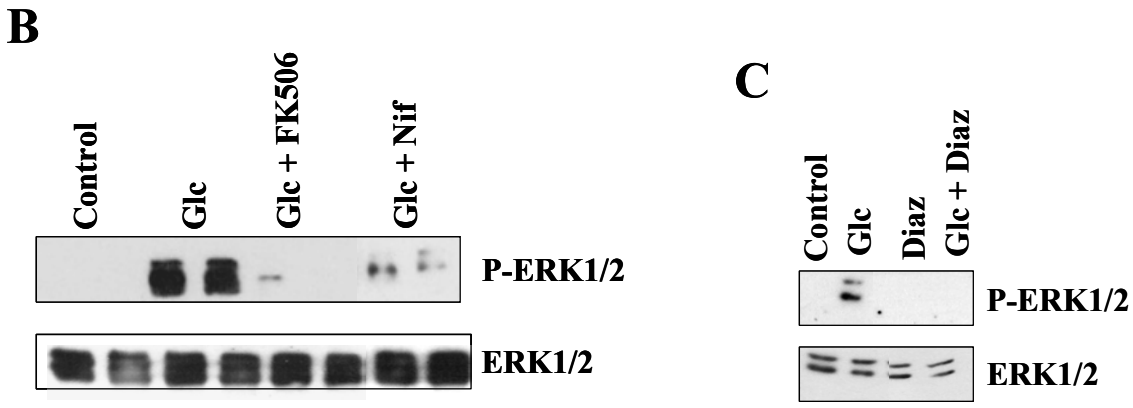



**Figure 2.5. Role of calcium influx in ERK1/2 activation. B.** Cells treated as in Fig. 1 were pre-incubated in 5 μM nifedipine or 100 nM FK506 for 15 min and then stimulated with 15 mM Glc for 30 min. **C.** Cells treated as in Fig. 1 were incubated with 1 mM diazoxide for 15 min and then stimulated with 15 mM Glc for 30 min. ERK1/2 activities were analyzed as above. In B, duplicate lanes show samples from independent replicates. Data shown are representative of 3 or more similar experiments.

with these agents. Diazoxide is a thiazide which maintains ATP-sensitive potassium channels in the open state, and is used therapeutically to inhibit insulin release from insulin-secreting tumors. This agent at millimolar concentrations blocked glucose activation of ERK1/2 (Fig. 5C); high micromolar diazoxide caused a modest reduction in ERK1/2 activity (not shown). These results indicate that $Ca^{2+}$ influx is important for ERK1/2 activation. $Ca^{2+}$ that is necessary to trigger the kinase cascade could be that resulting from the influx of extracellular $Ca^{2+}$ or that released from intracellular stores. To determine the impact of the release of $Ca^{2+}$ from intracellular stores on activation of ERK1/2 by glucose, cells were pretreated with 1 μM thapsigargin for 15 min, a time sufficient for this ATPase inhibitor to exhaust the intracellular $Ca^{2+}$ pool (43). Thapsigargin itself did not increase ERK1/2 activity following 5, 10 or 15 min of exposure (not shown). However, activation of ERK1/2 by glucose was completely blocked by thapsigargin (Fig. 6A), suggesting that glucose causes release of intracellular $Ca^{2+}$ to promote ERK1/2 activation. To compare the effects of glucose to membrane depolarization induced by KCl, we determined if the effects of KCl were also blocked by thapsigargin. Thapsigargin significantly reduced but did not completely block ERK1/2 activation by KCl (Fig. 6B), consistent with the idea that the $Ca^{2+}$ that activates ERK1/2 is the intracellular pool. For comparison, we also examined the effects of thapsigargin on stimulation of ERK1/2 by insulin (Fig. 4D) and GLP-1 (Fig. 6C). No blockade of insulin-increased ERK1/2 activity was detected, but GLP-1-induced activity was inhibited.

$Ca^{2+}$ influx can induce the release of this pool through ryanodine receptors and glucose-induced production of inositol trisphosphate ($IP_3$) may activate $IP_3$ receptors (44-48). Thus, we tested dantrolene and 2-aminoethoxy-diphenyl borate (2-APB), inhibitors of $Ca^{2+}$ release mediated by ryanodine and $IP_3$ receptors respectively (49;50), to seek independent evidence that intracellular stores are required (Fig. 6D). Both blocked ERK1/2 activation by glucose.

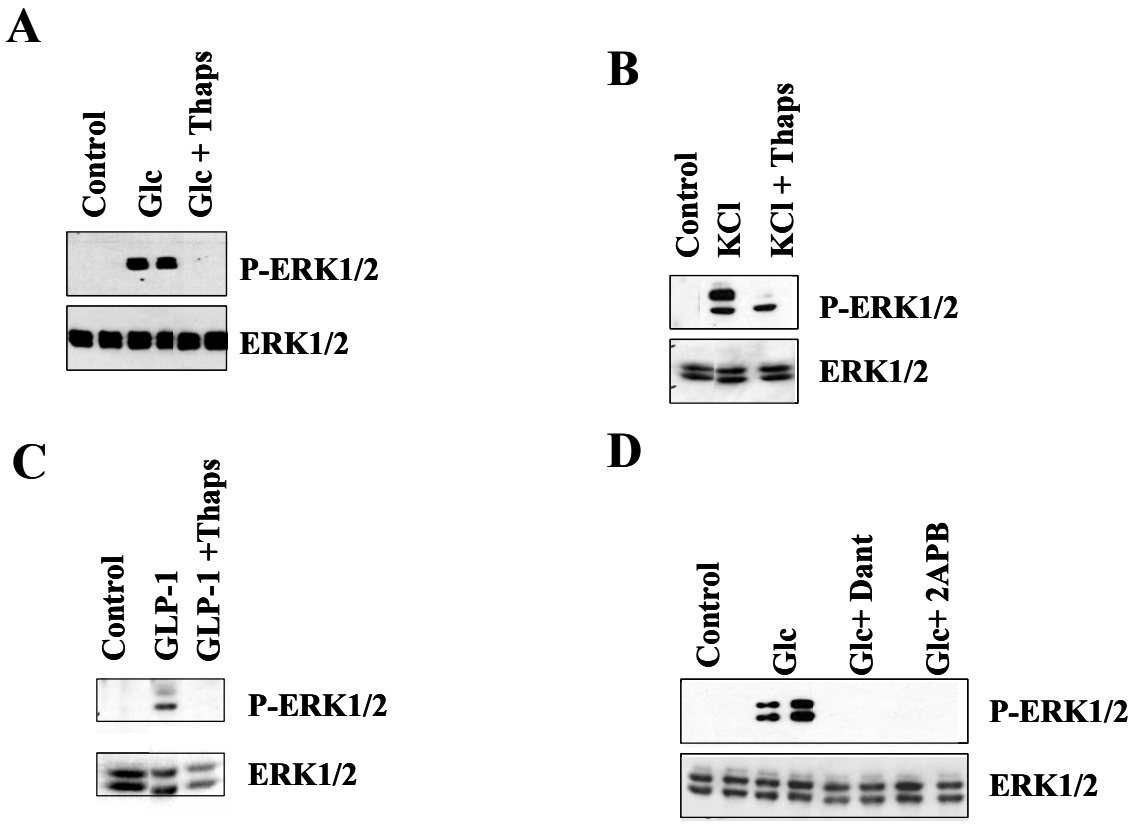



**Figure 2.6. Activation of ERK1/2 by glucose requires release of intracellular calcium.** INS-1 cells pre-incubated as in Fig. 1 were untreated or pre-treated with 1 μM thapsigargin for 15 min (**A-C**) and then unstimulated or stimulated with: **A.** 15 mM Glc for 30 min; **B.** 25 mM KCl for 10 min; **C.** 1 μM GLP-1 for 5 min; or **D.** Cells were pre-treated with 50 μM dantrolene or 50 μM 2-ABP for 15 min and then stimulated with 15 mM Glc for 30 min. ERK1/2 activities were analyzed as above. In **A** and **D**, duplicate lanes show samples from independent replicates. Data are representative of at least 3 similar experiments.

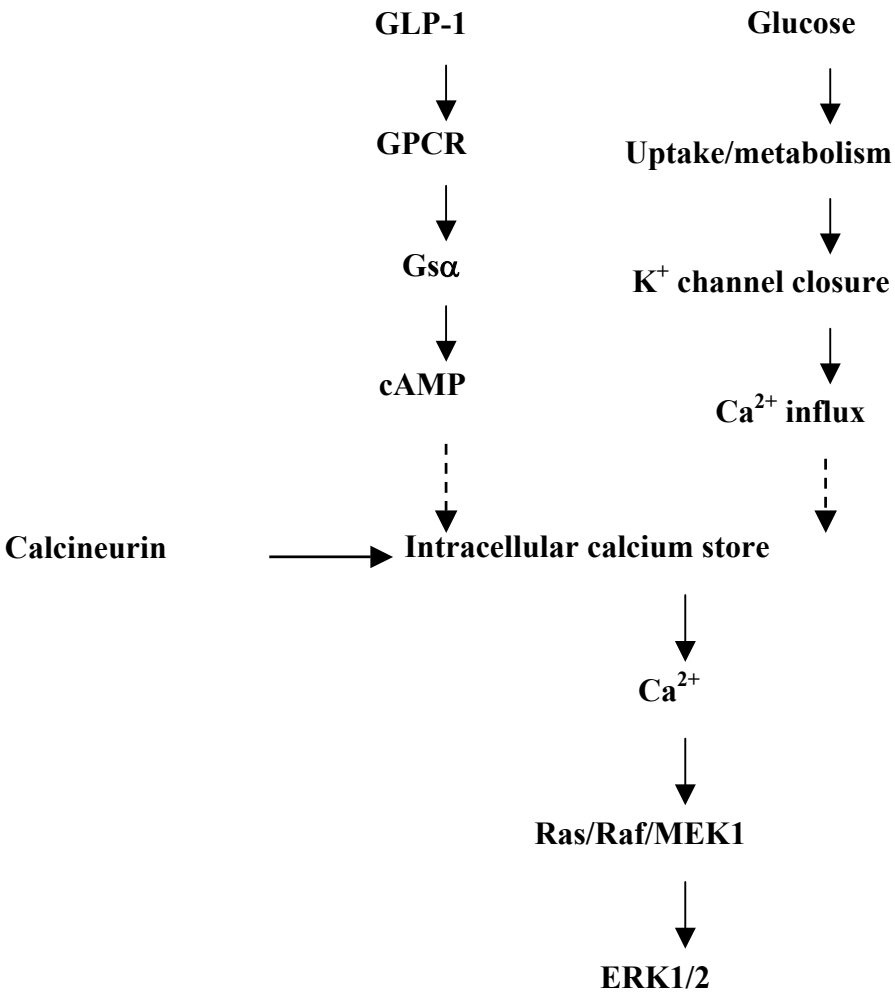



**Figure 2.7. Model of regulation of ERK1/2 in pancreatic beta cells.**

**Discussion**

Glucose causes the rapid and continuous activation of ERK1/2 in beta cells. The effects of GLP-1 and PACAP, while also very rapid, are short-lived. In comparison, ERK1/2 activation by forskolin is slower but prolonged, perhaps due to actions independent of its ability to increase cAMP or to the much greater accumulation of cAMP it elicits than GLP-1. Because drugs that block GLP-1 are variably effective in blocking forskolin, forskolin may bypass some otherwise essential steps, thereby forcing a cAMP-dependent pathway that does not normally occur in pancreatic beta cells in response to hormones that produce cAMP as a second messenger.

Our results further suggest that glucose and GLP-1 converge on a common mechanism of action. In contrast, insulin is generally a weaker ERK1/2 stimulus, and clearly regulates ERK1/2 through a different mechanism. Agents that block glucose- and GLP-1-induced ERK1/2 activity, including calmodulin antagonists and thapsigargin, are ineffective in blocking induction of kinase activity by insulin. Glucose-induced secretion of insulin does not require the release of intracellular $Ca^{2+}$ stores (51;52). In addition, FK506 appears to have little effect on insulin secretion within the first few hours of exposure (34;53-55), although it inhibits glucose-stimulated ERK1/2 activation. These observations further support the idea that the control of ERK1/2 activity exerted by glucose has little to do with the autocrine action of insulin on these cells.

The terminal components of the signaling pathway implicated by the use of interfering mutants include several of the usual suspects. Most clear, MEK1

activity is required; pharmacological inhibitors as well as an inhibitory mutant of MEK1 block ERK1/2 activation, consistent with the finding that MEK1 but not MEK2 is responsive to glucose (9). A small G protein, most likely Ras, is also required, based on the inhibitory effects of a dominant-interfering Ras mutant and of an N-terminal fragment of Raf1, which is thought to act by sequestering activated Ras, preventing it from binding to endogenous Raf proteins. The work of Bos and colleagues suggests that Rap cannot directly activate Raf, in spite of the fact that the Rap effector domain can bind to Raf (56). The inhibitory effect of the Src inhibitor PP2 suggests that a Src family or other tyrosine kinase leads to stimulation of Ras. Glucose-induced tyrosine phosphorylation has been documented in pancreatic beta cells (20). In contrast to previous reports, we find no evidence for a role of conventional isoforms of PKC, which might act upstream of Ras (11;17). Furthermore, phorbol ester stimulation of ERK1/2 is insensitive to inhibition by the immunosuppressants that completely block the action of glucose on the kinases.

Less clear is the MAP kinase kinase kinase (MAP3 kinase) involved in ERK1/2 activation. Kinase-dead MEK1 would probably interfere with any relevant MAP3 kinase, and the inhibitory N-terminal Raf fragment which contains the Ras binding domain most likely blocks the pathway by targeting Ras, not a Raf family member. In numerous experiments we have found inconsistent activation of Raf-1 and even less evidence for B-Raf activation by glucose in INS-1 cells. However, our findings do not yet convince us that glucose or GLP-1 employs a Raf-independent mechanism, as was recently suggested (12). This is

not the only setting in which Raf activity has not been well correlated with ERK1/2 activation (57). Aside from the difficulties of the assay itself, two possibilities seem worthy of consideration. First, cAMP may suppress phosphatase activity that normally inactivates MEK1. And second, cAMP may enhance the formation of Raf1-MEK1 complexes. In either case the efficiency of MEK1 activation would be increased, in spite of minimal Raf activation.

Two conclusions about mechanism are straightforward from our findings. First, the essential mediator of ERK1/2 activation by glucose and GLP-1 is $Ca^{2+}$. Inhibiting $Ca^{2+}$ signaling prevents ERK1/2 activation by either type of agent. Epac2, a cAMP-dependent guanine nucleotide exchange factor for Ras family small GTPases (58;59), is present in pancreatic beta cells and may mediate the actions of cAMP, perhaps directly on Ras. However, our findings suggest that, if this factor is involved, it likely acts upstream not downstream of $Ca^{2+}$; this conclusion is consistent with a report from Holz and coworkers who suggest that Epac2 activates $Ca^{2+}$ release in beta cells through effects on the related small G protein Rap (59). Second, an important source of $Ca^{2+}$ for glucose activation of ERK1/2 is the intracellular storage compartment. By blocking a $Ca^{2+}$ ATPase, thapsigargin depletes $Ca^{2+}$ from this pool. Furthermore, dantrolene blocks calcium-induced release from this pool. The impaired release of $Ca^{2+}$ from the storage compartment completely prevents ERK1/2 activation by glucose or GLP-1, strongly suggesting that the intracellular release of $Ca^{2+}$ is an essential part of the mechanism of ERK1/2 activation by these agents.

Given that both glucose and cAMP cause release of intracellular $Ca^{2+}$ from this pool, perhaps via ryanodine receptors (2;44;59;60), $Ca^{2+}$-stimulated $Ca^{2+}$ release may be the key process on which these agents converge. Inhibition of glucose stimulation of ERK1/2 by dantrolene supports this conclusion. However, 2-APB also blocks glucose activation. This inhibitor also reportedly interferes with $Ca^{2+}$ entry (61); thus, we can only speculate that the IP3 receptor is also involved in this process. These $Ca^{2+}$ release receptors are reportedly regulated by calcineurin, suggesting that the requirement for calcineurin in activating ERK1/2 may come from its effects on $Ca^{2+}$ release (37;38). If this is correct, calcineurin may be viewed as a gatekeeper, rather than acting directly as a participant, in ERK1/2 activation. Some evidence suggests that the $Ca^{2+}$ release pool may be linked directly to the plasma membrane (62). This juxtaposition may facilitate compartmentalized signaling from receptor and channel complexes; if so, that may account for the rapid effects of GLP-1 and PACAP relative to forskolin.

Inhibitor studies have implicated CaMK II, which is known to be activated by glucose in beta cells (31). It may act downstream of the release of intracellular $Ca^{2+}$ stores, as suggested below. The CaMKII inhibitors have been reported to influence not only other CaMKs, but also $Ca^{2+}$ channels (63); thus, the inhibition we observed may have been caused by effects on $Ca^{2+}$ signaling that is not mediated by CaMKs at all. This remains to be determined. Because so many glucose-stimulated components have been implicated in multiple ways by inhibitor studies, it has been difficult to define the signaling pathway leading to

stimulation of ERK1/2. We can envision two scenarios that might account for the apparent complexity of our findings. One is that ERK1/2 act as a coincidence monitor in this system in that more than one type of signal must be triggered for activation of the cascade by glucose; for example, based on the comments above, calcineurin might provide the conincident signal. A second is that the kinases are activated by a complex, but linear series of events that have not been previously defined for this pathway.

For future investigation and with the many caveats discussed above, we propose the following pathway (Fig. 7) which incorporates all of the signaling molecules implicated by our inhibitor studies. Glucose metabolism is coupled to $Ca^{2+}$ influx which causes $Ca^{2+}$ release from intracellular stores, through ryanodine and perhaps also IP3 receptors (44). GLP-1 through cAMP also induces $Ca^{2+}$ release via Epac2 from intracellular stores (2;59;64). The actions of GLP-1 and glucose converge at this $Ca^{2+}$ release step, and potentiation of the response may occur as a consequence of the mechanisms by which each agent works on $Ca^{2+}$ release. Depending on calcineurin activity $Ca^{2+}$ release will occur or be prevented. Release of intracellular $Ca^{2+}$ in a discrete location is coupled to activation of a CaM kinase family member. The CaM kinase then employs a tyrosine kinase (e.g., EGF receptors (65;66)) to activate the Ras/ERK1/2 cascade. We are currently devising experiments to test the key steps in this pathway.

ERK1/2 are most frequently implicated in cell proliferation programs. Nevertheless, they are highly expressed in terminally differentiated cells including

neurons (67). They play a role in long term potentiation and in synaptic modulation, providing a means of storing signaling information on a longer time scale than individual action potentials (68-71). Their actions in beta cells may be analogous in that they offer a means of integrating the complex and ever changing nutrient and hormonal signals that acutely control insulin secretion to ensure that beta cells maintain their secretory capacity.

## Chapter 3: Effects of Free Fatty Acids on ERK1/2

### Abstract

Chronic exposure to elevated free fatty acids (FFAs) has been shown to alter insulin secretion, transcription, induce insulin resistance, and decrease β-cell mass. We have previously shown that ERK1/2 are activated by glucose and are involved in insulin transcription in pancreatic β-cells. Here we examine the effects of elevated FFAs on ERK1/2 activity in INS-1 pancreatic β-cells. The pretreatment of INS-1 cells for 72 hours with a mixture of oleate and palmitate causes constitutively increased ERK1/2 activity and decreased the net activation of ERK1/2 by glucose. We also find that this constitutive activity disrupts normal ERK1/2 nuclear localization in response to glucose stimulation. Following a 24 hour pretreatment with FFAs, insulin gene transcription was also significantly decreased. In contrast, no effects of FFAs were observed on c-Jun N-terminal kinase. The effects of FFAs on ERK1/2 are reversible; after 24h the glucose sensitivity of ERK1/2 is restored. We also evaluated signaling events which lead to this constitutive activity. We find that the FFA-induced constitutive activity of ERK1/2 is PKC independent, and this activity cannot be relieved with either administration of antioxidants or agents that act on peroxisome proliferator-activated receptors (PPARs).

## Introduction

Type 2 diabetes involves both impaired insulin secretion and peripheral insulin resistance (57). Diabetes can lead to chronic hyperglycemia and hyperlidemia, which can both have negative effects on pancreatic β-cell function. The effects of hyperglycemia on β-cell function have become better understood while the deleterious effects of hyperlipidemia on β-cells are less well defined. The negative effects of hyperlidemia were suggested by studies on Zucker diabetic fatty (ZDF) rats, an animal model that combines obesity and type 2 diabetes. These studies revealed that β-cell function was impaired, but functional impairment was preceded by an increase in the plasma concentration of FFAs and the accumulation of triglycerides in pancreatic islets (85), leading to the proposal that FFAs contribute to β-cell pathology. Obesity and type 2 diabetes are associated with the chronic elevation of circulating free fatty acids (FFA) in humans (86).

The deleterious effects that FFAs exert on the β-cell have been documented and include a decrease in glucose-stimulated-insulin secretion (GSIS) (71), decreased mitochondrial membrane potential, and decreased ATP content (87;88). It has also been suggested that elevated FFAs decrease insulin gene expression in the presence of high glucose concentrations (89;90). However, the hypothesis that the negative effects of FFAs on insulin gene expression require elevated glucose has been challenged. A recent study reported that lipidss cause

decreased GSIS, insulin content, and increased triglyceride content in human islets independently of the negative effects of glucotoxicity (91). It remains unclear whether glucotoxicity is required for the manifestation of the negative effects of FFAs on insulin gene expression but it is clear that long-term exposure to FFAs results in the impairment of β-cell function.

We have previously demonstrated that the mitogen-activated protein kinases ERK1/2 are components of the mechanism by which glucose stimulates insulin gene expression. ERK1/2 are required for glucose-dependent insulin transcription. The inhibition of ERK1/2 activity inhibits glucose stimulation of the rat insulin I promoter and the E2A3/4 element. Glucose-induced insulin transcription requires the phosphorylation of certain transcription factors, Beta2/NeuroD1 and PDX-1, by ERK1/2. The phosphorylation of these transcription factors results in an increase in their functional activity (84). ERK1/2 help to transduce the glucose signal to insulin gene transcription. In the present study we examined the effects of FFAs and other nutrients on ERK1/2 activity and function.

**Material and Methods**

*Materials*- PKC inhibitors were purchased from Calbiochem. Glucose, forskolin, oleate, and palmitate were purchased from Sigma. Rosiglitazone and GW7845 were kind gifts from Dr. Joyce Repa, Assistant Professor Department of

Physiology UTSW. JNK antibodies were purchased from Santa Cruz and phospho-JNK antibodies were purchased from Cell Signaling.

*Cells and FFA Incubations* - INS-1 cells, either from early passages or subclones selected for increased glucose-stimulated insulin secretion kindly provided by Chris Newgard (6), were grown in RPMI 1640 medium containing 10% fetal bovine serum, 0.5 mM Hepes, pH 7.4, 10.2 mM L-glutamine, 50 mM sodium pyruvate, and 2.5 mM β mercaptoethanol {25}. Cells that were 60-80% confluent were pre-incubated for 1-2 h in Krebs-Ringer-bicarbonate-Hepes (KRBH) with 0 or 2 mM glucose prior to treatment. After treatment with the agents indicated in figure legends, the medium was removed and cells were washed with cold phosphate-buffered saline and harvested in 0.2 ml cold lysis buffer (50 mM Hepes, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.2 μg/ml phenylmethylsulfonyl fluoride, 0.1 M NaF, 2 mM $Na_3VO_4$, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, Frodin, 1995). After 20 min on ice, supernatants were collected following centrifugation for 10-30 min at 14,000 rpm in an Eppendorf microfuge and were stored at -80$^{o}$ C. FFAs bound to BSA were added directly to the medium of the cells that were to be preincubated at the specified concentrations. Because FFAs were bound to BSA, the corresponding amounts of BSA alone were added to a second group of cells as controls.

*Measurement of ERK1/2 Activity* - Equal amounts of lysate proteins (20-40 μg) were resolved in 10% polyacrylamide gels in sodium dodecyl sulfate (SDS) and subjected to electrophoresis. Proteins were transferred to nitrocellulose at 700

mA for 1.5 h at 4°C. Membranes were incubated in 5% nonfat milk/0.05% Tween in Tris-buffered saline (TBS) for 1 h, then in 1:3000 anti-phosphoERK1/2 antibody (BioSource or Sigma) in 1% nonfat milk/1% bovine serum albumin/0.05% Tween-TBS for 2 h, and finally in 1:5000 anti-rabbit IgG in 1% nonfat milk/1% BSA/0.05% Tween-TBS for 1 h at room temperature. Membranes were washed twice in 0.05% Tween-TBS and twice in TBS. After detection of phosphorylated ERK1/2 bands (43 kD and 41 kD, respectively) by enhanced chemiluminescence and autoradiography, membranes were stripped in 62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS for 30 min at 60°C, rinsed with 0.05% Tween-TBS, and immunoblotted as above with 1:5000 Y691 anti-ERK1/2 rabbit polyclonal antibody

*Transfections and Insulin Transcription Assays*- Cells that were to be transfected were rinsed once with PBS and then with serum-free culture medium containing DNA and transfection reagents (Fugene). The cells were exposed to this transfection cocktail for 16 h. The transfection cocktail was removed and replaced with normal cell culture medium. Following 2 h equilibration in culture medium, the solution was replaced with a solution composed of RPMI 1640 and 0.1% fetal bovine serum (FBS). After overnight incubation, the medium was removed and replaced with KRBH and the cells were exposed to the indicated pharmacological agents for 4 h. The cells were then lysed with passive lysis buffer and and assayed for luciferase-catalyzed photoemissions using a luciferase assay kit (Promega).

*Nuclear Localization Experiments*- INS-1 cells from 60 mM dishes were pelleted by centrifugation at 1500 x g for 5 minutes. The pellet was resuspended in 200 ul cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1mM DTT; 0.5 mM PMSF). The cells were placed on ice for 15 minutes; then 12.5 ul of 10% Nonidet NP-40 was added and the cells were vigorously vortexed for 10 seconds. The homogenate was centrifuged for 30 seconds in a microfuge. The supernatant containing the cytoplasm and RNA was transferred to another tube. The nuclear pellet was resuspended in 50 ul of ice cold buffer C (20 mM HEPES pH7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM DTT; 1 mM PMSF) (Schreiber and Schaffner 1989).

*Preparation of FFA/BSA Complex Solution* -A 10 mM solution stock of oleate and a 5 mM stock of palmitate were prepared as follows. The oleate and palmitate were added to 0.8 ml of ethanol and mixed well. 5 M NaOH (40µl) was added, and ethanol in the solution was evaporated with a stream of $N_2$ until the ethanol was evaporated. 4 ml of 0.9% NaCl was added to the solid material. The material was mixed with a stir bar under $N_2$. Once the solution started stirring, 4.16 ml of ice cold 24% BSA was added. The solution was covered and stirred for 10 minutes at room temperature. The solution was brought to a final volume of 10 ml with the addition of 1.84 ml of 0.9% NaCl. The solution was then stored in 0.2 ml aliquots under $N_2$ at –20° C.

**RESULTS**

*Nutrients activate ERK1/2 in INS-1 cells*- We have previously shown that ERK1/2 can be activated by glucose and this activation requires the metabolism of glucose (76). With this in mind, we wanted to determine if there were any metabolites that could induce ERK1/2 activity. The effects of citrate and pyruvate, two major products of glucose metabolism, on ERK1/2 were observed. In these experiments ERK1/2 activity was monitored in cell lysates with antibodies that selectively recognize the doubly phosphorylated, active forms of the kinases. Citrate and pyruvate were both able to cause ERK1/2 activation in a manner similar to glucose (Figure 3.1).

This result caused us to investigate the idea that ERK1/2 activation was a direct result of the increased ATP production, which is an effect of glucose metabolism that regulates β-cell function. We used a pharmacological approach to address this question. Phenylacetic acid (PAA), an inhibitor of pyruvate carboxylase, was used. PAA has been shown to inhibit glucose-stimulated insulin secretion (92). PAA inhibited glucose-induced ERK1/2 activation, as well as the activation of ERK1/2 by citrate and pyruvate. We also attempted to interrupt an arm of the tricarbozylic-acid cycle that would not interfere with ATP production. We attempted to disrupt the production of malonyl-CoA. This was achieved by the use of gemfibrozil and clofibrate. Gemfibrozil and clofibrate, peroxisome proliferator activated receptor agonists and commonly used hypolipidemic drugs, rapidly activate AMP-activated protein kinase (93). The AMP-activated protein kinase phosphorylates

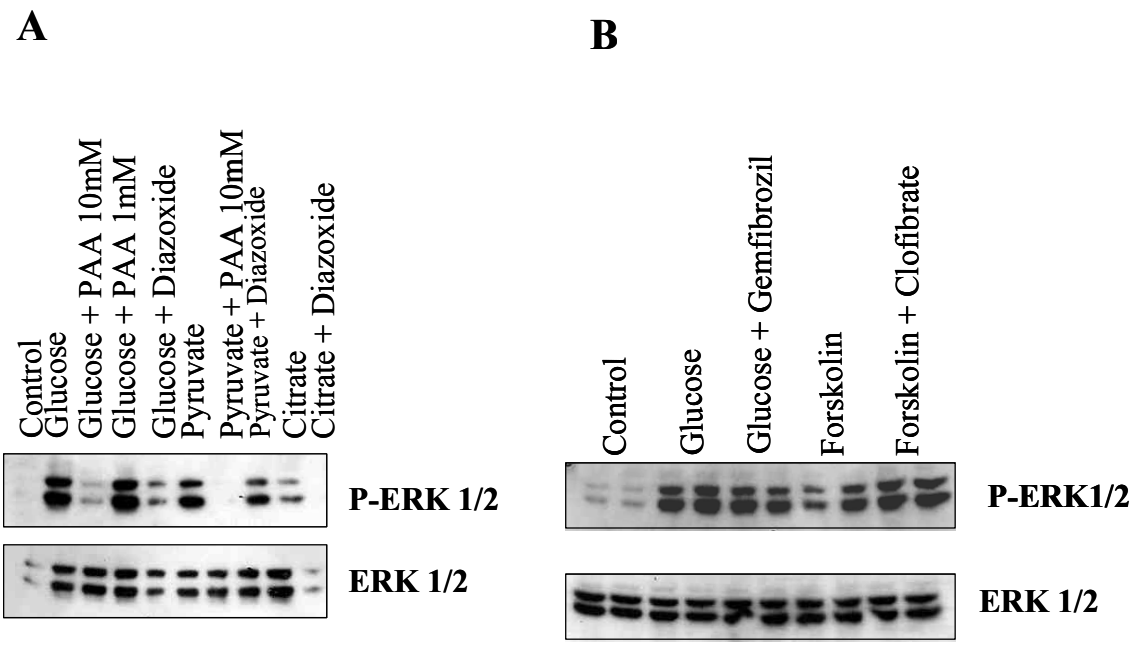



**Figure 3.1. Glucose metabolites activate ERK1/2.** INS-1 cells were preincubated in KRBH for 2 h and either untreated or exposed to either 15 mM glucose, 5 mM citrate, or 5 mM pyruvate. A. 10 mM phenylacetic acid (PAA) or 1 mM diazoxide was added for 15 min before the addition of glucose, citrate, or pyruvate. Lysate proteins were immunoblotted as described in Methods. B. INS-1 cells were treated with 5mM clofibrate or gemfibrozil prior to glucose stimulation. Experiments were repeated a minimum of 2-3 times.

acetyl coA carboxylase (ACC) (92), which causes it to be inactive. Inactivation of ACC prevents the conversion of acetyl CoA to malonyl-CoA. When this metabolic step was inhibited, it did not affect glucose-induced activation of ERK1/2. This is consistent with the idea that ATP production is the crucial product of glucose metabolism that is required for ERK1/2 activation.

*FFAs cause constitutive ERK1/2 activity*- Initially we wanted to confirm that FFAs were having direct effects on INS-1 cells. We stained cells with Oil Red O to visualize intracellular lipids. Oil Red O staining was performed on INS-1 cells after 24 h, 48 h, 72 h and 7 days incubation with FFAs. After 24 hours a small amount of intracellular lipid was detectable. The amount of intacellular lipid appeared to increase linearly and there was not a significant difference in the amount of intracellular lipid when observing 72 h or 7 days of FFA exposure (data not shown).

Once it was confirmed that the FFAs had led to intracellular lipid accumulation, we examined the ERK1/2 activity in cells that had been pretreated with FFAs for 72 hours. In these experiments ERK1/2 activity was monitored in cell lysates with antibodies that selectively recognize the doubly phosphorylated, active forms of the kinases. Total ERK1/2 immunoreactivity demonstrated that roughly equal amounts of the two proteins were present in each lane. INS-1 cells that were treated with BSA displayed no detectable ERK1/2 activity after two hours of starvation but did exhibit ERK1/2 activity in response to glucose stimulation. This behavior is consistent with cells that have not had any

pretreatment (Figure 3.2). However, cells that were pretreated with FFAs showed an increase in basal ERK1/2 activity. In addition, these cells did not exhibit a significant increase in ERK1/2 activity in response to glucose treatment. This was apparently because the basal activity was high, and it was difficult to observe a substantial increase in ERK1/2 activity over that level.

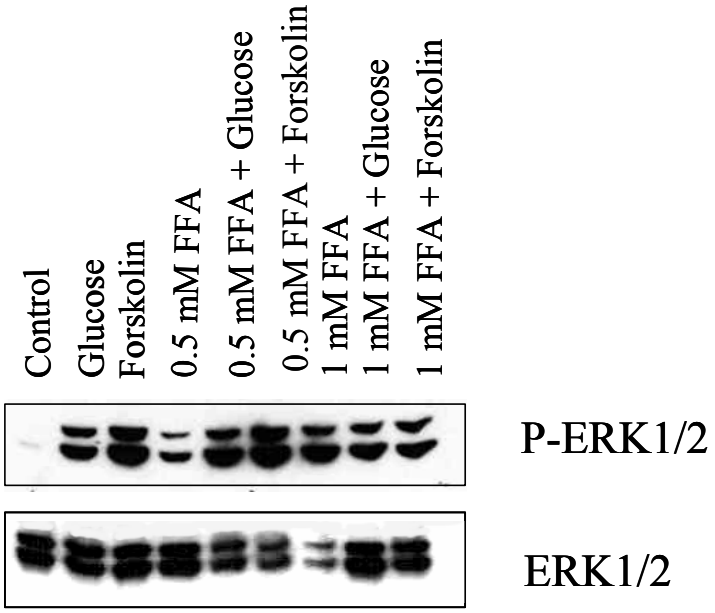



**Figure 3.2. FFAs induce constitutive ERK1/2 activity.** INS-1 cells were preincubated with the indicated concentration of FFAs for 72 h. The cells were incubated in KRBH for 2 hours following the FFA preincubation. The cells were then stimulated for 20 minutes with 15 mM glucose or 10 uM forskolin. Lysate proteins were immunoblotted as described in Methods. Experiments were repeated a minimum of 3 times.

These data show that FFAs have a long term effect on ERK1/2 activity. To determine if the effects of FFAs were specific for ERK1/2, we examined the activity of another MAP kinase group, the c-Jun amino-terminal kinases (JNKs). These kinases are thought to play a role in obesity and insulin resistance (94). JNK activity was examined in INS-1 cell lysates which had been preincubated with FFAs for 72 hours. In these experiments JNK activity was monitored in cell lysates with antibodies that selectively recognize the phosphorylated, active forms of the JNK (Figure 3.3). FFAs had no effect on JNK activity in these cells following longterm exposure. This demonstrates the specificity of FFA action on ERK1/2 in INS-1 cells.

*Free fatty acids have negative effects on insulin transcription*- It has been shown that FFAs can impair insulin secretion (71). Here we show that FFAs also inhibit insulin transcription. It has been reported that insulin gene expression is inhibited by 0.5 mM palmitate in the presence of 16.7 mM glucose (90). We used 1mM FFAs (2:1 oleate to palmitate) (Unger laboratory protocol) in the presence of 11 mM glucose due to the fact that INS-1 cells are cultured in 11mM glucose. After 48 hours of incubation with FFAs, we were unable to detect any insulin promoter activity (Figure 3.4a). We shortened the FFA incubation from 48 hours to 24 hours and a significant decrease in promoter activity was observed (Figure 4b). We also wanted to determine if some of the PPARγ agonists which are used clinically as antidiabetic agents could prevent the negative effects of FFAs on insulin transcription. We used the PPARγ agonists rosiglitazone and GW7845.

These PPARγ agonists did not prevent the negative effects of FFAs on insulin transcription. There was an obvious decrease in insulin gene expression as a direct result of FFA incubation; cells treated with U0126 also displayed decreased in insulin gene transcription.

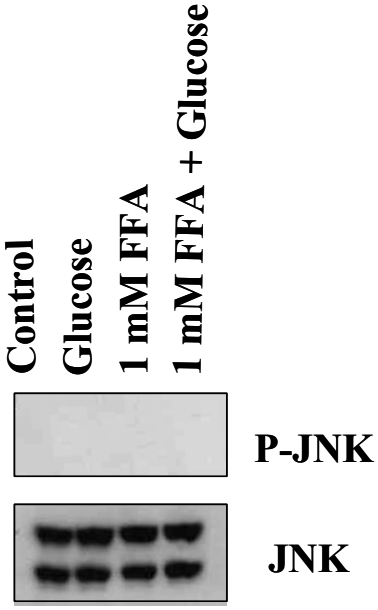



**Figure 3.3. FFAs do not increase JNK activity in INS-1 cells.** INS-1 cells were treated as in figure 3. The lysates were probed with an antibody, which detects the active form of JNK.

**A**

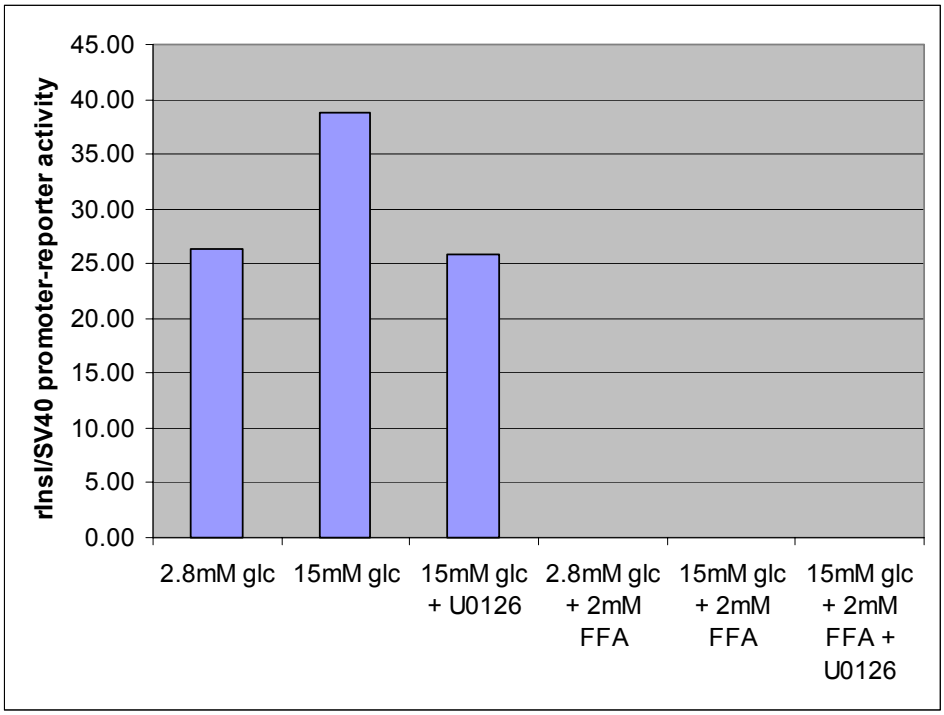



**Figure 3.4. FFAs inhibit insulin gene transcription.** INS-1 cells were transfected with pGL3-rINSI and then incubated as described in Materials and Methods. A. Cells were preincubated in FFA for 48 hours at the indicated concentration. Cells were treated with U0126 (MEK inhibitor), rosiglitazone, and GW7845 for 30 minutes as indicated prior to the stimulation by glucose for 4 hours.

**B**

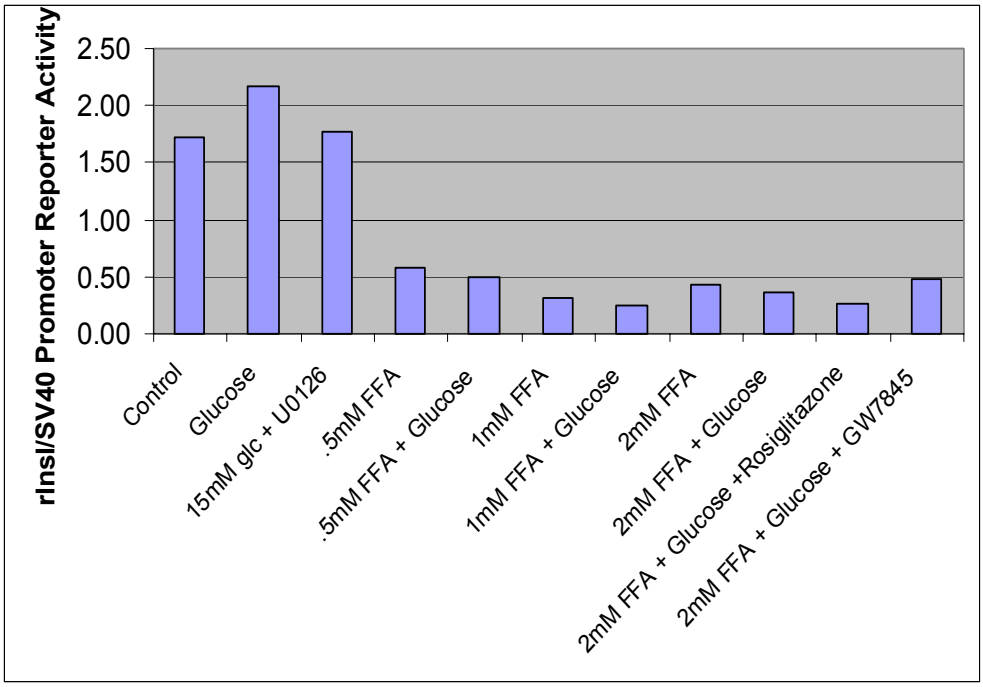



**Figure 3.4. FFAs inhibit insulin gene transcription.** INS-1 cells were transfected with pGL3-rINSI and then incubated as described in Materials and Methods. B. INS-1 cells were transfected as indicated in panel A and preincubated with FFAs for 24 hours; rosiglitazone and GW7845 were also added to some assays 30 minutes prior to glucose stimulation for 4 hours.

*Effects of FFA on ERK1/2 are reversible*- It was of great interest to us to determine if the effects of FFAs on ERK1/2 activity were reversible or they resulted in part from irreversible damage to the cells. Thus, we examined ERK1/2 activity in INS-1 cells following the removal of the preincubated FFAs and compared this ERK1/2 activity with the ERK1/2 activity in cells that remained in FFAs (figure 3.5). We were able to determine that at 24 hours the constitutive activity of ERK1/2 had dissipated and glucose responsiveness was restored. Perhaps this result is not surprising considering some diabetic patients can improve their glucose homeostasis with changes in diet.

*FFAs alter normal ERK1/2 nuclear localization*- We next wanted to determine whether or not the constitutive activity of ERK1/2 that was caused by FFAs altered the normal localization of ERK1/2. In many cases, ERK1/2 nuclear localization is necessary so that ERK1/2 can phosphorylate its substrates. The phosphorylation of some of the transcription factors required for insulin gene expression by ERK1/2 occurs in the nucleus (84). Furthermore we showed previously that glucose promotes the nuclear localization of ERK1/2.

With this in mind we wanted to examine ERK1/2 nuclear localization in INS-1 cells following preincubation with FFAs. Nuclear fractionation experiments were performed on the INS-1 cell lysates, which were preincubated without or with FFAs. The results showed that FFAs severely altered the normal

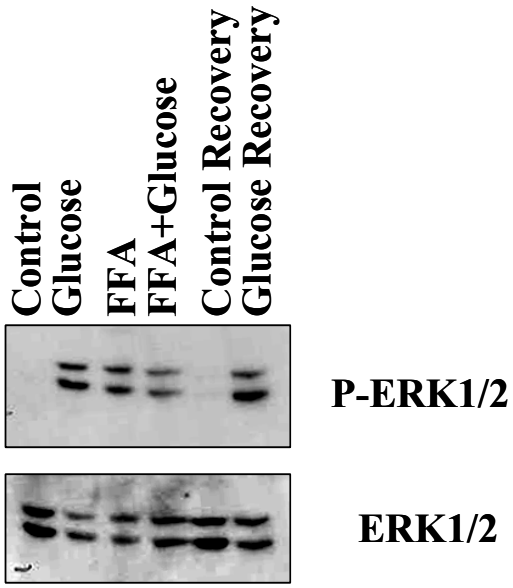



**Figure 3.5. FFA-induced constitutive activity of ERK1/2 is reversible.** INS-1 cells were preincubated with FFAs for 72 hours. The medium containing FFAs was replaced with normal culture medium for 24 hours. Cells were incubated in KRBH for 2 hours then stimulated with 15mM glucose. Lysate proteins were immunoblotted as described in Methods. This experiment was repeated three times.

pattern of glucose-induced ERK1/2 nuclear localization (Figure 3. 6). Cells preincubated with BSA contained no detectable amounts of active ERK1/2 in the nucleus without stimulation with glucose. Once these cells were stimulated with glucose, there was a dramatic increase in the amount of nuclear ERK1/2. However, in the cells that were preincubated with FFAs, this dramatic change in active ERK1/2 distribution was not observed. The cells that were preincubated with FFAs exhibited a significant amount of active ERK1/2 in the nucleus in the unstimulated state. Also, upon stimulation of the cells that were preincubated with FFAs with glucose, there was no longer a detectable increase in the amount of active ERK1/2 in the nucleus.

*FFA-induced constitutive activity of ERK1/2 is PKC independent-* Diacylglycerol (DAG) is a lipid second messenger and activates PKC (95), and DAG can also be produced indirectly from FFAs. Therefore we considered the possibility that there may be a potential role for a PKC isoform in the pathway which leads to FFA-induced constitutive activity of ERK1/2. Phosphatidylcholine, which contains both saturated and mono-unsaturated fatty acids, can be a source of DAG (96). DAG can directly activate PKC by binding to the C1 domain of PKC (95).

To determine if PKC was involved in FFA induced ERK1/2 activity. I used pharmacological inhibitors of several PKC isoforms. As a control I wanted to confirm that PKC was not involved in glucose-induced ERK1/2 activation in INS-1 cells. When using pharmacologic agents to determine if PKC is involved in a signaling pathway, it is very important to determine what concentration will best inhibit PKC while minimizing the inhibition of other enzymes. We

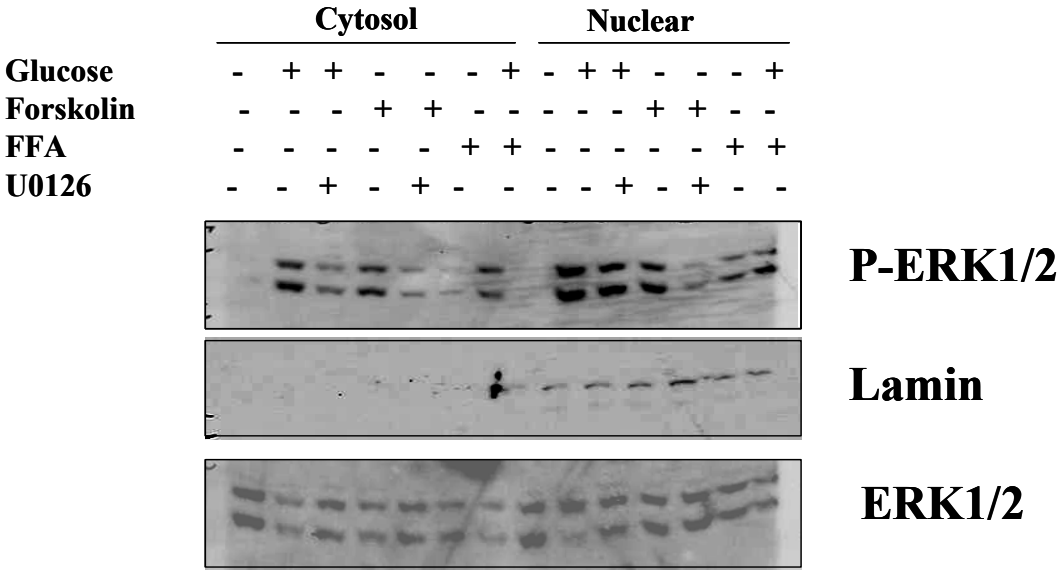



**Figure 3.6. FFAs alter normal ERK1/2 nuclear localization.** INS-1 cells were preincubated in FFAs for 72 hours then incubated in KRBH for two hours. Cytosolic and nuclear fractions were separated as described in Methods. The nuclear and cytosolic fractions were then were immunoblotted as described in Methods. Experiments were repeated a minimum of 3 times.

determined the minimum concentration of PKC inhibitors needed to inhibit PMA-induced ERK1/2 activity and used that concentration to evaluate mechanisms of other stimuli. We used a panel of inhibitors with distinct specificities among PKC isoforms (Table 3.1). Prior to glucose stimulation we treated the cells with PKC inhibitors for 30 minutes (Figure 3.7a). I used glucose and forskolin as control stimuli to compare to PMA-induced ERK1/2 activation. Our data suggest that PKC is not involved in glucose-induced ERK1/2 activation. We then tested the idea that FFA-induced ERK1/2 activation was regulated by PKC. Once again INS-1 cells were preincubated with FFAs and then treated with a variety of PKC inhibitors. The results that we obtained were a bit surprising. None of the PKC inhibitors inhibited FFA induced ERK1/2 activity (igure 3.7b).

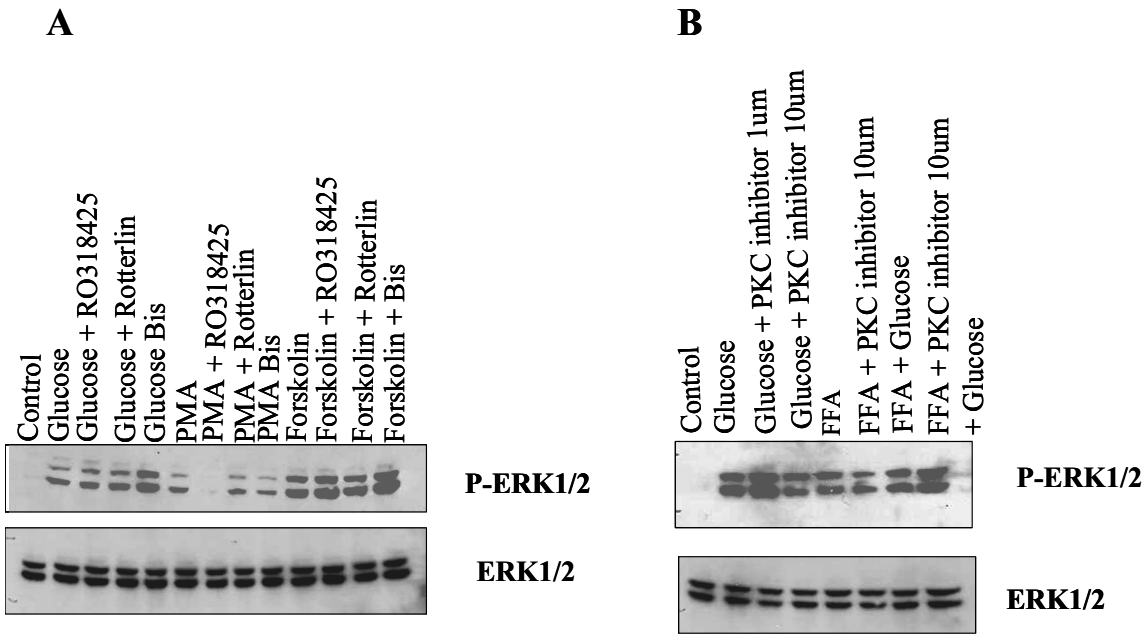



**Figure 3.7. FFA-induced constitutive activity and glucose-induced ERK1/2 activity is PKC independent. A.** INS-1 cells were incubated in KRBH for 2 hours. Then the PKC inhibitors were added 20 minutes prior to the addition of PMA, glucose, or forskolin. Lysate proteins were immunoblotted as described in Methods. B. INS-1 cells were preincubated with FFAs as described in figure 3. PCK Inhibitor, R0318425, was added 20 minutes prior to glucose stimulation. The data are representative of at least three similar experiments.

**Table 3.1**

Table 1: Isozyme Specificities of Selected Protein Kinase C Inhibitors ($IC_{50}$ values are in µM)

| Product | Cat. No. | $PKC_{\alpha}$ | $PKC_{\beta}$ | $PKC_{\beta I}$ | $PKC_{\beta II}$ | $PKC_{\gamma}$ | $PKC_{\delta}$ | $PKC_{\varepsilon}$ | $PKC_{\zeta}$ | $PKC_{\mu}$ | $PKC_{\eta}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Bisindolylmaleimide I (Gö 6850) | 203290 | 0.008 | – | 0.018 | – | – | 0.21 | 0.132 | 5.8 | – | – |
| CGP41251 | – | 0.024 | – | 0.017 | 0.032 | 0.018 | 0.360 | 4.50 | >1000 | – | 0.060 |
| Gö 6976 | 365250 | 0.0023 | – | 0.006 | – | – | – | – | – | 0.02 | – |
| Gö 6983 | 365251 | 0.007 | 0.007 | – | – | 0.006 | 0.01 | – | 0.06 | 20 | – |
| LY333531 | – | 0.360 | – | 0.0047 | 0.0059 | 0.400 | 0.250 | 0.600 | $>10^6$ | – | 0.052 |
| Ro-31-7549 | 557508 | 0.053 | | 0.195 | 0.163 | 0.213 | – | 0.175 | – | – | – |
| Ro-31-8220 | 557520 | 0.005 | – | 0.024 | 0.014 | 0.027 | – | 0.024 | – | – | – |
| Ro-31-8425 | 557514 | 0.008 | – | 0.008 | 0.014 | 0.013 | – | 0.039 | – | – | – |
| Ro-32-0432 | 557525 | 0.009 | – | 0.028 | 0.031 | 0.037 | – | 0.108 | – | – | – |
| Rottlerin | 557370 | 30 | 42 | – | – | 40 | 3 - 6 | 100 | 100 | – | – |
| Staurosporine | 569397 | 0.028 | – | 0.013 | 0.011 | 0.032 | 0.028 | 0.025 | >1.5 | – | – |
| UCN01 | – | 0.029 | – | 0.034 | – | 0.030 | 0.590 | 0.530 | – | – | – |

Calbiochem, Inhibitor SourceBook, EMD Biosciences 2003/2004

**DISCUSSION**

FFAs have negative effects on insulin secretion and insulin transcription. Here we have shown that FFAs directly impact ERK1/2. The exact mechanism of action of FFAs is not yet defined. Some groups have suggested that ceramide synthesis causes the decrease in insulin transcription as a result of chronic exposure of FFAs to pancreatic β-cells (89). Ceramide has also been shown to inhibit ERK1/2 activity in human embryonic kidney 293 cells (HEK 293) through the inhibition of PKC (97). This is a very interesting hypothesis, but we do not think that this is the mechanism that leads to constitutive ERK1/2 activity in INS-1 cells. This is because all of our data suggest that PKC isoforms are not involved in the constitutive activity of ERK1/2 that is observed as a result of the chronic exposure to FFAs. It is also important to keep in mind that ceramide inhibits ERK1/2 activity in 293 cells, while a stimulatory effect is observed in INS-1 cells.

The apparent lack of a role for PKC suggests that the FFA-induced constitutive activity of ERK1/2 utilizes a novel pathway. This idea is consistent with our findings. ERK1/2 constitutive activity induced by FFA required 24 hours to be reversed and could not be relieved with the use of common antidiabetic drugs, these drugs have been shown to reverse certain other inhibitory effects of FFAs. The reversibility of ERK1/2 activation by FFAs is consistent with the idea that glucose homeostasis can be improved in diabetic patients with a change in diet, that reduces fat intake.

The effects of FFAs have on insulin transcription may be a result of decreased

transactivation activity of the insulin promoter. Our nuclear fractionation experiments detected altered nuclear localization of ERK1/2 in response to elevated glucose. We have previously reported that ERK1/2 phosphorylate insulin transcription factors that are required for maximal glucose-induced insulin transcription. Since FFAs alter ERK1/2 nuclear localization, one must consider the idea that this altered nuclearlocalization may have a direct effect on the phosphorylation of one or more of E47 and/or NeuroD1, insulin transcription factors needed for maximum transactivating activity of the insulin promoter. It is possible that prolonged exposure cues to active ERK1/2 induces the degradation of the phosphorylated transcriptions factors.

In conclusion, activation of ERK1/2 are most commonly associated with but not limited to cell proliferation. Here we have demonstrated that FFAs alter ERK1/2 activity and its nuclear localization in INS-1 cells and our data suggest the effects of FFAs on ERK1/2 result in impaired insulin transcription. The mechanism by which FFAs alter ERK1/2 is not defined. Perhaps once this pathway is defined, it may offer novel drug targets which will aid in the treatment of type 2 diabetes.

## Chapter 4: Conclusions

These studies have aided in the delineation of the mechanism of glucose-induced ERK1/2 activation. One of the key players that was determined to be part of the glucose-induced ERK1/2 pathway was calcineurin. When the activity of calcineurin is inhibited, there is no detectable ERK1/2 activity in response to elevated glucose concentrations. These data are very interesting when one considers the number of transplant patients that develop diabetes after surgery. One of the immunosupressants that is used to prevent organ rejection is FK506, the calcineurin inhibitor that we found blocks glucose-induced ERK1/2 activity. There are many steps in the glucose-induced ERK1/2 activity pathway that remain to be defined. Currently, it is known that glucose metabolism leads to membrane depolarization and calcium influx, both of which are required for insulin secretion as well as insulin transcription. These events are also required for glucose-induced ERK1/2 activity.

The target of calcineurin still eludes us. Calcineurin may be acting on the ryanodine or the $IP_3$ receptor, which would cause intracellular calcium release. This observation is supported by data that we have collected which shows intracellular calcium release is required for ERK1/2 activation. Calineurin may be part of a calcium induced calcium release system. Upon depolarization of the β-cell, the calcium influx that follows may cause calcineurin activation which then stimulates intracellular calcium release.

Another issue that was addressed in this report was the action of FFAs on ERK1/2 activity. We had previously shown that ERK1/2 phosphorylate certain

transcription factors whose activity is required for maximal insulin gene transcription. Chronic exposure of pancreatic β-cells to elevated level of FFAs leads to decreased insulin secretion and insulin transcription. Our experiments have shown that this chronic exposure alters the normal glucose-induced nuclear localization of ERK1/2 and causes constitutive activity of ERK1/2. The direct effect of ERK1/2 constitutive activity is not well understood. This constitutive activity may result in a negative feedback loop, which actually signals the cell to stop producing insulin. This constitutive activity also may be a direct result of extracellular signals that inform the cell that the levels of circulating of nutrients are high and insulin production is needed. Even though the cells sense the need for increased insulin production, the chronic exposure to FFAs may exhaust the insulin-producing pathway.

The altered nuclear localization of ERK1/2 is perhaps the result of the constitutive activity of ERK1/2. When the nuclear localization of ERK1/2 is altered it is plausible to assume that the normal phosphorylation of ERK1/2 targets is also altered. The FFA-induced constitutive activity of ERK1/2 may directly inhibit the phosphorylation of E47 and Beta2. Future experiments should examine the phosphorylation of the previously mentioned transcription factors. It should also be determined if the chronic exposure of FFAs results in a decrease in the transactivating activity of the factors that regulate insulin promoter.

Type 2 diabetes involves both insulin resistance and decreased insulin secretion. The data reported in this study is related to the decrease in insulin secretion observed in type 2 diabetes. Although ERK1/2 do not have a direct

effect on insulin secretion, the role it appears to play in insulin transcription may result in decreased insulin production in pathophysiological settings. This decrease in insulin production could lead to the decrease in insulin secretion.

It is possible that the simplest explanation for the negative effects of FFA on the β-cell and ERK1/2 activity is the appropriate one: all cells have checks and balances that work well within certain ranges, however, once the range has been exceeded, the appropriate regulatory control is lost.

In this study key components of the pathway regulating glucose-induced activation of ERK1/2 were defined. The effects of FFAs on ERK1/2 activity were also reported. Since ERK1/2 activity is required for maximal insulin gene transcription, this suggests that ERK1/2 are vital members of the system which regulates glucose homeostasis. Further studies should define the pathway of glucose-induced ERK1/2 activation; determine the targets of calcineurin; elucidate the mechanism by which chronic exposure of FFAs leads to decreased insulin transcription. These insights will contribute to the understanding of the pathogenesis of type 2 diabetes and possibly reveal novel targets for antidiabetic drugs.

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# Vita

Don Arnette was born in New Orleans, Louisiana on July 28, 1973 the son of Don and Katie Arnette. After completing his high school education at Red Oak High School, he attended the University of Texas at Arlington. It was not long before his basketball endeavors took him from UTA to a junior college to West Virginia University and back to Texas. He obtained his Bachelor of Science degree with a major in Biology from the University of North Texas in 1997. During the next two years he worked as a technician for Dr. Melanie Cobb. In August of 2000 he entered the Graduate School of Biomedical Sciences at the UT Southwestern in Dallas.

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