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IL-1 β Amplification of Nitric Oxide Production and Its Inhibitory Effects on Glucose Induced
Early Growth Response-1 Expression in INS-1 Cells

A thesis
presented to
the faculty of the Department of the Biology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biology

by
Ada D. Young
August 2012

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Keywords: egr-1, glucose stimulation, interleukin- 1 β , insulin dependent diabetes mellitus

ABSTRACT

IL-1 β Amplification of Nitric Oxide Production and Its Inhibitory Effects on Glucose Induced Early Growth Response-1 Expression in INS-1 Cells

by

Ada Young

The pathophysiology of cytokines released by infiltrating white blood cells upon pancreatic beta cells is not fully understood. Early growth response gene-1 (Egr-1) expression is specifically and transiently up regulated in pancreatic beta cells in response to glucose. We hypothesized that interleukin-1 beta (IL-1 β) induction of nitric oxide alters glucose induced Egr-1 transcription levels. Egr-1 levels were assessed via western blot, nitric oxide was measured with a Griess Reagent kit and insulin levels via ELISA. Glucose induced both insulin and Egr-1 production in INS-1 cells. IL-1 β dose dependently increased nitric oxide production over time and significantly attenuated glucose induced Egr-1 expression. Sodium nitroprusside dose dependently reduced glucose induced Egr-1 production. The data suggest a strong relationship between IL-1 β induced nitric oxide production and the reduction of glucose stimulated Egr-1 production. The pathways altered by this cytokine could provide a better understanding of the pathophysiology leading to pancreatic beta cell death.

DEDICATION

This thesis is dedicated to Peggy Jenca for being one of the most persevering and inspiring women I've ever known.

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CONTENTS

	Page
ABSTRACT.....	2
DEDICATION.....	3
ACKNOWLEDGEMENTS.....	4
LIST OF FIGURES.....	7
Chapter	
1. INTRODUCTION.....	8
Development of Insulin Dependent Diabetes Mellitus.....	10
Role of Macrophages and IL-12 Production in the Development of IDDM.....	10
Role of Immune Cell Cytokine Signaling in the Development of IDDM.....	12
Interleukin 1-beta (IL-1 β) Induces Nitric Oxide Production.....	13
Role of Egr-1 in the Pancreatic Beta Cell.....	15
2. PRETREATMENT OF INS-1 CELLS WITH IL-1B INHIBITS GLUCOSE- STIMULATED EXPRESSION OF EGR-1.....	18
Abstract.....	19
Introduction.....	21
Research Design and Methods.....	22
Cell Culture.....	22
Sample Collection and Preparation.....	23
Western Blot.....	23
Measurement of Nitrite and Insulin.....	24

Chapter	Page
Experiment 1. Effect of Glucose on Insulin Production and Expression of Egr-1 In INS-1 Pancreatic Beta Cells.....	24
Experiment 2. Effect of IL-1 β Treatment on Nitric Oxide Production Over Time In INS-1 Cells.....	25
Experiment 3. Effect of IL-1 β Treatment Over Time On Glucose’s Ability to Induce Egr-1 Expression.....	25
Experiment 4. Effect of Increasing Sodium Nitroprusside Concentrations on Glucose’s Ability to Induce Egr-1 Expression.....	26
Statistical Analysis.....	26
Results.....	27
Discussion.....	33
References.....	37
REFERENCES.....	41
APPENDICES.....	49
Appendix A: Cell Processing.....	49
Appendix B: Protein Assay.....	50
VITA.....	51

LIST OF FIGURES

Figure	Page
1.1 Glucose stimulated insulin production and exocytosis.....	9
1.2 Pathways induced by IL-1 β and Glucose affecting Egr-1.....	17
2.1 Glucose significantly stimulates insulin production from INS-1 cells.....	27
2.2 Effect of glucose on the expression of Egr-1 over time.....	28
2.3 Administration of IL-1 β (U/ml) dose-dependently increases nitric oxide production from INS-1 cells.....	29
2.4 Pre-treatment of INS-1 cells with IL-1 β attenuates the glucose stimulated increase in Egr-1 expression.....	30
2.5 Sodium nitroprusside dose-dependently inhibits glucose stimulation of Egr-1 expression from INS-1 cells.....	32

CHAPTER 1

INTRODUCTION

The pancreas is a glandular organ that is intricately involved in the gastrointestinal digestive process. It regulates the uptake of carbohydrates, lipids, and proteins by releasing insulin to bind to specific insulin responsive tissues (Boron and Boulpaep 2003). The major targets of insulin include liver, muscle, and adipose tissue (Nussey and Whithead 2001). The regulation of metabolism through the pancreas occurs via the binding of specific neurotransmitters by the exocrine gland cells and glucose by the endocrine gland cells, respectively (Boron and Boulpaep 2003). The endocrine portion of the pancreas contains beta cells found within the islets of Langerhans. Increased blood glucose stimulates the pancreatic beta cells to synthesize and secrete insulin into the blood stream (Boron and Boulpaep 2003). Once in circulation, insulin binds to specific target tissues, making them more receptive to glucose uptake (Nussey and Whithead 2001). Insulin also acts in an autocrine and paracrine fashion, binding to the beta cells it is released from and/or adjacent beta cells (Boron and Boulpaep 2003). It is the flux of islet beta cell binding to glucose and insulin that regulates insulin production and secretion within the pancreas.

Receptors specific for glucose (ex. GLUT 2) can bind and passively transport the sugar molecules into the cell depending upon the concentration gradient between the inside and the outside of the cell. Once glucose enters the cell, a key enzyme, glucokinase, initiates the process of glycolysis. This metabolic process affects production and secretion of insulin (Ashcroft 1980; German 1993). Oxidative glucose metabolism increases ATP within the cell, closing ATP

sensitive potassium channels leading to cellular depolarization via calcium influx (Figure 1.1). The increased concentration of cytosolic calcium is a very important component in the glucose-stimulated exocytosis of insulin granules (Szollosi et al. 2007). The breakdown of glucose for energy and the influx of calcium alter the concentrations of a number of key regulatory enzymes and in turn activate pathways that help promote insulin production and release and overall beta cell health. It is critical to the health of the pancreas and the organism as a whole for these pathways and molecules to interact in a balanced and consistent manner. A disruption or imbalance can lead to beta cell dysfunction and even affect the use of glucose throughout the body.

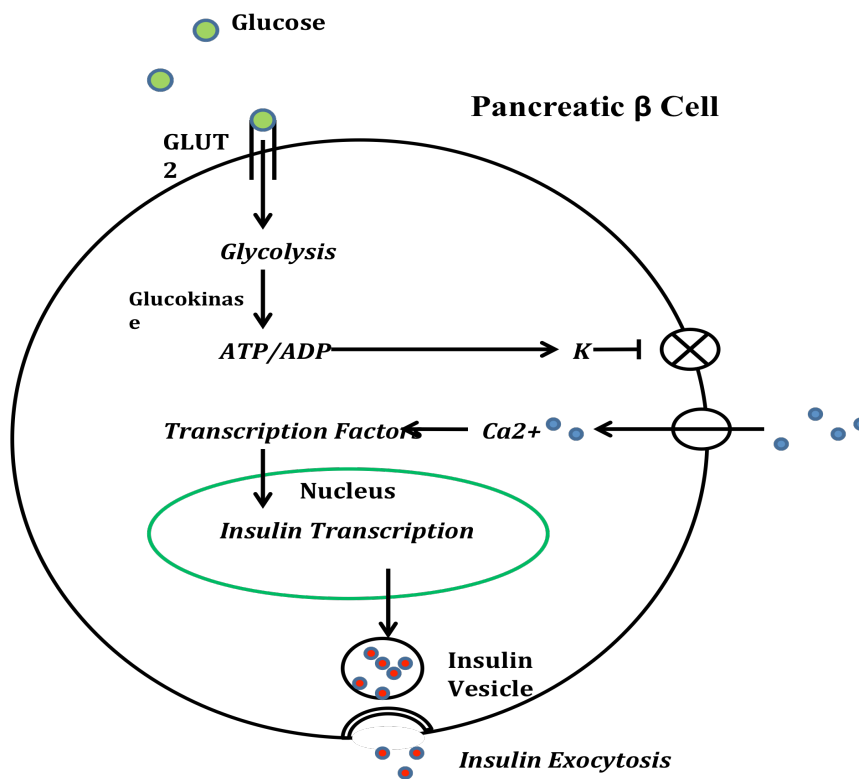


Figure 1.1 – Schematic model of glucose stimulated insulin production and exocytosis in a healthy pancreatic β cell (Adapted from Gottlob et al. 2001; Srinivasan et al. 2002; Szollosi et al. 2007).

Development of Insulin Dependent Diabetes Mellitus

Insulin-dependent diabetes mellitus (IDDM or Type 1 diabetes) is one of the most frequent chronic childhood diseases with a prevalence estimated to affect approximately 120,000 individuals less than 19 years-of-age and 300,000 to 500,000 individuals of all ages (LaPorte et al. 1995). The risk of devastating complications associated with this disease remain high and include such things as heart disease, stroke, high blood pressure, blindness, kidney disease, amputations, etc. Although great strides have been made towards understanding the pathogenesis of IDDM, therapies for its prevention have remained elusive.

Insulin-dependent diabetes mellitus is a macrophage-dependent, T Cell-mediated autoimmune disease characterized by the infiltration of the islet cells of the pancreas by immune cells (insulinitis) followed by massive destruction of insulin-producing pancreatic beta cells. Mediation of islet beta cell destruction involves the participation of a variety of immune/inflammatory cells in the insulinitis lesion, notably dendritic cells, macrophages and T lymphocytes, as well as cytokines produced by these cells (Thomas et al. 2000; Yoon and Jun. 2005; Lehuen et al. 2010). It is believed that the autoimmune response in IDDM involves some disturbance(s) in the immunoregulatory balance that leads to a dominance of Th1 cells and their respective cytokine production (Thivolet. 2002; La Torre et al. 2010; Lehuen et al. 2010).

Role of Macrophages and IL-12 Production in the Development of IDDM

A large body of evidence has been produced suggesting macrophages are a key part of the initial steps in the development of IDDM (Lee et al. 1988; Yoon and Jun. 1999; Alleva et al. 2000; Lehuen et al. 2010). Much of this evidence has been obtained through the use of mouse

and rat models. BioBreeding (BB) rats and nonobese diabetic (NOD) mice have been used extensively in this area of research due to their spontaneous development of autoimmune type 1 diabetes. It has been found in both BB rats and in NOD mice that macrophages infiltrate the pancreas during the initial phase of insulinitis (Karges et al. 1995; Lehuen et al. 2010). The recruitment of auto-reactive T-cells is dependent upon the activated macrophages and is necessary for the development of the disease (Jun et al. 1999; Lehuen et al. 2010). The pertinence of macrophages in the development of diabetes has been reproduced in a number of other studies and is thought to be accomplished through cytokine signaling between the T-helper cells (Th), macrophages, and beta cells (Karges et al. 1995; Jun et al. 1999; Thomas et al. 2000; Yoon and Jun. 2005; Eizirik et al. 2009; Lehuen et al. 2010).

The balance of Th1/Th2 cell populations has been observed to be an important factor in the development of immune dysfunction (Delovitch et al. 1997). The cytokine IL-12, released by macrophages is understood to be a key regulator in the promotion of Th1 development and has been found to be expressed at high levels in NOD mice macrophages (Alleva et al. 2000). Jun et al. (1999) found that macrophage depleted NOD mice had lower IL-12 expression which prevented diabetes from developing. When they added IL-12 back into the macrophage depleted NOD mice, they found a drastic increase in diabetes development. IL-12 has not only been found to stimulate Th1 activation but it has also been found to cause direct damage to beta cells (Lehuen et al. 2010). Based on these and other findings, it appears that the essential role of macrophages in the development of IDDM was due, in part, to their production of IL-12.

Role of Immune Cell Cytokine Signaling in the Development of IDDM

A substantial amount of evidence from studies using BB rats and NOD mice supports a critical role for T cells and macrophages in the development of IDDM (Rabinovitch and Suarez-Pinzon. 1998; Jun et al. 1999; Yoon et al. 2005; Leheun et al. 2010). Insulinitis is characterized by leukocyte infiltration of the pancreatic islets by a mixture of CD4⁺ and CD8⁺ lymphocytes, B-lymphocytes, macrophages, and dendritic cells in NOD mice and humans recently diagnosed with IDDM (Thomas et al. 2000; Yoon and Jun. 2005; Lehuen et al. 2010). As was previously described, macrophage secretion of IL-12 directs T helper cells to differentiate along the Th1 pathway and stimulates the production of a variety of proinflammatory (IL-1 β and TNF α) and Th1 cytokines (IL-2 and IFN- γ) (Jun et al. 1999; Alleva et al. 2000; Yoon and Jun. 2005). The potential role of these proinflammatory and Th1 cytokines in the development of diabetes has been the primary focus of numerous research studies over the past few decades (Jun et al. 1999; Alleva et al. 2000; Thomas et al. 2000; Yoon and Jun. 2005; Lehuen et al. 2010).

Activation of CD4⁺T cells and CD8⁺T cells to pancreatic autoantigens is induced by the binding of macrophages and dendritic cells and their consequential release of IL-12 (Yoon and Jun. 2005). Macrophage activated Th1 cells release cytokines, including IL-2, which signal white blood cells to migrate and respond to pancreatic beta cells (Rabinovitch and Suarez-Pinzon 1998). CD4⁺ T cells release IFN γ , acting in a cyclical fashion, inducing macrophages to become cytotoxic (Yoon and Jun 2005). The combined release of IFN γ by T-cells and cytotoxic macrophages activates other resting macrophages (Yoon et al. 1998). The large population of cytotoxic macrophages and active dendritic cells then release a series of detrimental cytokines to the beta cells including interleukin-1beta (IL-1 β), Tumor Necrosis Factor (TNF), and free

radicals (Uno et al. 2007). A number of studies have found IL-1 β and TNF damage islet cells and induce apoptosis (Thomas et al. 2000; Yoon and Jun. 2005; Uno et al. 2007; Schwarznau et al. 2009).

Tumor Necrosis Factor is a cytokine mainly produced by macrophages and T-helper cells. Green et al. (1998) found that the expression of TNF α in the beta cells of neonatal NOD mice caused diabetes development in all mice. Kagi et al. (1999) produced a TNFR-1 deficient NOD mouse model that developed insulinitis but not diabetes. Studies have found TNF α combined with IFN γ are very important factors in the destruction of beta cells and have often been studied in congruence with a macrophage-derived cytokine, IL-1 β (Campbell et al. 1988; Suk et al. 2001; Wachlin et al. 2003; Kim et al. 2007). Cumulatively, these findings support the involvement of many immune cells and their respective cytokines in the pathogenesis of IDDM. They support the concept posed by Rabinovitch (1998) and many other researchers that the deletion of a single “pathogenic” cytokine is not likely to be sufficient for complete prevention of diabetes.

Interleukin 1-beta (IL-1 β) Induces Nitric Oxide Production

The cytokine IL-1 β is released by macrophages and pancreatic beta cells depending upon the type of stress the cells are under (Maedler et al. 2002; Donath et al. 2010; Venieratos et al. 2010). Once bound to the cell, IL-1 β in high doses activates pathways leading to cell dysfunction and eventually to apoptosis. Nuclear factor kappa B (NF κ B), a ubiquitous transcription activator, is activated by glucose and IL-1 β . Activation of NF- κ B via IL-1 β alters its role, inducing beta cell apoptosis (Eldor et al. 2006; Ortis et al. 2008). The damage and eventual death of the beta

cell is in part due to the activation of iNOS and production of the reactive oxygen species NO via activation of NF- κ B (Evans et al. 2003; Aktan 2004). Chen et al. (2000) found when selecting for INS-1 cells resistant against IL-1 β that the cells had a disruption in the NF- κ B/iNOS pathway. Eldor et al. (2006) found with the use of a transgenic mouse model expressing a degradation resistant NF κ B protein inhibitor that the effects of cytokine attack could be prevented. The negative effect of IL-1 β on healthy pancreatic beta cell pathways has been reported to affect the PI3/Akt pathway as well (Aikin et al. 2004; Elghazi et al. 2006; Martinez et al. 2006).

The enzyme Phosphoinositide 3-kinase and one of its main targets, protein kinase B (Akt), are known to be directly affected by glucose and IL-1 β in beta cells (Elghazi et al. 2006; Martinez et al. 2006). Increased expression of this kinase participates in beta cell survival through inhibition of the JNK pathway, a stress responsive pathway (Aikin et al. 2004; Elghazi et al. 2006). Aikin et al. (2004) also found inhibition of PI3K made beta cells more susceptible to cytokine mediated death. The reduction of Akt/PKB activity has been shown to exhibit defective insulin secretion but normal beta cell mass (Bernal-Mizrachi et al. 2004). When the islet cells are bound by IL-1 β , the JNK pathway is up regulated as are the reactive nitrogen species produced by that pathway (Kang et al. 2009; Schwarznau et al. 2009). One of the more notorious and detrimental effects of IL-1 β and the pathways it alters is the up regulation of inducible nitric oxide synthase (iNOS) and thus nitric oxide (NO) production, inhibiting mitochondrial glucose oxidation (Ortis et al. 2008, Ortis et al. 2010).

The reactive nitrogen species produced by IL-1 β are known to be a major inducer of pancreatic beta cell apoptosis (Eckhardt et al. 1999; Eldor 2006; Friberg et al. 2010). The enzyme iNOS, once up-regulated by this cytokine and related MAPKs, produces nitric oxide

(NO) leading to beta cell dysfunction (Larsen et al. 1998; Eckhardt et al. 1999). Nitric oxide is so detrimental because it reacts with prosthetic groups such as those found in transcription factors, structures in DNA, and other proteins (Bogdan. 2001). Adaptability to nitric oxide imbalance along with other causes of beta cell dysfunction has provoked the investigation of transcription factors affected by cytokine and anti-oxidant pathways (Henderson et al. 1994; Kronke et al. 2011).

Role of Egr-1 in the Pancreatic Beta Cell

The effect of cytokines and other compounds on beta cells in IDDM directly influences transcription factors and the products they create, including insulin. Early growth response 1 (Egr-1) is a ubiquitous, zinc-finger, transcription factor activated by many pathways and is therefore involved in a number of disease states (Pagel & Deindl. 2011). The role of Egr-1 in pancreatic beta cells has not been fully discerned. Based upon current research, it appears to have a role in protecting beta cells and promoting cell viability in part through insulin production (Josefsen et al. 1999; Eto et al. 2006; Kang et al. 2009; Chang et al. 2011).

Eto et al. (2006) found that varying levels of Egr-1 expression regulated insulin promoter activity by controlling another transcription factor, PDX-1. Complimenting this study, Muller et al, 2012 found that a doxycycline induced, Egr-1 double negative mouse model had significantly decreased expression levels of PDX-1 and insulin mRNA compared to controls. Garnett et al. (2005) silenced Egr-1 activity with small interfering RNA (siRNA) in INS-1 cells and also found that glucose stimulation of insulin was not affected. Beta cell proliferation, however, was inhibited in a glucose-independent manner. Recent studies focusing on anti-oxidant compounds and beta cells have shown that Egr-1 is expressed and activates the transcription of γ -

glutamylcysteine ligase (GCLC), a rate-limiting enzyme for glutathione (GSH) (Kang et al. 2009; Chang et al. 2011). Upregulation of GSH was found to protect the beta cell from NO damage in INS-1 cells (Kang et al. 2009). These studies suggest Egr-1 plays an integral role in the protection and health of pancreatic beta cells.

Proinflammatory cytokines released by immune cells drastically alter the pathways found in normal, healthy pancreatic beta cells (Gurzov et al. 2008; Eizirik et al. 2009; Grunnet et al. 2009). The release of IL-1 β by macrophages induces the JNK pathway eventually leading to the upregulation of iNOS and NO production (Storling et al. 2005). Rabinovitch et al. (1996) determined both IL-1 β and IFN γ in part promote diabetes development through increasing iNOS expression. Henderson et al. (1994) found increased levels of NO reduced Egr-1 expression levels in rat lung macrophages. Kronke et al. (2001) further supported the Henderson et al. (1994) observation. They treated Sp1 and Egr-1 with a NO donor, s-nitrosocysteine, and found that it dose-dependently inhibited DNA binding. The increase of nitric oxide is just one of many altered components when beta cells are affronted by cytokines (Figure 1.2). Glucose is a very important component that binds beta cells and also has a strong effect on Egr-1 expression (Figure 1.2).

Josefsen et al. (1999) found that glucose dose dependently stimulated Egr-1 expression strongly in glucose responsive cell lines MIN6, INS-1, and β TC-3, weakly in RINm5F cells, and not at all in HIT-T15 hamster beta cells. Egr-1 is a transcription factor regulated by its level of expression rather than by cellular location (Kang et al. 2007). Induction of Egr-1 via glucose appears to be regulated by calcium levels, c-AMP levels, ternary complex factors, and kinases all induced by glucose binding (Frodin et al. 1995; Bernal-Mizrachi et al. 2001; Mayer et al. 2009). Mayer et al. (2009) determined Ca²⁺, PKC and activation ERK, a

kinase, are integral to glucose's ability to upregulate Egr-1 expression. These studies and many others suggest Egr-1 plays an important part in beta cell function that is yet to be fully understood (Figure 1.2). Attaining a fundamental comprehension of how Egr-1 is affected by positive and negative compounds binding the beta cell could lead to a more clear understanding of how IDDM develops.

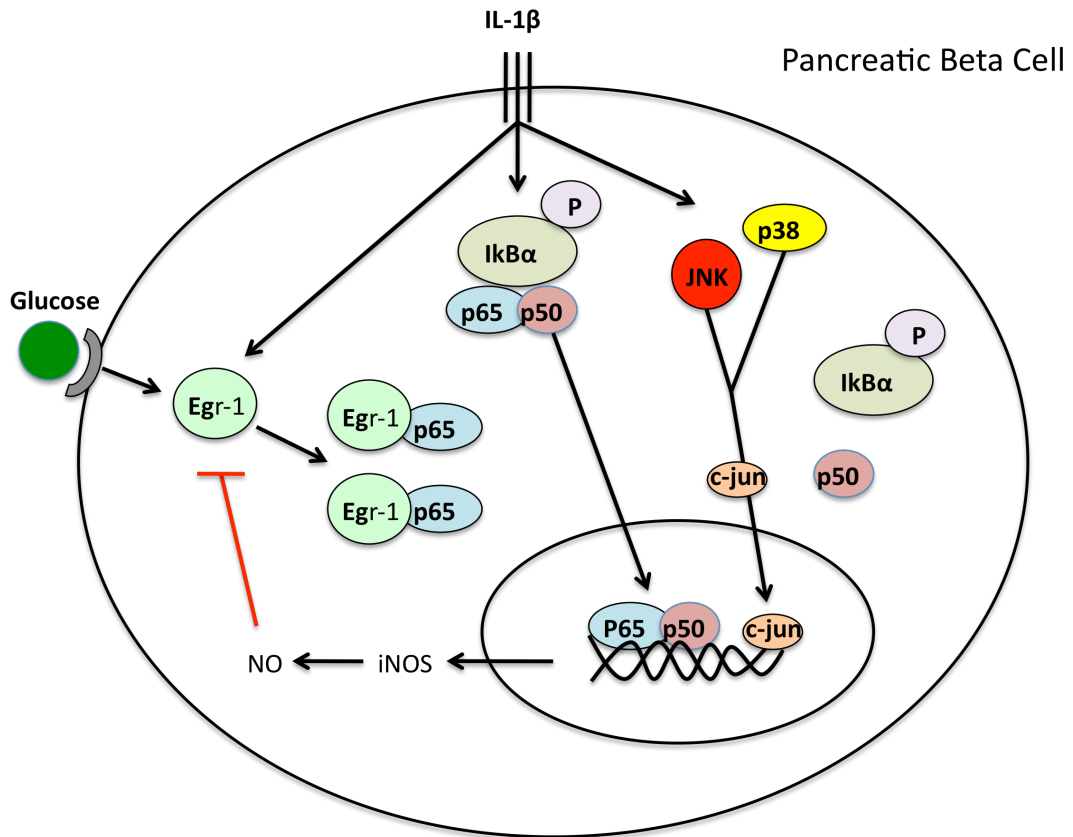


Figure 1.2 – Schematic model of pathways induced by IL-1 β and Glucose affecting Egr-1.

CHAPTER 2

PRETREATMENT OF INS-1 CELLS WITH IL-1B INHIBITS GLUCOSE-STIMULATED EXPRESSION OF EGR-1.

Ada Young, W. Keith De Ponti, John J. Laffan,
Chaunfu Li, Leonard Robertson, and Kevin F. Breuel

Keywords: egr-1, glucose stimulation, interleukin- 1 β

ABSTRACT

Objective: Inflammatory Early growth response gene-1 (Egr-1) expression is specifically and transiently upregulated in pancreatic beta cells in response to glucose. It has been suggested that genes transcriptionally regulated by Egr-1 may be important in normal beta cell function.

Recently, we reported that interleukin-1 β (IL-1 β) stimulates Egr-1 expression in pancreatic beta cells and that co-administration of sodium nitroprusside, a nitric oxide donor, inhibits the IL-1 β -stimulated increase in Egr-1. The objective of this study was to determine if pre-treatment of beta cells with IL-1 β alters the ability of glucose to stimulate Egr-1 expression.

Research Design and Methods: Rat insulinoma cells (INS-1) were cultured for 24 hours in glucose free RPMI 1640 media supplemented with fetal calf serum, L-glutamine, antibiotics, sodium pyruvate and 2- β -mercaptoethanol (GFCM). In experiment 1, INS-1 cells were treated with GFCM alone or GFCM supplemented with 20 mM glucose, 1 mM IBMX and 1 μ M forskolin and cultured for 0, 30, 60 and 120 minutes. After culture, media and cells were collected and stored for subsequent determination of insulin levels and Egr-1 protein expression, respectively. In experiment 2, cells were treated with GFCM alone or GFCM supplemented with 100 U/ml of IL-1 β . After 24 hours, media was aspirated and replaced with GFCM alone or GFCM supplemented with 20 mM glucose, 1 mM IBMX and 1 μ M forskolin. After culture for 2 hours, cells were collected and stored for subsequent analysis of Egr-1 protein expression by western blot.

Results: In experiment 1, administration of glucose significantly ($P < 0.05$) increased insulin levels at 60 (36.9 ng/ml) and 120 (51.6 ng/ml) minutes over that observed at time 0 (7.5 ng/ml). Additionally, glucose treatment for 60 and 120 minutes significantly increased ($P < 0.05$) Egr-1 protein expression in INS-1 cells as compared to those treated with GFCM alone. In experiment

2, administration of glucose resulted in a 55 fold increase in Egr-1 protein expression as compared to control levels. Pre-treatment with IL-1 β completely inhibited ($P<0.01$) the glucose-stimulated increase in Egr-1 protein expression.

Conclusions: Based on these results we speculate that IL-1 β stimulation of nitric oxide and the subsequent inhibition of glucose stimulated Egr-1 expression is detrimental to normal pancreatic beta cell function.

Introduction

Insulin-dependent diabetes mellitus (IDDM) is a macrophage-dependent, T cell-mediated autoimmune disease characterized by the infiltration of the islet cells of the pancreas by immune cells (insulinitis) followed by massive destruction of insulin-producing pancreatic beta cells (Bach 1994). It is now generally accepted that pro-inflammatory and Th1 cytokines play a key role in the destruction of the beta cells. Interleukin-1 β (IL-1 β) initiates a cascade of events that leads to the activation of inducible nitric oxide synthase, the production of nitric oxide (NO) and the subsequent destruction of the pancreatic beta cells by an apoptotic process (Corbet & McDaniel 1996; McDaniel et al. 1997). In addition, the administration of IL-1 β inhibits glucose-stimulated insulin secretion from rat islets (Hughes et al. 1990).

A major part of normal pancreatic beta cell function is the binding of glucose and the subsequent stimulation of insulin production and secretion. In order to induce insulin production, glucose must activate a series of transcription factors. Specifically, glucose has been reported to induce the expression of early growth response gene (Egr-1) (Josefsen et al. 1999). Changes in Egr-1 expression levels regulate other beta cell specific transcription factors including the pancreas duodenum homeobox-1(PDX-1) and therefore insulin production (Eto et al. 2006). The role of glucose stimulated Egr-1 expression and its effect upon insulin production could play a very important part in understanding the development of IDDM.

During its development, a series of cytokines and chemokines are released by white blood cells. Interleukin (IL) 1 β is one of the cytokines released by macrophages in IDDM development and is known to be a direct contributor to the induction of pancreatic β cell death. It regulates a number of transcription factors including nuclear factor (NF)- κ B and AP-1 (Cahill and Rogers 2008; Liu and Khachigian. 2009). In human and rat aortic smooth muscle cells, IL-

1 β has been reported to stimulate histone 3 acetylation and phosphorylation at the Egr-1 promoter site (Wang et al. 2010). IL-1 β activation of the inhibitor of DNA binding proteins is directly mediated by Egr-1 binding in vascular smooth muscle cells (Zhu et al. 2007). IL-1 β also induces iNOS and thus nitric oxide production in rodent beta cells. In glomerular mesangial cells, nitric oxide has been found to inhibit growth through impairing Egr-1 function (Rupprecht et al. 2000). Further elucidation of the IL-1 β and Egr-1 relationship could greatly benefit understanding the process and thus the prevention of apoptosis in pancreatic β cells.

The role of glucose and IL-1 β binding of the pancreatic β cell appear to affect each other's behavior in beta cells. Glucose metabolism is a normal part of beta cell function and is generally believed to assist in the promotion of beta cell survival. IL-1 β is a cytokine that mediates abnormal beta cell function and generally assists in the progression of beta cell death. Each of these molecules enacts similar pathways and both regulate Egr-1 expression. The role of Egr-1 during the development of IDDM, when both glucose and IL-1 β are bound to the pancreatic beta cell, is not fully understood. Herein, we demonstrate that IL-1 β stimulated nitric oxide production does inhibit glucose induced Egr-1 expression.

Research Design and Methods

Cell Culture

The rat insulinoma cell line INS-1 used in this work was obtained from Dr. Phillippe Halban. Cells were grown in continuous culture in 175-cm² flasks containing RPMI 1640 medium supplemented with 10% inactivated fetal calf serum, 2 mM L-glutamine, 1 mM

pyruvate, 50 μ M β -mercaptoethanol, 100 I.U. penicillin, and 100 μ g/ml streptomycin penicillin and streptomycin. Cells were cultured at 37°C in a humidified environment of 5% CO₂ in air.

Sample Collection and Preparation

INS-1 cells were scraped and pipetted to detach from the wells and centrifuged at 3000 rpm for 5 minutes. The cell pellet was lysed by resuspending in 200 μ l of lysis buffer (4% SDS, 50 mM Tris at pH6.8) and then boiled for 6 minutes. Protein concentration was determined by the BCA (Sigma, St. Louis, MO) method with BSA as a standard. Samples were adjusted to equal protein concentration by addition of lysis buffer. Glycerol (10%), BME (5%), and bromophenol blue (0.025%) were added to each sample.

Western Blot

Forty μ g total protein aliquots from each sample were loaded onto a 10% SDS-PAGE (BioRad minigel) and run until the dye front reached the end of the gel. The gel was then blotted to nitrocellulose for 1 hour at 100V in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol) After blotting the nitrocellulose was dried at room temperature and then blocked overnight in blotto (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20, 5% nonfat dry milk) at 4°C. The blot was then treated with rabbit polyclonal anti-Egr-1 (1:1,500, Santa Cruz, Santa Cruz, CA) in blotto for 1 hour at room temperature. The blot was washed 6 times (5 min/wash) in wash buffer (10 mM tris pH 8.0, 150 mM NaCl, 0.05% Tween-20). The blot was then incubated with goat anti-rabbit IgG)-HRP second antibody 1:6,700, Santa Cruz, Santa Cruz, CA)

for 1 hour at room temperature. The blot was then washed as previously described and reacted with ECL (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech Inc., Piscataway, NJ) for less than 10 minutes.

Measurement of Nitrite and insulin

Concentrations of nitrite, a stable metabolite of nitric oxide, in the culture media were determined using a colorimetric procedure (Greiss Reagent). Insulin levels were determined using a commercially available ELISA kit (Alpco, Windham, NH).

Experiment 1. Effect of Glucose on Insulin Production and Expression of Egr-1 in INS-1

Pancreatic Beta Cells

INS-1 cells were collected and plated (1×10^7 cells/well) in 6-well tissue culture plates and cultured in 5 ml of media for 24 hours. Culture media was aspirated and replaced with glucose free RPMI 1640 media (GFM) and cultured for an additional 24 hours. Media was aspirated and cells were cultured in GFM supplemented with 20 mmol/L glucose, 1 mM IBMX and 1 μ M Forskolin (GFM+) for 0, 30, 60, and 120 minutes respectively. After the appropriate time, media from the sample was collected and stored at -70°C for subsequent determination of insulin levels. Cells were collected and snap frozen for subsequent determination of Egr-1 levels by western blot.

Experiment 2. Effect of IL-1 β Treatment on Nitric Oxide Production Over Time in INS-1 Cells.

INS-1 cells were collected, plated (2×10^5 cells/well) in 96-well tissue culture plates and cultured in 200 μ l of media. After 0, 3, 6, 12, 18, and 24 hours, media was aspirated and cells were cultured in RPMI media alone or supplemented with 100 U/ml of IL-1 β . After 0, 1, 2, 3, 6, 9, 12, and 24 hours cells were collected, processed, and stored at -70°C for subsequent determination of Egr-1 protein expression by western blot.

Experiment 3. Effect of IL-1 β Treatment Over Time on Glucose's Ability to Induce Egr-1 Expression.

INS-1 cells were collected and plated (1×10^7 cells/well) in 6-well tissue culture plates and cultured in 5 ml of media. After 24 hours, the media was aspirated and replaced with GFM alone or GFM supplemented with 100 U/ml IL-1 β at various times following media exchange (0, 6, 12, and 18 hours). Twenty four hours after the addition of GFM, media was aspirated and replaced with GFM alone or GFM supplemented with 20 mmol/L glucose, 1 mM IBMX and 1 μ M Forskolin. After 2 more hours cells were collected, processed, and stored at -70°C for subsequent determination of Egr-1 protein expression by western blot.

Experiment 4. Effect of Increasing Sodium Nitroprusside Concentrations on Glucose's Ability to Induce Egr-1 Expression.

INS-1 cells were collected and plated (1×10^7 cells/well) in 6-well tissue culture plates and cultured in 5 ml of media. After 24 hours, media was aspirated and the cells were cultured in GFM for an additional 24 hours. Media was aspirated again and cells were treated with GFM alone, GFM supplemented with 20 mmol/L glucose, 1 mM IBMX and 1 μ M Forskolin (GFM+), or GFM+ containing various levels of sodium nitroprusside (1.25, 2.5, 5, and 10 mM). After 2 hours cells were collected, processed, and stored at -70°C for subsequent determination of Egr-1 protein expression by western blot.

Statistical Analyses

ANOVA and pair-wise contrasts were used to test differences among time point means.

Results

Experiment 1.

Glucose stimulated insulin production in INS-1 cells. We examined the effect of glucose on insulin production in the INS-1 pancreatic beta cells. INS-1 cells were treated with glucose (20 mM) and cultured for 30, 60, and 120 minutes. The administration of glucose to the INS-1 cells significantly ($P<0.05$) increased insulin levels at 60 (36.9 ng/ml) and 120 (51.6 ng/ml) minutes over that observed at time 0 (7.5 ng/ml) (Figure 2.1).

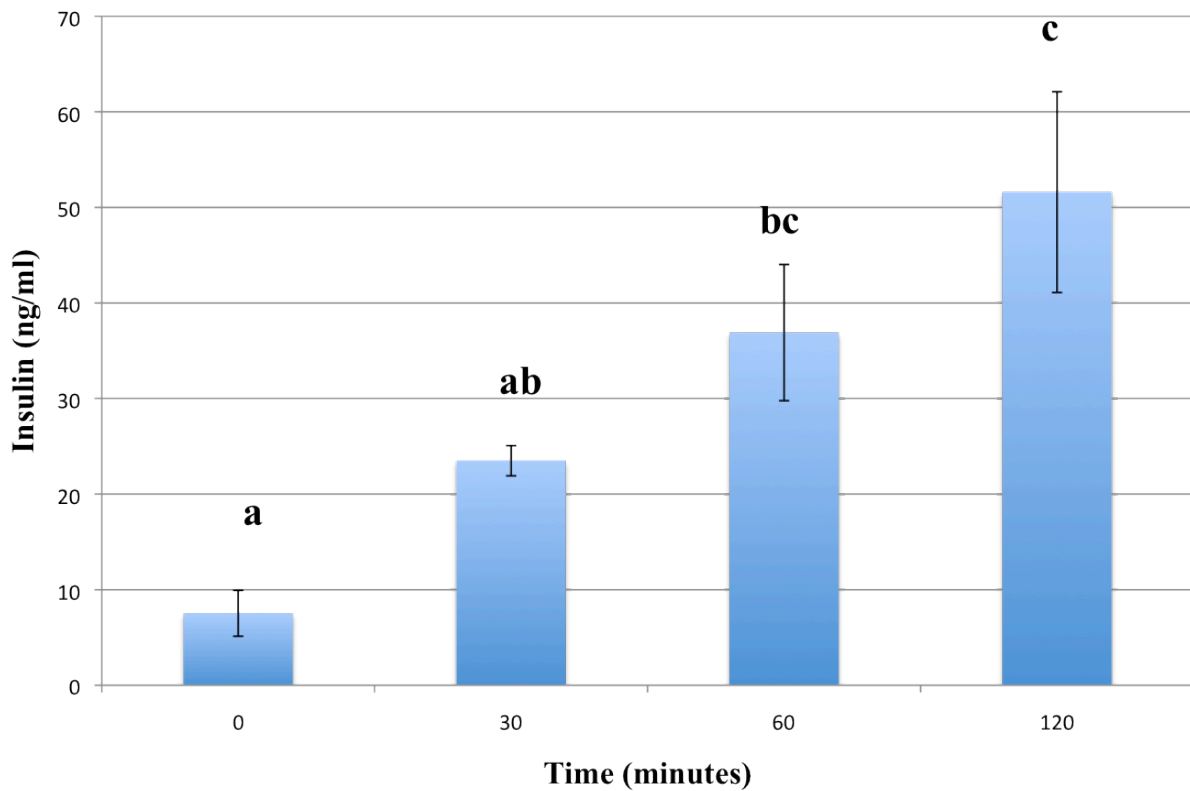


Figure 2.1: Glucose significantly stimulated insulin production from INS-1 cells cultured *in vitro* at 60 and 120 minutes. Data are expressed as mean \pm SEM of 3 experiments.

Experiment 2.

Glucose increased expression of Egr-1 in INS-1 cells. We examined the effect of glucose on the expression of Egr-1 in INS-1 pancreatic beta cells. INS-1 cells were treated with glucose (20 mM) and cultured for 30, 60, and 120 minutes. Treatment of the INS-1 cells with glucose significantly ($P < 0.05$) increased the expression of Egr-1 at 60 and 120 minutes of culture as compared to the 0-hour time group (Figure 2.2).

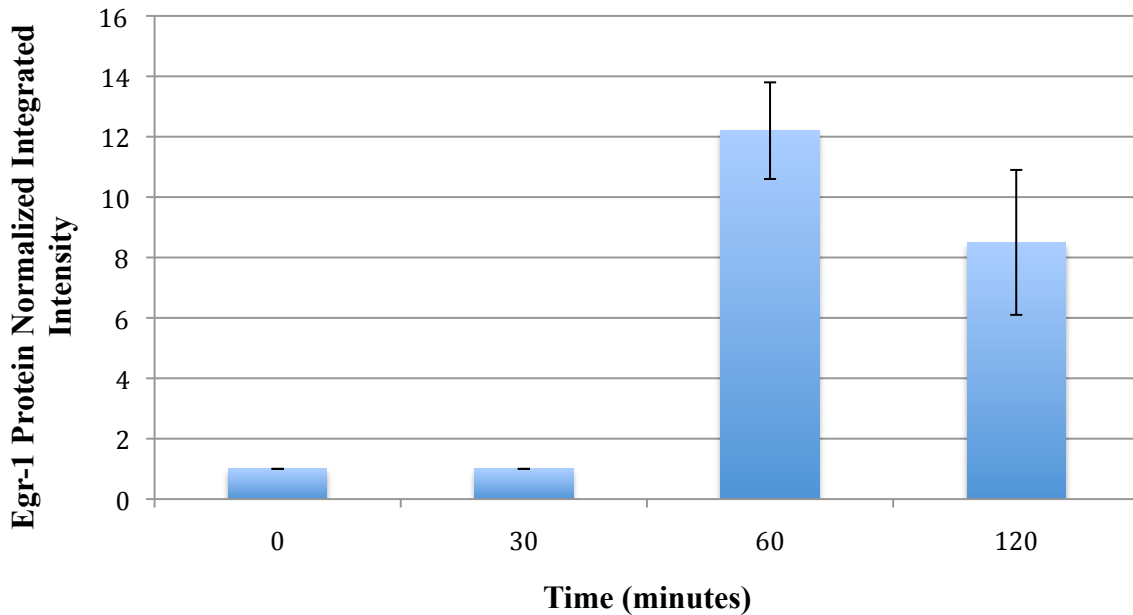


Figure 2.2: Effect of glucose on the expression of Egr-1 over time. Data are normalized to the 0-minute time period which was set at 1.0. Data are expressed as mean \pm SEM of 3 experiments.

Experiment 3.

Interleukin-1 β dose-dependently increases nitric oxide in INS-1 cells. We examined the effect of IL-1 β (0, 1, 5, 50, and 100 U/ml) on nitrite production in INS-1 pancreatic beta cells over time (0, 3, 6, 12, 18, and 24 hours). Administration of IL-1 β dose-dependently increased

nitrite production over time in the INS-1 pancreatic beta cells (Figure 2.3.). The figure also shows that the administration of 50 and 100 U/ml of IL-1 β increased nitrite production from INS-1 cells after 6 hours of culture and continued to further increase nitrite levels through 24 hours of culture. These data show a direct relationship between IL-1 β and Nitric oxide production. The time lag of six hours indicates the possibility of several intermediates in the pathway between IL-1 β stimulation and NO production in the INS-1 pancreatic beta cells.

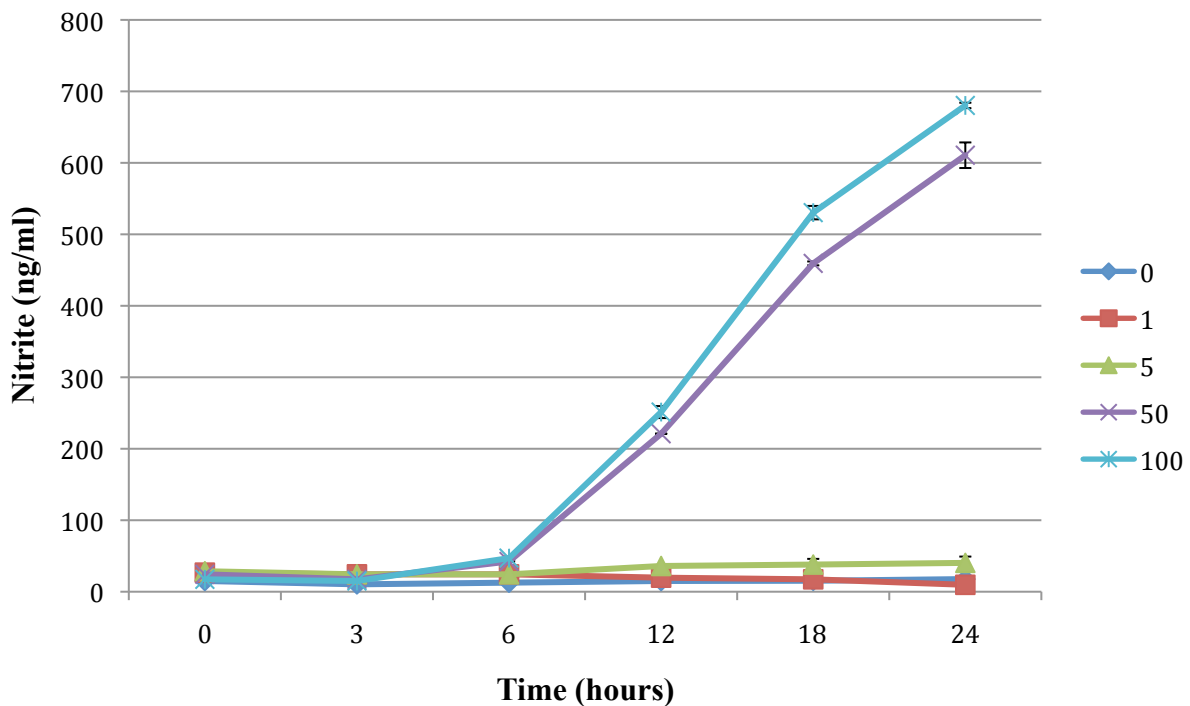


Figure 2.3: Administration of IL-1 β (U/ml) dose-dependently increased nitric oxide production from INS-1 cells cultured *in vitro* for 0, 3, 6, 12, 18, and 24 hours. Data are expressed as a mean for each of the three experiments.

Experiment 4.

Pre-treatment of INS-1 cells with IL-1 β attenuates the glucose-stimulated increase in Egr-1 protein expression in a time dependent manner. We examined the effect of glucose on Egr-1 expression in INS-1 pancreatic beta cells that had been previously treated with IL-1 β . Pre-

treatment of INS-1 cells with IL-1 β (100 U/ml) inhibited the glucose-induced increase in Egr-1 protein expression in INS-1 cells cultured *in vitro* in a time dependent manner (Figure 2.4). Specifically, treatment of INS-1 cells with IL-1 β for 24, 18, and 12 hours before the administration of glucose, significantly ($p < 0.05$) attenuated glucose-stimulated expression of Egr-1 by 24, 51, and 62 percent, respectively (Figure 2.4). In contrast, pre-treatment of INS-1 cells with IL-1 β for 6 hours before the administration of glucose did not alter glucose-stimulated expression of Egr-1 significantly (Figure 2.4). This is possibly because only after 6 hours was a high amount of nitric oxide stimulation from IL-1 β treatment of the INS-1 cells seen (Figure 2.3).

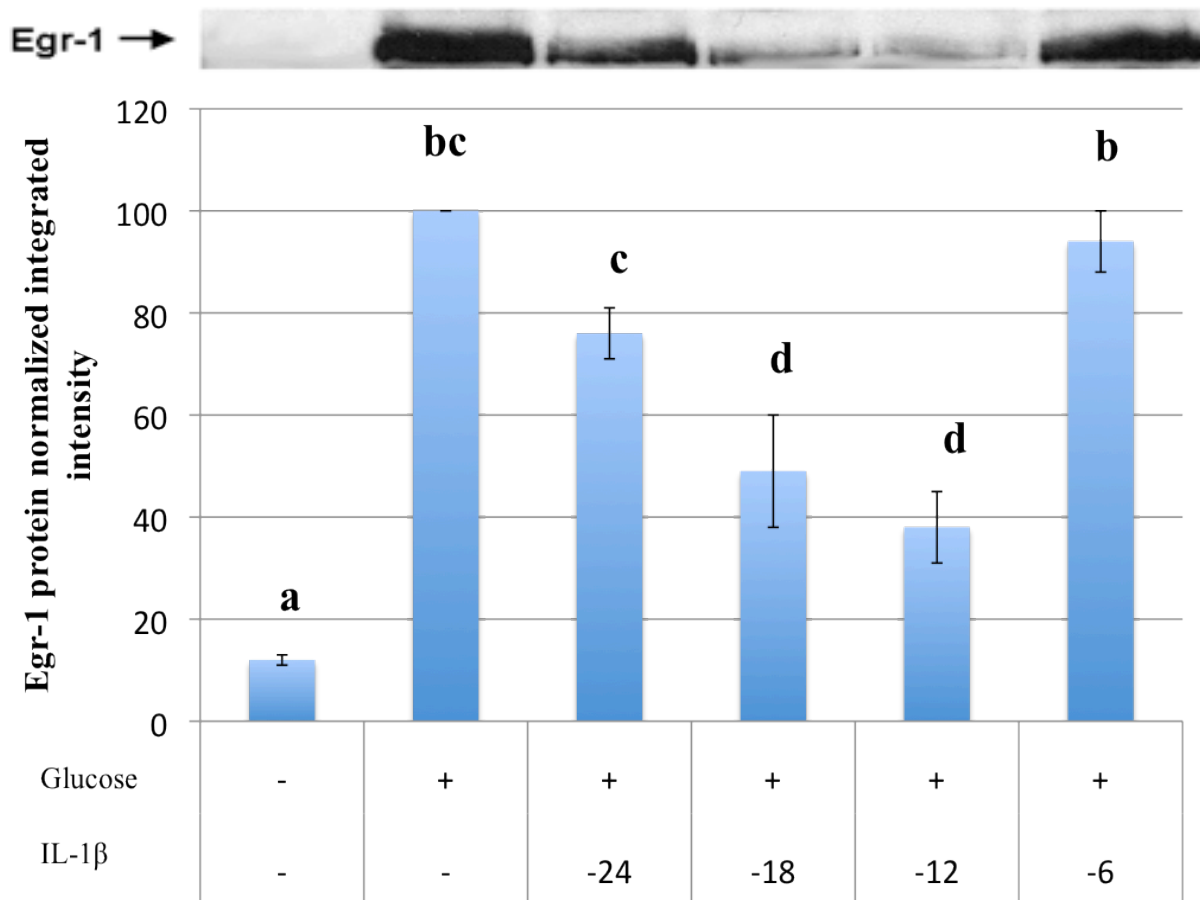


Figure 2.4: Pre-treatment of INS-1 cells with IL-1 β attenuated the glucose-stimulated increase in Egr-1 expression in a time dependent manner. Data are normalized to the glucose treatment group which was set at 100%. Data are expressed as mean \pm SEM for 3 experiments Experiment 5.

Sodium Nitroprusside attenuates glucose-stimulation of Egr-1 expression in INS-1 cells.

To further verify the hypothesis that IL-1 β alters glucose-stimulated expression of Egr-1 in INS-1 cells via the upregulation of nitric oxide, we examined the effect of sodium nitroprusside, a nitric oxide donor, on glucose-stimulated expression of Egr-1 in INS-1 pancreatic beta cells. INS-1 cells were treated with glucose (20 mM) alone or in the presence of increasing concentrations of sodium nitroprusside (0, 1.25, 2.5, 5, and 10 mM) for 2 hours. Glucose significantly ($p < 0.05$) increased the expression of Egr-1 in INS-1 pancreatic beta cells cultured for 2 hours (Figure 2.4). Co-administration of sodium nitroprusside, dose-dependently, inhibited

the glucose-stimulated ($P < 0.05$) increase in Egr-1 protein expression by INS-1 pancreatic beta cells cultured *in vitro* for 2 hours (Figure 2.5). Administration of 2.5, 5, and 10 mM of sodium nitroprusside decreased the glucose-stimulated increase ($P < 0.05$) of Egr-1 protein expression by 15, 52, and 66 percent, respectively (Figure 2.5).

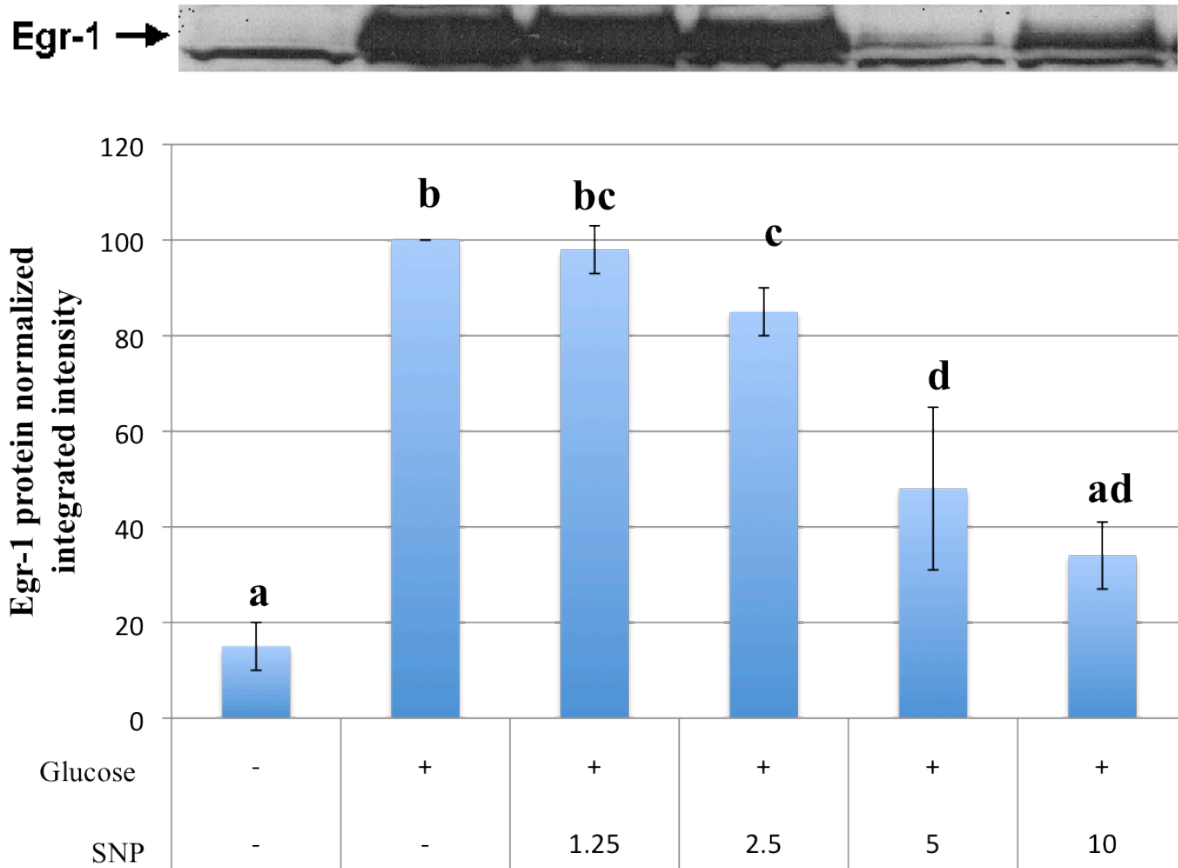


Figure 2.5: Administration of sodium nitroprusside dose-dependently inhibited glucose stimulation of Egr-1 expression from INS-1 cells cultured *in vitro* for 2 hours. Data are normalized to the glucose treatment group which was set at 100%. Data are expressed as mean \pm SEM for 3 experiments.

Based on these findings we conclude that nitric oxide is an important negative regulatory intermediate of stimulated Egr-1 protein expression in INS-1 pancreatic beta cells. Our findings

support our hypothesis that exposure of pancreatic beta cells to IL-1 β initiates a cascade of events that subsequently alters the ability of glucose to promote normal beta cell function. Furthermore, our findings suggest that IL-1 β 's ability to alter glucose stimulation of Egr-1 protein expression involves the production of nitric oxide.

Discussion

Our long-range goal is to understand the pathophysiology of IDDM and to develop new therapeutic strategies for prevention and/or treatment of this disease. In these studies we investigated the role of nitric oxide to alter the glucose-stimulated increase in the expression of the transcription factor, Egr-1. The importance of Egr-1 in normal beta cell function has been emphasized by previous reports of its role in insulin mRNA expression and its paralleled increase with insulin production in insulinoma cells (Frodin et al. 1995; Josefsen et al. 1999; Bernal-Mizrachi et al. 2001; Eto et al. 2006; Muller et al. 2012). Our observation that glucose stimulates the production of insulin and increases the expression of Egr-1 from cultured INS-1 pancreatic beta cells further supports those studies. Recently, Muller et al. (2012) concluded that Egr proteins are important regulators of pancreatic beta cell function, influencing insulin biosynthesis and glucose homeostasis.

Proinflammatory cytokines, such as IL-1 β and IFN- γ released by infiltrating immune cells are thought to be key mediators of apoptosis in pancreatic beta cells (Rabinovitch and Suarez-Pinzon 1998; Eizirik et al. 2001; Grunnet et al. 2009). Administration of IL-1 β alone, or in combination with IFN- γ has been shown to induce markers of apoptosis and cell death in INS-1 cells and rat and human islets (Grunnet et al. 2009; Wu et al. 2012). Administration of IL-1 β to INS-1 cells significantly increased caspase-3 activity, a specific marker for apoptosis, by 7.6 fold over that observed in INS-1 cells cultured in media alone (Wu et al. 2012). Furthermore, Wu and

coworkers found that treatment of INS-1 cells with IL-1 β increased cell death by 49% over untreated INS-1 cells.

Neutralization of IL-1 β by the administration of anti-IL-1 β antibody reduced the incidence of cyclophosphamide-induced diabetes (34%) in non-obese diabetic (NOD) mice as compared to controls (100%) (Cailleau et al.1997). Similarly, administration of 0.2 mg/kg and 2 mg/kg of a soluble IL-1 receptor to cyclophosphamide-treated NOD mice decreased the incidence of diabetes (53% and 7%, respectively) as compared to controls (65%). Neutralization of IL-1 did not alter insulinitis to a great degree. These findings are consistent with the concept that IL-1 β is integral to the process leading to IDDM and that it likely participates in the effector phase because neutralization has little affect on insulinitis (Rabinovitch and Suarez-Pinzon 1998). Collectively these findings demonstrate that proinflammatory cytokines, in particular IL-1 β , activate a series of events that ultimately lead to destruction of the pancreatic beta cells and onset of IDDM. However, the exact mechanisms activated by the release of proinflammatory cytokines in the pancreas, which lead to cell death, and IDDM remain to be determined.

A detrimental compound induced by IL-1 β that, in part, leads the endocrine pancreas to cell death and IDDM is nitric oxide (NO). Treatment of pancreatic beta cells with IL-1 β has been shown to increase inducible nitric oxide synthase (iNOS) and thus NO concentrations (Corbett et al. 1991; Rabinovitch et al. 1996; Aktan 2004; Storling et al. 2005). In our studies, administration of IL-1 β increased nitric oxide production over time in a dose dependent manner. The role of nitric oxide as a key mediator of proinflammatory cytokine-induced beta cell death is further supported by ablation/replacement studies using pharmacological inhibitors or donors of nitric oxide production (Storling et al. 2005). Rat insulinoma cells, INS-1, incubated for 24 hours with IL-1 β , IFN γ and then treated with NOS blocker NMA had a 90% reduction in

apoptosis (Storling et al. 2005). When a nitric oxide donor SNAP was introduced into the culture media of the same INS-1 cells over a 24-hour period, it dose-dependently induced apoptosis (Storling et al. 2005). Kacheva et al. (2011) found similar results when treating INS-1E cells with an iNOS blocker. They studied the NF- κ B- iNOS pathway and found an iNOS blocker inhibited IL-1 β induced NF- κ B activation. The detrimental effect of IL-1 β induced NO on the viability of pancreatic beta cells is suggested by these studies.

The increased concentration of NO due to IL-1 β may be causing beta cell dysfunction through altering transcription factor function. Transcription factors play a pivotal role in producing and regulating products necessary for pancreatic beta cells to function properly. They are strongly affected by the type of compound binding the cell whether it is glucose or pro-inflammatory cytokines, such as IL-1 β . The importance of Egr-1 in pancreatic beta cell health and function is supported in a number of studies (Frodin et al. 1995; Josefsen et al. 1999; Bernal-Mizrachi et al. 2000; Garnett et al. 2005; Eto et al. 2006; Muller et al. 2012). It participates in these positive functions through glucose stimulation (Josefsen et al. 1999; Eto et al. 2006; Muller et al. 2012). NO production inhibits normal pathways enacted by glucose including insulin secretion (Corbett et al. 1993; Tsuura et al. 1998; Henningson et al. 2002). Henningson et al. (2002) treated mouse islets with 20mM of glucose for 60 minutes and found exogenous or endogenous NO donors suppressed insulin secretion but increased it dramatically with nitric oxide inhibitors. One target of NO in the glucose pathway that has been identified is phosphofruktokinase (Tsuura et al. 1998). Another target of NO is the zinc finger transcription factor Egr-1 (Henderson et al. 1994; Kroncke et al. 2001). Interference of glucose stimulated Egr-1 via IL-1 β induced NO production could play an important part in IDDM development.

A number of studies using pharmacological donors of NO, including the present study, suggests NO plays an important role in IDDM development through Egr-1. We found sodium nitroprusside, a NO donor, dose dependently inhibited glucose induced Egr-1 production. Henderson et al. (1994) treated rat lung macrophages with low doses of NO donor SNAP (0.1-1mM), before treatment with IFN and LPS and found a decrease in Egr-1 transcription by 70%. A study treating glomerular mesangial cells (MC) with S-nitrosoglutathione (GSNO) suppressed Egr-1 binding sites and ultimately inhibited MC proliferation (-84%) (Rupprecht et al. 2000). Kroncke et al. (2001) found treatment of recombinant Egr-1 with NO donor S-nitrosocysteine (SNOC) dose-dependently inhibited DNA binding by the transcription factor.

The role of IL-1 β induced NO in pancreatic beta cell death is not fully understood. It is widely recognized as playing a large part in beta cell destruction. Our studies confirmed that IL-1 β increased NO levels beginning at the 6-hour marker. This time frame coordinated with the time it took for IL-1 β to begin inhibiting glucose induced Egr-1 production. These 2 studies show that IL-1 β interferes with glucose's ability to function normally in part through inhibition of Egr-1. In order to confirm that it was nitric oxide and not another compound, we treated the INS-1 cells with increasing concentrations of SNP and found Egr-1 was inhibited dose-dependently. This reconfirms previous studies suggesting NO directly affects Egr-1 expression. Our study has shown that IL-1 β alters glucose's ability to induce Egr-1 production through NO. Our findings provide evidence for the first time that the increase in nitric oxide in pancreatic beta cells during the onset of IDDM may alter the ability of glucose to promote cell viability and inhibit insulin production, therefore leaving the cells more susceptible to cell death.

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APPENDICES

APPENDIX A

Cell Processing

Cell Lysis Buffer

10% SDS	4ml
1M Tris-HCL, 6.8pH	500 μ l
diH ₂ O	<u>5.5ml</u>
	10ml

APPENDIX B

Protein Assay

Diluted Albumin(BSA) Standards: Thermo Scientific BCATM Protein Assay Kit

<u>Vial</u>	<u>Volume of Diluent</u>	<u>Volume & Source of BSA</u>	<u>Final BSA Concentration</u>
A	0 μ l	300 μ l of stock	2000 μ g/ml
B	125 μ l	375 μ l of stock	1500 μ g/ml
C	325 μ l	325 μ l of stock	1000 μ g/ml
D	175 μ l	175 μ l of vial B dilution	750 μ g/ml
E	325 μ l	325 μ l of vial C dilution	500 μ g/ml
F	325 μ l	325 μ l of vial E dilution	250 μ g/ml
G	325 μ l	325 μ l of vial F dilution	125 μ g/ml
H	400 μ l	100 μ l of vial G dilution	25 μ g/ml
I	400 μ l	0	0 μ g/ml=Blank

BCA Working Reagent:

BCA Reagent A (50 parts) + Reagent B (1 part)

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