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dissertation entitled

Low-potency Poly(ADP-ribose) Polymerase Inhibitors (lp-PARPi) Induce Insulin Gene Expression through the Upregulation of MafA in INS-1 Pancreatic β-cells

presented by

Diana Zi Ye

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of the requirements for the

Doctoral degree in Pharmacology/Toxicology

Major Professor's Signature

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Date

Low-potency Poly(ADP-ribose) Polymerase Inhibitors (lp-PARPi) Induce Insulin Gene Expression through the Upregulation of MafA in INS-1 Pancreatic β-cells

By

Diana Zi Ye

A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

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Abstract

Low-potency Poly(ADP-ribose) Polymerase Inhibitors (lp-PARPi) Induce Insulin Gene Expression through the Upregulation of MafA in INS-1 Pancreatic β-cells

By

Diana Zi Ye

Chronic hyperglycemia impairs pancreatic β-cell function, in part, through changes in gene expression profile. It leads to decreased expression of β-cell specific genes including insulin, and pancreatic duodenal homeobox factor (PDX-1). Nicotinamide (NAM), a low-potency poly(ADP-ribose) polymerase inhibitor (lp-PARPi), increases insulin biosynthesis in human and porcine fetal islets. NAM and another lp-PARPi, 3-aminobenzamide (3-AB), attenuate diabetes in several diabetic animal models. The mechanisms whereby these compounds regulate insulin biosyntheis were not well understood. The aim of this thesis project was to investigate the mechanisms of lp-PARPi in the regulation of insulin gene expression.

Exposure of INS-1 pancreatic β-cells to high glucose (16.7 mM) suppressed insulin gene expression, in part, through the decreased insulin promoter activity. Lp-PARPi-10 mM NAM, 10 mM 3-AB and 500 μM PD128673 (PD), stimulated insulin promoter activity and mRNA level in INS-1 cells. Consistent with these findings, lp-PARPi also increased cellular insulin content, and partially reversed glucose-mediated impairment of glucose-stimulated insulin secretion (GSIS). The present study identified that lp-PARPi-induction of insulin promoter activity was through the increase of MafA binding to C1 and A5/core elements. The lp-PARPi enhanced MafA binding to insulin promoter was due to increase of MafA protein levels in INS-1 cells cultured with high glucose. These data point at MafA as the target of lp-PARPi in INS-1 cells. Measuring

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MafA mRNA indicated that lp-PARPi increase MafA mRNA levels in cells cultured with high glucose. Inhibition of protein translation by cycloheximide showed that lp-PARPi did not affect MafA stability. Inhibition of RNA synthesis showed that lp-PARPi did not delay MafA mRNA degradation. These data indicate that lp-PARPi upregulate MafA protein level through the induction of MafA promoter activity.

In summary, this project provides evidence that lp-PARPi modulate glucose-mediated phenotypic changes of pancreatic β-cell. This project is the first to show that MafA gene expression is downreguated by elevated glucose in INS-1 β-cells. Lp-PARPi-induction of insulin promoter activity is through the increased MafA gene expression in INS-1 cells cultured in high glucose. Consistent with increased insulin gene expression, lp-PARPi also partially improve INS-1 cell function under high glucose.

The heavens tell of the glory of God. The skies display his marvelous craftsmanship.

-Psalm 19:1

To my father (Jizhi Ye), mother (Deshi Yang Ye), brothers (Ning Ye and Hong Ye), husband (Khon T. Au), daughter (Liyun H. Au), and family

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3-AB

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ACC

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AMPK

AP-2

ATP

BER

BETA2

bHLH

bp

BRCT

C/EBPβ

cAMP

CBP

CHOP

CPT-1

CRE

List of Abbreviations

3-AB	3-aminobenzamide
p38 MAPK	p38 mitogen-activated protein kinase
ACC	acetyl-CoA carboxylase
ADP	adenosine 5'-diphosphate
ADPr	ADP-ribose
AG	aminoguanidine
AGE	glycosylation end product
AMD	automodification domain
AMPK	AMP-activated protein kinase
AP-2	adaptor protein complex 2
ATP	adenosine 5'-triphosphate
BER	base excision repair
BETA2	beta-cell E box transcription factor 2
bHLH	basic helix-loop-helix
bp	base-pair
BRCT	breast cancer susceptibility protein, C-terminus
C/EBPβ	CCAAT/enhancer-binding protein β
cAMP	3', 5'-cyclic adenosine monophosphate
CBP	CREB binding protein
CHOP	C/EBP homologous protein
CPT-1	carnitine palmitoyl transferase-1
CRE	cAMP responsive element

CRI

CRI

CtB

DBI

DNA

DN-

DSB

EMS

ER

FFA

FAS

GAPI

GFA

GK ra

GLU

GLU

GSIS

HDA

HEB

HMG

Hnf

HR

hTAF

CREB	cyclic AMP-response element binding protein
CREM	CRE modulator
CtBP	C-terminal binding protein
DBD	DNA binding domain
DNA	deoxyribonucleic acid
DN-JNK	dominant-negative JNK
DSB	double-strand-break
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
FFA	free fatty acid
FAS	fatty acid synthase
GAPDH	glyceraldehyde-phosphate dehydrogenase
GFAT	glutamine:fructose-6-phosphate aminotransferase
GK rats	Goto-Kakizaki rats
GLUT2	glucose transporter 2
GLUT4	glucose transporter 4
GSIS	glucose-stimulated insulin secretion
HDAC	histone deacetylase
HEB	human E-protein
HMGI(Y)	high-mobility-group protein I(Y)
Hnf	hepatic nuclear factor
HR	homologous recombination
$hTAF_{II}130$	human TBP-associated factor II130

HTH

hUbc9

IAPP

ICC

ICER

IDDM

IDX1

IEF1

IEF1

IFNγ

IL-1β

iNOS

INS-1

INSCA

IPF1

IRAP

Isl-1

IUF1

JNK

KD

Lhx

Lim

HTH	helix-turn-helix
hUbc9	human ubiquitin-conjugating enzyme 9
IAPP	islet amyloid polypeptide
ICC	islet-like cell cluster
ICER	inducible cAMP early repressor
IDDM	insulin-dependent diabetes mellitus
IDX1	islet duodenum homeobox-1
IEF1	insulin enhancer factor 1
IEF1	insulin enhancer factor 1
IFNγ	interferon γ
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
INS-1	rat insulinoma cell line
INSCAT	insulin promoter regulating chloramphenical acetyltransferase (CAT) reporter gene
IPF1	Insulin Promoter Factor 1
IRAP	insulin-responsive amino peptidase
Isl-1	islet factor 1
IUF1	Insulin upstream factor 1
JNK	c-Jun N-terminal kinase
KD	kilo Dalton
Lhx	LIM-homeobox protein
Lim	LIM-homeodomain protein

LA

Lmx

lp-PAR

L-PK

LXR

LZ

mM

Maf

MARE

MMS

MODY

MRC

MVP

NAD^+

NAC

NAM

NF-Y

Ngn3

NHEJ

NIDD

NLS

NO

NOD

LA	lipoic acid
Lmx	LIM-homeodomain protein
lp-PARPi	low-potency poly(ADP-ribose) polymerase inhibitor
L-PK	liver-pyruvate kinase
LXR	liver X receptor
LZ	leucine-zipper
mM	milimolar
Maf	musculoaponeurotic fibrosarcoma
MARE	Maf recognition element
MMS	methylmethanesulfonate
MODY	mature onset diabetes of young
MRC	multiprotein DNA replication complex
MVP	major vault protein
NAD^+	β-nicotinamide adenine dinucleotide
NAC	N-acetyl-L-cysteine
NAM	nicotinamide
NF-Y	nuclear factor Y
Ngn3	neurogenin 3
NHEJ	nonhomologous end joining
NIDDM	non-insulin dependent diabetes mellitus
NLS	nuclear localization signal
NO	nitric oxide
NOD	non-obese diabetic

NRE

Oct-1

pADPr

PARG

PARP

Pax

PC2/3

PCAF

PCNA

PD

PDX-1

PFK-1

PKB

PKC

PPAR

pTKCA

Pur-1

Q

RIPE-3

RNA

RNP

RPA

NRE	negative regulatory element
Oct-1	Octamer transcription factor 1
pADPr	poly(ADP-ribose)
PARG	pADPr glycohydrolase
PARP	poly(ADP-ribose) polymerase
Pax	paired-box containing transcription factor
PC2/3	proinsulin-processing endopeptidase 2/3
PCAF	p300-associated factor
PCNA	proliferating-cell nuclear antigen
PD	PD128763
PDX-1	pancreatic duodenal homeobox-1
PFK-1	phosphofructokinase-1
PKB	protein kinase B
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
pTKCAT	thymidine kinase promoter regulating chloramphenical acetyltransferase (CAT) reporter gene
Pur-1	purine-binding protein-1
Q	quercetin
RIPE-3b-1	rat insulin promoter element-3b-1
RNA	ribonucleic acid
RNP	ribonulceoprotein
RPA	replication protein A

ROS

RSV

RXRα

SCE

SDS-PAG

Sir2

SRE

SREBP-1

SSB

STF1

STZ

T0

TANK

TBP

TFP1

TEF-1

TG

TFIII

TNF

TRE

TRF1

TSA

μM

ROS	reactive oxygen species
RSV	Rous sarcoma virus
RXRα	retinoic X receptor alpha
SCE	sister chromatid exchange
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sir2	silent information regulator 2
SRE	sterol response element
SREBP-1c	sterol response element binding protein-1c
SSB	single-strand-break
STF1	somatostatin transcription factor 1
STZ	strepzotocin
T0	T0901317
TANK	tankyrase
TBP	TATA box-binding protein
TFP1	telomerase associated protein-1
TEF-1	transcriptional enhancer factor-1
TG	triglyceride
TFIID	transcription factor IID
TNF-α	tumor necrosis factor-α
TRE	12-O-tetradecanoate-13-acetate-responsive element
TRF1	telomeric repeat binding factor-1
TSA	trichostatin A
μM	micromolar

UCP2

USF

VPARP

XRCC1

YY1

ZDF rats

UCP2	uncoupling protein 2
USF	upstream stimulatory factor
VPARP	vault-PARP
XRCC1	X-ray cross-complementing factor-1
YY1	Yin-Yang 1
ZDF rats	Zucker diabetic fatty rats

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Chapter 1. Introduction

Diabetes mellitus has reached epidemic proportions, affecting more than 194 million people worldwide in the year of 2003. It has been estimated that there will be an approximately 42% increase of the diabetic population by year 2025 with the greatest increase in developing countries (Source: International Diabetes Federation (http://www.eatlas.idf.org)). Diabetes is a metabolic disorder and it is associated with relative or absolute insufficiency of insulin secretion. There are two types of diabetes: type 1 (also called juvenile diabetes or insulin-dependent diabetes mellitus (IDDM)), and type 2 (also called adult-onset diabetes or non-insulin-dependent diabetes mellitus (NIDDM)). Type 1 diabetic patients have an absolute insulin insufficiency due to the immunological destruction of pancreatic β-cells, which are responsible for the synthesis and secretion of insulin. Type 2 diabetes is characterized by insulin resistance and a relative insulin deficiency. About 85% to 95% of diabetic individuals have type 2 diabetes in developed countries, and it accounts for even higher percentage of diabetes in developing countries (Source: International Diabetes Federation (http://www.eatlas.idf.org)). Type 2 diabetes is polygenic and alterations of several gene products have been identified and most of them are normally involved in insulin secretion or insulin sensitivity (reviewed in (1)). Decreased insulin secretion and insulin sensitivity lead to hyperglycemia and hyperlipidemia in diabetic individuals. It is believed that chronic hyperglycemia and hyperlipidemia induce β-cell damage and play essential roles in the progression of type 2 diabetes.

The current therapeutic agents targeting pancreatic β-cell function include sulfonylurea derivatives and meglitinides (2). Both of these agents act by binding to the

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sulphonylurea receptor 1, and enhance insulin secretion (2). Sulfonylurea derivatives and meglitinides, however, do not increase insulin biosynthesis or β-cell differentiation. Low-potency poly(ADP-ribose) polymerase inhibitors (lp-PARPi) including nicotinamide (NAM), 3-aminobenzamide (3-AB), and PD128763 (PD) are a group of compounds known to have low affinity to poly(ADP-ribose) polymerase (PARP). The effects of NAM and 3-AB have been evaluated in animal models of type 1 and type 2 diabetes, and even in human for type 1 diabetes (3-9). In type 1 diabetic animal models and hyperglycemic animal models, NAM or 3-AB treatment decreases blood glucose level, increases pancreatic insulin content, improves glucose tolerance and increases β-cell regeneration (3, 5, 6). In type 2 diabetic Zucker diabetic fatty (ZDF) rats, which have the mutation in the leptin receptor, NAM reduces plasma free fatty acid (FFA) levels, prevents triglyceride accumulation in islets and improves glucose-stimulated insulin secretion (GSIS) (4). NAM and 3-AB also stimulated insulin biosynthesis, β-cell proliferation and differentiation, and improved GSIS in cultured porcine and human fetal pancreas (10-15). Therefore, in comparing to sulfonylurea derivatives and meglitinides, which only increase secretion from β-cells, lp-PARPi can improve β-cell function, possibly through the increased insulin biosynthesis and enhanced β-cell differentiation. The mechanisms whereby lp-PARPi increase insulin biosynthesis and enhance β-cell differentiation have not been well studied. The focus of this thesis project is on studying the mechanisms whereby lp-PARPi regulate insulin biosynthesis in INS-1 cells cultured with elevated glucose for a prolonged period.

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MafA gene

Overall hypothesis:

Chronic hyperglycemia leads to reduced insulin gene expression, in part, through the decreased insulin promoter activity (16-20). The human insulin promoter contains several cis-elements, which mediate glucose regulation of insulin promoter activity. Chronic hyperglycemia reduces pancreatic duodenal homeobox factor 1 (PDX-1) binding to the A elements, and this is associated with downregulation of PDX-1 gene expression (17-19, 21). Several lines of evidences, however, indicate that increased PDX-1 expression does not restore defective insulin gene expression (22-24), indicating that loss of PDX-1 does not account for glucose-suppression of insulin promoter activity. Glucose-suppression of insulin promoter activity is also mediated by reduced binding to the C1 element (17, 20). The identity of the C1-activator lost in β-cells exposed to elevated glucose was unknown. Recently, MafA has been shown to bind to the C1 element and induce insulin promoter activity upon acute glucose stimulation (25-28). In the first section of my thesis, I present a series of experiments investigating **the hypothesis that loss of MafA protein and binding to insulin promoter plays a major role in defective insulin promoter activity in β-cells cultured in high glucose (Fig. 1).**

Culturing of human and porcine fetal islets with lp-PARPi, NAM and 3-AB, increased insulin biosynthesis, insulin content, and improved GSIS (10, 12, 13, 15). The mechanisms whereby these compounds increase insulin biosynthesis were unknown. My preliminary data showed that lp-PARPi markedly induced insulin promoter activity in INS-1 cells cultured in high glucose. One of the possibilities is that lp-PARPi restore MafA gene expression. Therefore, **I hypothesized that lp-PARPi increased insulin**

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promoter activity through the enhanced MafA gene expression and MafA binding to insulin promoter in INS-1 cells exposed to elevated glucose (Fig. 1).

Specific Aims:

Specific aim 1: To determine the effects of chronic hyperglycemia on MafA gene expression and binding to insulin promoter. (Chapter 4)

Specific aim 2: To determine whether lp-PARPi increase insulin promoter activity through the enhanced MafA gene expression and MafA binding to insulin promoter in INS-1 cells cultured in high glucose. (Chapter 5)

Specific aim 3: To study the effects of lp-PARPi on transcription factors, coactivators and repressors action at insulin promoter. (Chapter 6)

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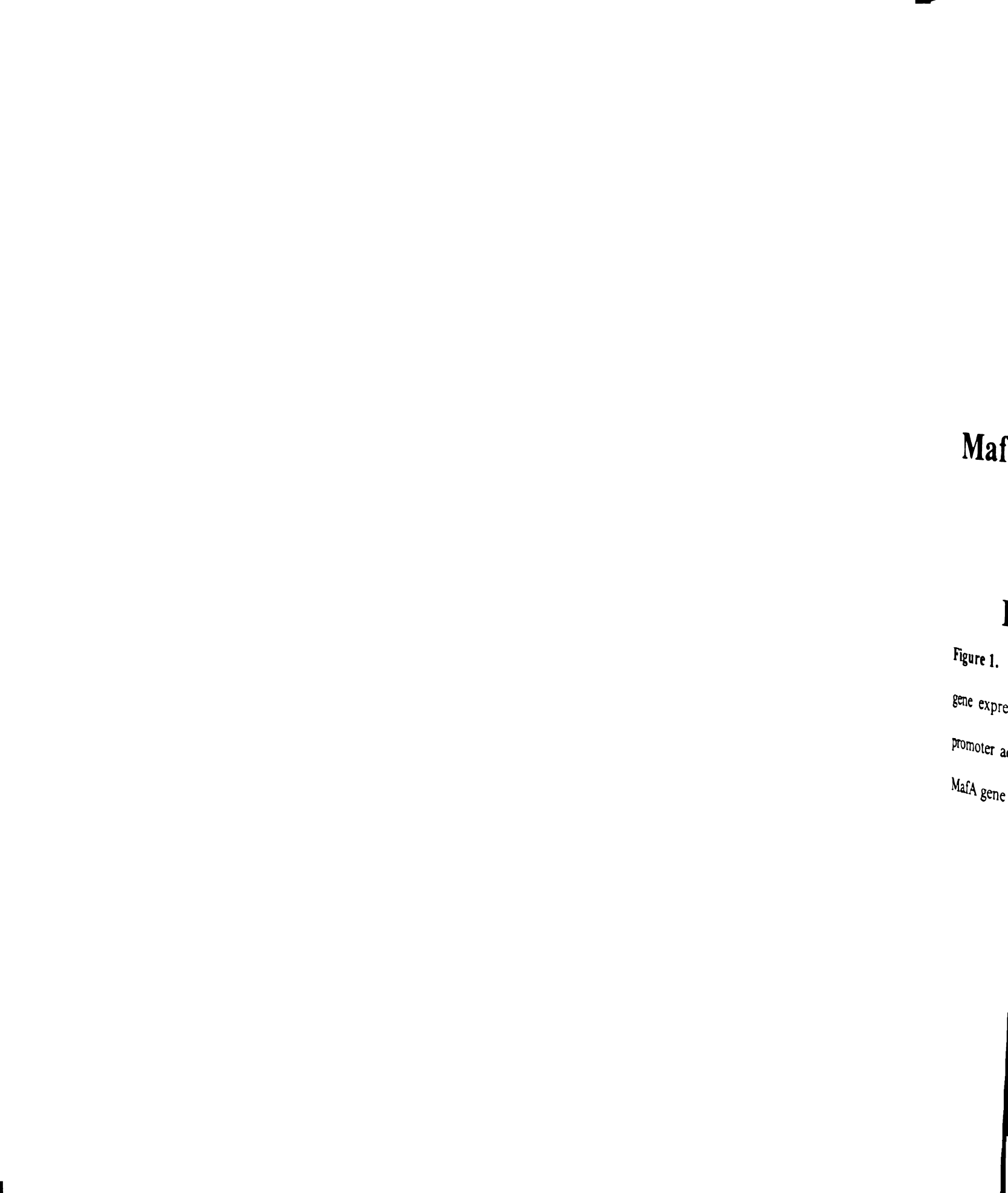

Figure 1.

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promoter a

MafA gene

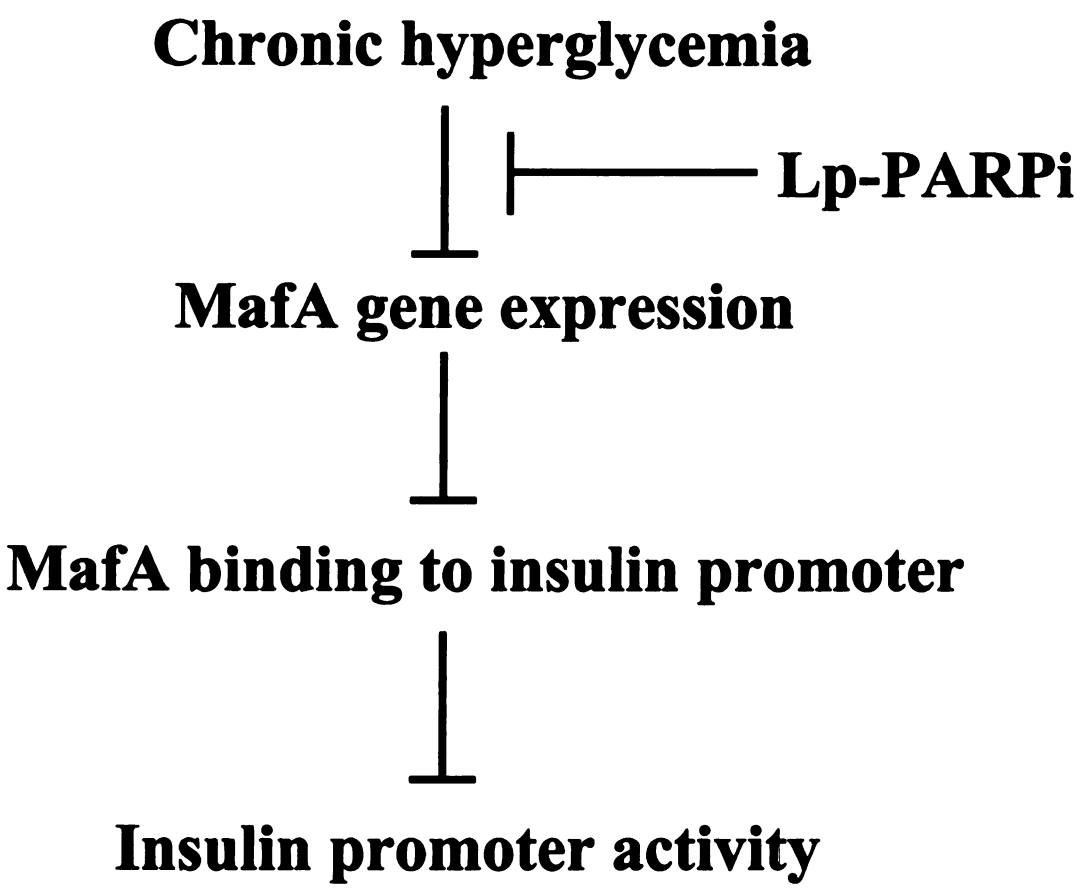

Figure 1. Hypothesis of the thesis project. Chronic hyperglycemia reduces MafA gene expression, leading to decreased MafA binding to insulin promoter and insulin promoter activity. Lp-PARPi stimulate insulin promoter activity through the increased MafA gene expression and binding to insulin promoter.

Chapter 2. Literature Review

I. Insulin biosynthesis and secretion

1. Insulin biosynthesis

Insulin is synthesized in pancreatic β-cells, and plays an essential role in the metabolism of carbohydrates, proteins and fatty acids. Insulin mRNA is translated to a single chain precursor called preproinsulin, which contains the signal peptide, B-chain, connecting peptide (C peptide), and A-chain (Fig. 2). The signal peptide is responsible for the translocation of preproinsulin from cytoplasm across the rough endoplasmic reticulum (RER) membrane. Once preproinsulin enters the RER, the signal peptide is removed and proinsulin is generated. C peptide is important for the correct structural alignment and disulfide linkage of the insulin A and B chains. Proinsulin has a structure similar to insulin since the C peptide adds little secondary structure to the insulin molecule. This probably can explain why proinsulin also has some biological activity as insulin. There are two dibasic sites that flank the C peptide. These two sites are essential for the proteolytic conversion of proinsulin to insulin. Within the RER, proinsulin undergoes a folding process so that the disulfide bonds of A- and B- chains are correctly aligned. In Golgi, the proinsulin enters immature granules and is converted to biological active insulin. Two proinsulin-processing endopeptidases, PC2 and PC3, are responsible for excising C peptide. Once the immature granules become mature granules, they are ready to be secreted upon stimulation.

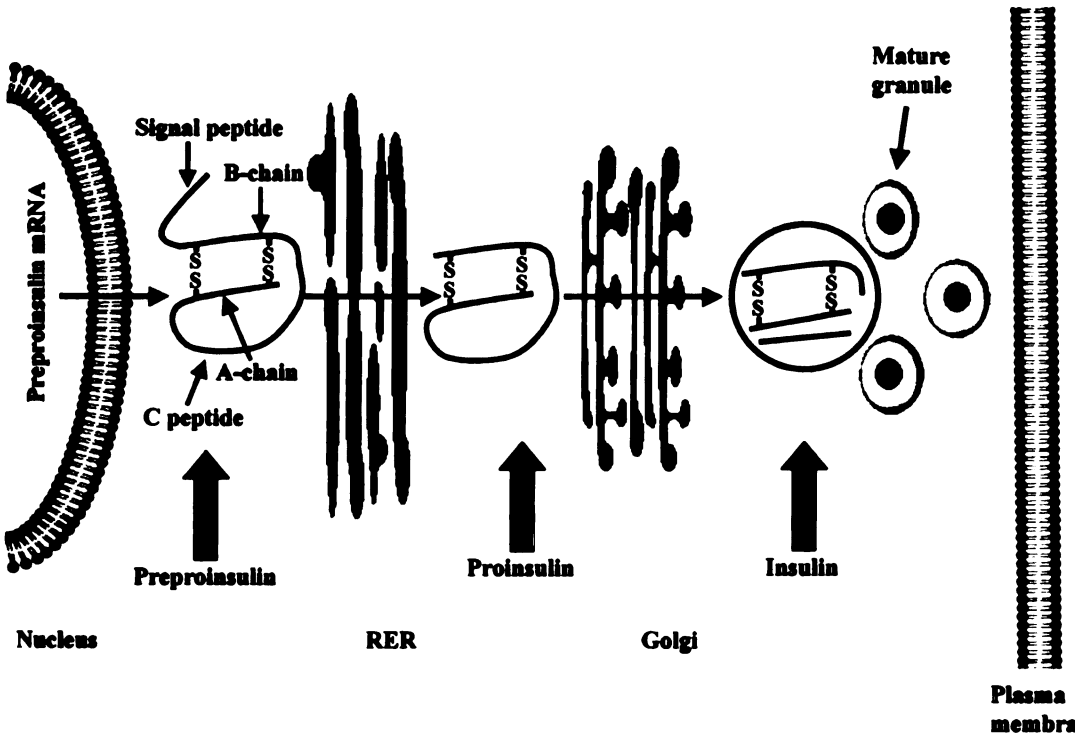

Figure 2. Insulin biosynthesis. Preproinsulin mRNA is transferred to cytosol. There, it is translated to preproinsulin. Preproinsulin is comprised of the signal peptide, B-chain, C peptide and A-chain. Preproinsulin is transferred to rough ER and the signal peptide is removed. Proinsulin is then generated and enters Golgi. In the Golgi, proinsulin is cleaved by proinsulin-processing endopeptidase PC2 and PC3 to generate mature insulin. Insulin is stored in mature granules and ready for release upon stimulation. This figure is modified from Figure 3-4 in *Diabetes Mellitus-A Fundamental and Clinical Text (29).*

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2. Glucose-stimulated insulin secretion (GSIS)

Insulin is the key hormone in regulating blood glucose level. Normoglycemia is reached by the interplay of insulin secretion and its action on the peripheral tissues. When blood glucose is elevated, glucose is taken up rapidly by the β-cell via the glucose transporter 2 (GLUT2). Once inside the β-cell, glucose is phosphorylated by glucokinase to glucose-6-phosphate, which is then further metabolized through glycolysis to pyruvate. In the mitochondria, the tricarboxylic acid (TCA) cycle uses pyruvate to produce ATP. ATP can also be produced through glycolysis and reduced nicotinamide dinucleotide phosphate (NADH) shuttle. The increased ATP/ADP ratio results in the closure of K_{ATP} channel. The β-cell membrane is then depolarized, leading to the opening of L-type voltage-gated Ca^{2+} channels. Influx of Ca^{2+} triggers insulin release from the preformed mature granules. Acute glucose stimulation can also stimulate insulin gene expression and increase insulin biosynthesis (Fig. 3).

Glucose-stimulated insulin secretion (GSIS) is biphasic. *In vitro*, when the rat islets are incubated in a stimulatory glucose concentration, the insulin secretion will increase within 10 minutes. The insulin release will reach a peak rapidly and then declines over the next 4 minutes. This is called the first phase of GSIS. After the first phase, the insulin secretion increases steadily until it reaches plateau within the following 40 minutes. This is termed the second phase of GSIS (reviewed in (30)).

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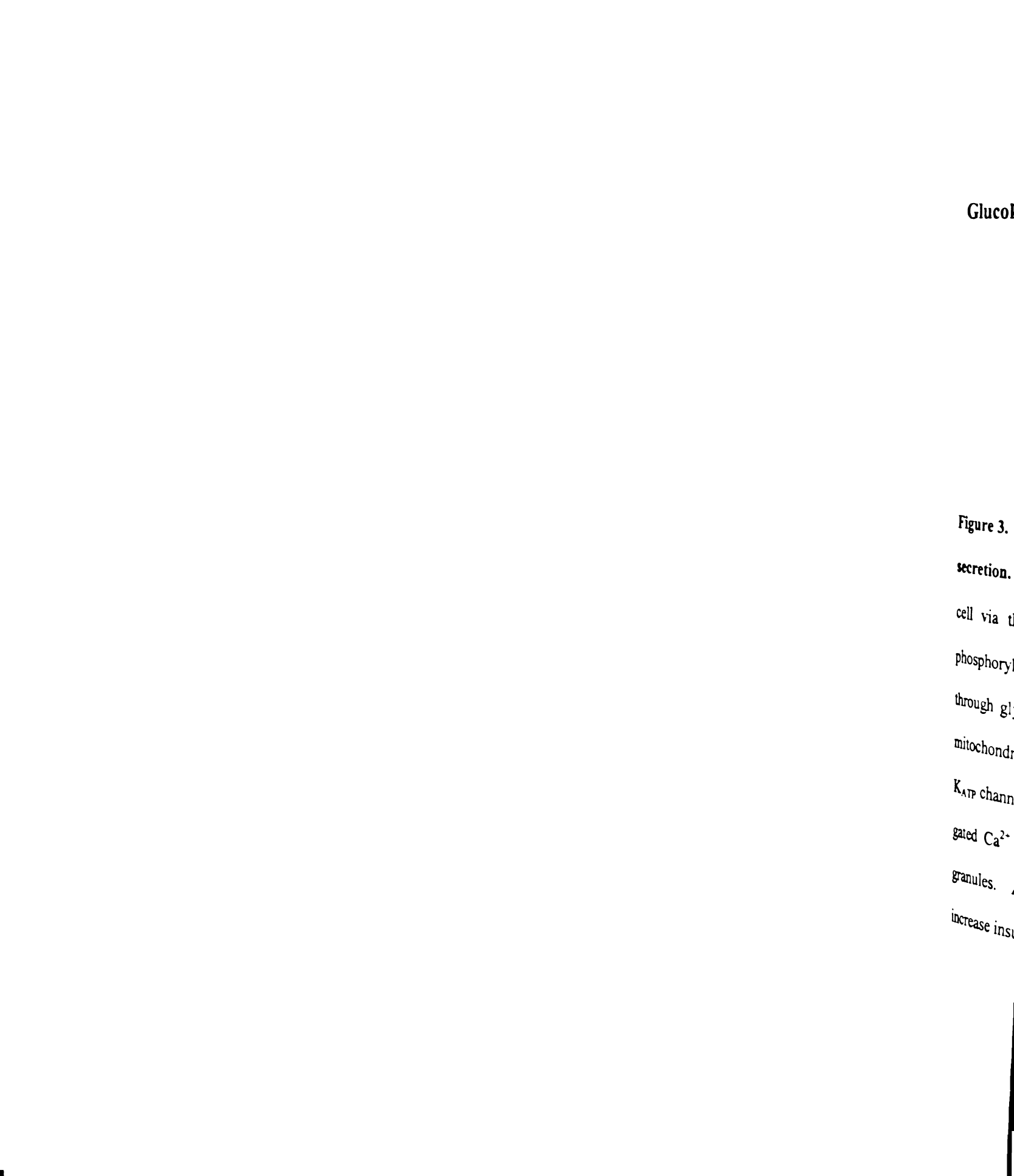

Figure 3.

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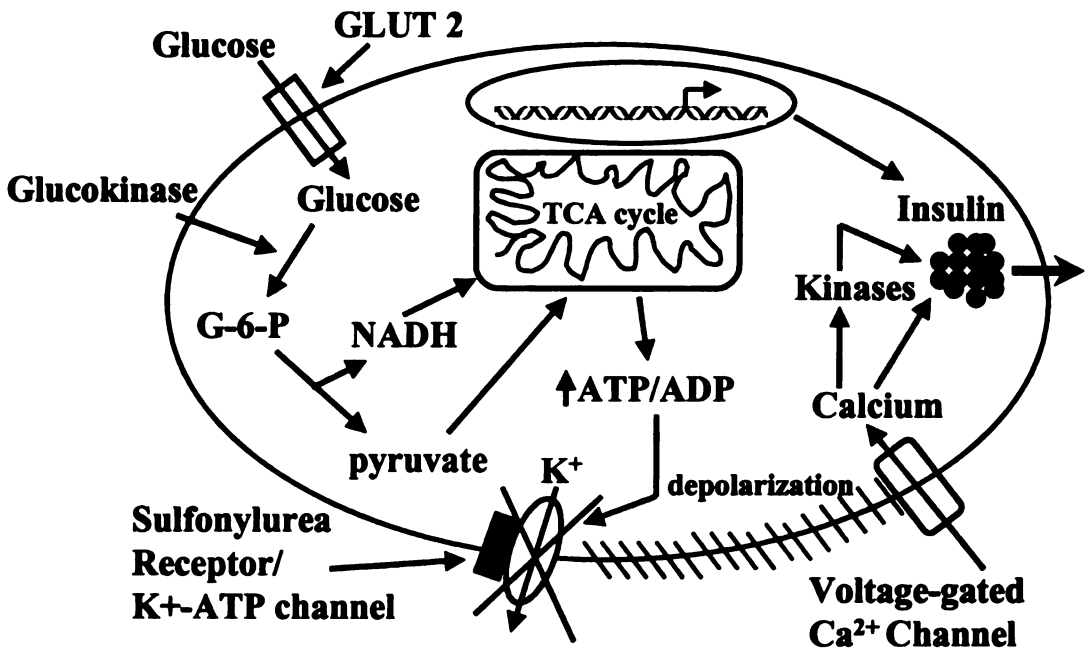

Figure 3. Schematic representation of acute glucose-stimulated insulin secretion. Acute increases of blood glucose leads to increased glucose uptake by the β-cell via the glucose transporter 2 (GLUT2). Once inside the β-cell, glucose is phosphorylated by glucokinase to glucose-6-phosphate, which is then further metabolized through glycolysis to pyruvate. The tricarboxylic acid (TCA) cycle uses pyruvate in mitochondria to produce ATP. The increased ATP/ADP ratio leads to the closure of K_{ATP} channel. The β-cell membrane is then depolarized, causing the opening of voltage-gated Ca^{2+} channels. Influx of Ca^{2+} triggers insulin release from preformed mature granules. Acute glucose stimulation can also stimulate insulin gene expression and increase insulin biosynthesis.

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II. Regulation of human insulin gene transcription by transcription factors and coactivators

Insulin is specifically expressed in pancreatic β-cells. Rats and mice have two non-allelic insulin genes, and human has one insulin gene. The human insulin gene is located on chromosome 11p15.5 and contains two introns and three exons (31). Preproinsulin mRNA is 446 bp in length. The insulin promoter consists of DNA sequences immediately upstream of the site of transcription initiation. Transgenic animals and transient transfection analyses have shown that the proximal 5'-flanking region of insulin promoter (-360 bp to +1 bp) is sufficient for directing β-cell specific insulin gene expression (32-34). Transcription of insulin gene is the rate-limiting step of insulin biosynthesis, and is regulated through cis-elements located within the proximal promoter. Figure 4 is the simplified version of human insulin promoter that shows the cis-elements and the transcription factors bound to them. Removal or mutation of these cis-elements results in altered promoter function and gene transcription. Important cis-elements within human insulin promoter include the A elements (A1, A2, A3 and A5), C1 element, E-box elements (E1, E2, and E3), cAMP responsive element (CRE), Z/NRE element, and A5/core elements. Transcription factors and coactivators can form binding complexes at the insulin promoter to control glucose-dependent insulin gene transcription (Fig. 5). In addition, nuclear proteins that modify chromatin structure also play roles in regulating insulin gene transcription. High glucose can acutely stimulate insulin promoter activity, whereas chronic hyperglycemia leads to suppression of insulin promoter activity. The review in this section will consider effects of acute glucose stimulation on insulin promoter activity. The effects of chronic hyperglycemia on

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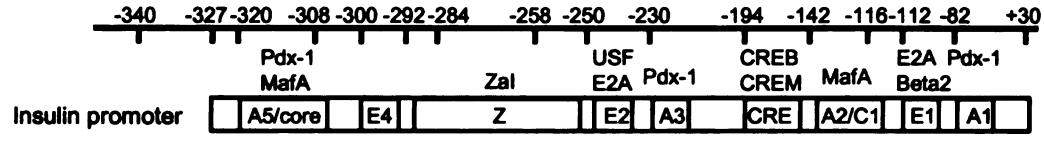

Figure 4. A simplified representation of human insulin promoter showing well characterized cis-elements and binding factors. The insulin gene transcription is regulated by the proximal 5'-flanking region of insulin promoter (-360 bp to +1 bp). The insulin promoter consists of several cis-elements, which are important for insulin promoter activity. Shown here are the A elements (A1 and A3), E-boxes (E1, E2, E3, and E4), A2/C1 elements, CRE, Z/NRE element, and A5/core elements. PDX-1 is the main transcription factor that binds to A elements. Basic helix-loop-helix proteins, BETA2 and E2A, are transcription factors that bind to E-boxes. MafA is the transcription factor that binds to both C1 and A5/core elements. CREB and CREM have been shown to bind to CRE.

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1. A elements and PDX-1

The A elements are adenosine/thymidine-rich sequences that contain a core TAAT sequence. The A2 is the exception to the rule and contains a GGAAAT sequence. There are four A elements on human insulin promoter: A1 (-82 bp to –77 bp), A2 (-134 bp to –129 bp), A3 (-215 bp to –210 bp) and A5 (-318 bp to –313 bp) (Fig. 4). A1 and A3 are both well conserved. The A3 element plays a major role in cell-specific and glucose-responsive transcription (35-37). Mutation of the A3 element on insulin promoter led to loss of the promoter activity in response to acute glucose stimulation in islets and β-cell lines (35-37). The A5 element and the adjacent highly conserved enhancer core sequence are termed A5/core, which mediates glucose-stimulation of the distal insulin promoter activity (38). The A2 element along with nearby C1 element forms an insulin minienhancer region called rat insulin promoter element 3b (RIPE3b), which mediates β-cell-specific and glucose-induced insulin gene transcription (39).

Homeodomain proteins bind to the A elements to regulate insulin gene transcription. There are five major homeoproteins in human pancreatic β-cells including islet factor-1 (Isl-1), LIM-homeodomain proteins-lmx1.1, lmx1.2, lmx2/lim1/lhx, and PDX-1 (also called IPF1, STF1, IDX1, IUF1 and GSF). Among all these homeodomain proteins, PDX-1 plays an essential role in insulin gene expression as well as pancreas development (34, 36, 37, 40).

PDX-1 binds to the A elements on insulin promoter and activates insulin promoter upon acute glucose stimulation (35, 37). The enhancer activity of PDX-1 depends on its

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interaction with transcription factors adjacent to it, namely BETA2 and E47, which bind to the E-boxes (41) (Fig. 5). The cooperative DNA binding of PDX-1 and BETA2/E47 can stabilize the binding complex formation at the promoter (42). It was suggested that the synergy between PDX-1 and BETA2/E47 was facilitated by the binding of high-mobility-group protein I(Y) (HMG I(Y)) to the A3 element (42). HMG I(Y) is a chromatin-associated protein and binds to the minor groove of AT-rich region of DNA. HMGI(Y) has been shown to bind to the A3/4 elements on the rat 1 insulin promoter and physically interact with PDX-1 through its homeodomain (42). Binding of HMG I(Y) itself to A3/4 elements, however, does not induce the promoter activity, suggesting that HMG I(Y) may facilitate binding complex formation by modifying chromatin structure (42) (Fig. 5).

The interaction of PDX-1 and BETA2/E47 recruits coactivators p300 and CBP to enhance insulin promoter activity (43, 44) (Fig. 5). The recruitment of p300 to the insulin promoter stabilizes PDX-1/BETA2/E47 complex formation at the promoter (44). p300 acts as the histone acetyltransferase at the insulin promoter to cause hyperacetylation of histone H4 and relaxation of chromatin structure, facilitating gene transcription (43). Glucose modulates PDX-1 binding activity to the insulin promoter in a phosphorylation-dependent manner (45, 46). Phosphorylated PDX-1 can be translocated to the nuclei and interact with p300 (43, 47). In contrast, dephosphorylated PDX-1 rapidly recruits histone deacetylases HDAC-1 and HDAC-2 to insulin promoter when the β-cells are switched from high glucose to low glucose (48) (Fig. 5). HDAC-1 and -2 may deacetylate histone H4, causing decreased insulin promoter activity in cells treated with low glucose (48). PDX-1 and coactivator p300 interaction also enhances the

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occupancy of RNA polymerase II (Pol II) at the insulin coding region, but not at the transcription initiation site of the promoter, suggesting that PDX-1/p300 interaction stimulates transcriptional elongation by Pol II (49).

2. C1 element and Maf proteins

The C1 (-124 bp to –116 bp) element is a cytosine-rich element. The C1 element and adjacent A2 element synergize with the E1 element to activate the insulin promoter (50, 51). In rat 2 insulin promoter, the C1 element mutation causes a dramatic loss of insulin promoter activity (39, 52). Transcription factor MafA binds to the C1 element and has been shown to increased insulin promoter activity when overexpressed (25-27).

MafA belongs to the Maf oncogene family, which was originally identified with the isolation of a viral oncogene, v-maf gene, from the genome of acute transforming retrovirus AS42 (53). MafA is a glucose-regulated and β-cell enriched transcription factor that activates insulin promoter (25, 54). Acute high glucose stimulation can lead to increase of MafA nuclear protein and mRNA levels (25, 55). MafA/C1 binding complexes formation can be induced at acute glucose stimulation, leading to increased insulin promoter activity (52, 56, 57).

MafA plays a crucial role on insulin gene expression by interacting with other transcription factors of insulin promoter including PDX-1 and BETA2 (Fig. 5) (58-60). Both *in vivo* and *in vitro* overexpression of MafA, PDX-1 and BETA2 together led to a marked increase of insulin gene expression, and this induction was less profound without the overexpression of MafA (58-60). In strepzotocin (STZ)-induced diabetic mice, triple infection of MafA, PDX-1 and BETA2 adenovirus increased blood insulin level and

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improved glucose tolerance (60). Unlike PDX-1 and BETA2, which recruit p300 and synergistically activate insulin promoter, MafA does not appear to activate insulin promoter by direct contacting with p300 (59). Overexpression of MafA, PDX-1, BETA2, and p300 together, however, significantly induced insulin promoter activity (59), indicating that MafA may stabilize PDX-1/BETA2/p300 binding complex formation on the insulin promoter.

3. E-box elements and the basic helix-loop-helix (bHLH) proteins

The E-box elements contain the core sequence CANNTG. They have been shown to be strong regulatory elements, and mutation in E-box elements completely abolished the insulin promoter activity (61). The E1 (-111 bp to –104 bp) element (also called IEB1) is well conserved in all of the known mammalian insulin promoters, whereas the E2 (-239 bp to –232 bp) element is poorly conserved. The human insulin promoter also contains a more distal E3 (-273 bp to –257 bp) element located within the Z element (62). An additional putative E element termed E4 (-300 bp to –294 bp) was recently identified located between A5/core elements and Z element (63).

Basic helix-loop-helix class (bHLH) transcription factors bind to E-box elements as dimers (64). There are two types of bHLH transcription factors, which are important in insulin gene transcription, class A and B. Class A bHLH transcription factors are found ubiquitously, and can bind to DNA as homodimers or heterodimers. Class A bHLH proteins found in β-cells are E2A proteins (E47 and E12), and human E-protein (HEB) (65-67). Class B bHLH proteins are tissue-specific and preferentially forming heterodimers with class A factors. The class B bHLH protein found in pancreatic β-cells

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is BETA2 (also known as NeuroD1) (50). Heterodimers of E47/BETA2 have been shown to regulate insulin promoter activity in β-cell lines (68).

BETA2/E47/12 regulate insulin promoter activity through their interactions with other transcription factors and coactivators (Fig. 4). BETA2/E47/E12 synergistically interact with MafA and PDX-1 to activate insulin promoter (41, 50, 51). Overexpression of BETA2, E47/12 and PDX-1 simultaneously dramatically stimulated insulin gene transcription when compared to each transcription factor alone (69). Coactivators p300 and CREB binding protein (CBP) interact with both BETA2 and E47 to enhance their transactivation activities of insulin promoter, possibly through increased interaction of transcription factors with RNA pol II of the basal transcription machinery (44, 70, 71). Bridge-1 is another coactivator that can potentiate BETA2/E47 activity upon glucose stimulation (72). Bridge-1 is a PDZ-domain protein that is expressed in a variety of tissues. It interacts with E47 and E12, but not BETA2 (72). Expression of Bridge-1 antisense RNA decreased rat 1 insulin promoter by 45% in INS-1 cells (72).

BETA2 posttranscriptional modification also enhances its activation of insulin promoter. p300-associated factor (PCAF) has been shown to associate and acetylate BETA2 at the loop region of bHLH to potentiate its activation of insulin promoter (73). Moreover, phosphorylation of E47/E12 by ERK1/2 facilitates their dimerization with BETA2, leading to activation of insulin promoter (74).

4. cAMP response elements (CRE) and their binding factors

There are four functional CREs on the human insulin gene and each of these CREs have sequences that differ from the consensus CRE motif (TGACGTCA) (75).

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Two of the CREs (CRE1: -221 bp to –195 bp, CRE2: –189 bp to –167 bp) are present on insulin promoter region (75). The other two CREs are located outside of insulin promoter region: one is located at the first exon, and the other one is located at the first intron (75). Site-directed mutation of the CREs led to a remarkable reduction of insulin gene transcription (75).

Several nuclear proteins have been shown to bind to CRE, including c-Jun, cAMP response element binding protein (CREBP), cAMP response element modulator (CREM) and MafA (28, 76). Increased levels of cAMP can induce insulin gene transcription, however, cAMP is only a modest activator of insulin gene transcription (76). Studies have shown that CREB is not as strong of an activator of the insulin promoter compared to CREM. CREM activates the insulin gene transcription by not only directly binding to the CREs, but by also recruiting transcription factor IID (TFIID) to the insulin promoter (76). Inada et al. have proposed that glucose stimulation might involve an intracellular signal transduction to enhance phosphorylation of CREM, which would increase CREM transactivation of insulin promoter (76). Another reason for the poor activity of CREB could be due to the overlapping of other proteins such as NF-Y, which binds to CCAAT element directly downstream of the CRE2 (77).

Another transcription factor that has been shown to bind to the CRE is c-Jun, a transcription factor activated by stress signals (75). Overexpression of c-Jun was found to repress cAMP-induced insulin gene transcription (75). c-Jun consensus element (TGACTCA) is very similar to the CRE, and c-Jun can form heterodimer with CREBP and bind to the CRE with high affinity. Glucose deprivation leads to induction of c-Jun

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mRNA level, indicating the possible role of c-Jun for suppression of insulin gene transcription (75).

More recently, Matsuoka et al. have proposed that the CRE2 element on the insulin promoter could be actually a MafA binding site (28). Direct binding of MafA to the CRE2 element has been shown, but the binding affinity is much weaker than MafA binding to the C1 element (28).

5. Negative regulatory element (NRE) and Z element

It has been shown by Docherty and associates that 5' deletion of -292 bp to –243 bp of the human insulin gene promoter results in a large (25-fold) increase of insulin gene transcription in HIT β-cells (78). This sequence has also been shown to inhibit transcription when linked to a heterologous promoter transfected into either non-β-cell or β-cell tumor lines (78). Therefore, this region was named negative regulatory element (NRE), and it was proposed that NRE mediated glucose-suppression of insulin gene transcription. The NRE, however, does not function as a repressor in INS-1 cells or primary β-cells (38, 62). Indeed, the NRE functions as a glucose-dependent activation element when it is placed upstream of a minimal insulin promoter (-85 bp to +30 bp) or a heterologous promoter in both fetal and adult islets (62). Therefore, NRE appears to be an activator *in vivo*, and was renamed the Z element by German et al. Several islet protein complexes bind to the Z element, including Za1 complex, which positively correlates with glucose activation of insulin promoter (62).

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6. A5/core elements and MafA and PDX-1

Deletion analysis has demonstrated that –327 bp to –261 bp of 5' insulin promoter region mediated glucose-suppression of insulin promoter activity (38, 62). Within this region, the A5/core elements (-323 bp to –309 bp) has been shown to be responsible, in part, for the glucose-suppression of the distal insulin promoter activity (63). Both MafA and PDX-1 have been identified to bind to the A5/core elements (63).

7. A model of integrated regulation of insulin transcription by nuclear proteins

Acute glucose activation of insulin gene transcription is regulated by transcription factors, chromatin modification proteins, and coactivators. Shown in figure 5 is a model of integrated regulation of insulin gene transcription.

HMG I(Y) is a chromatin-associated protein, and binds to the minor groove of AT-rich region such as A3/4 of insulin promoter. Under acute high glucose stimulation, HMG I(Y) binding to DNA changes the chromatin conformation and facilitates the PDX-1/ BEAT2/E47 binding complexes formation. High glucose also causes posttranslational modification of nuclear proteins, and these modifications include phosphorylation, acetylation and methylation. Phosphorylation of PDX-1 by ERK1/2 (74), phosphatidylinositol 3-kinase (PI3K) and stress-activated protein kinase 2 (SAPK2) (74), leads to PDX-1 translocation from cytosol to the nucleus where it binds to the promoter. BETA2 can also be phosphorylated by ERK1/2 (43, 47, 74). Phosphorylated PDX-1 and BETA2 lead to enhanced transactivation of insulin promoter. The interaction of PDX-1 with BETA2/E47 recruits p300/CBP, which can acetylate histone H4 (79) or H3 (80), making the chromatin more accessible for transcription binding complexes. Meanwhile,

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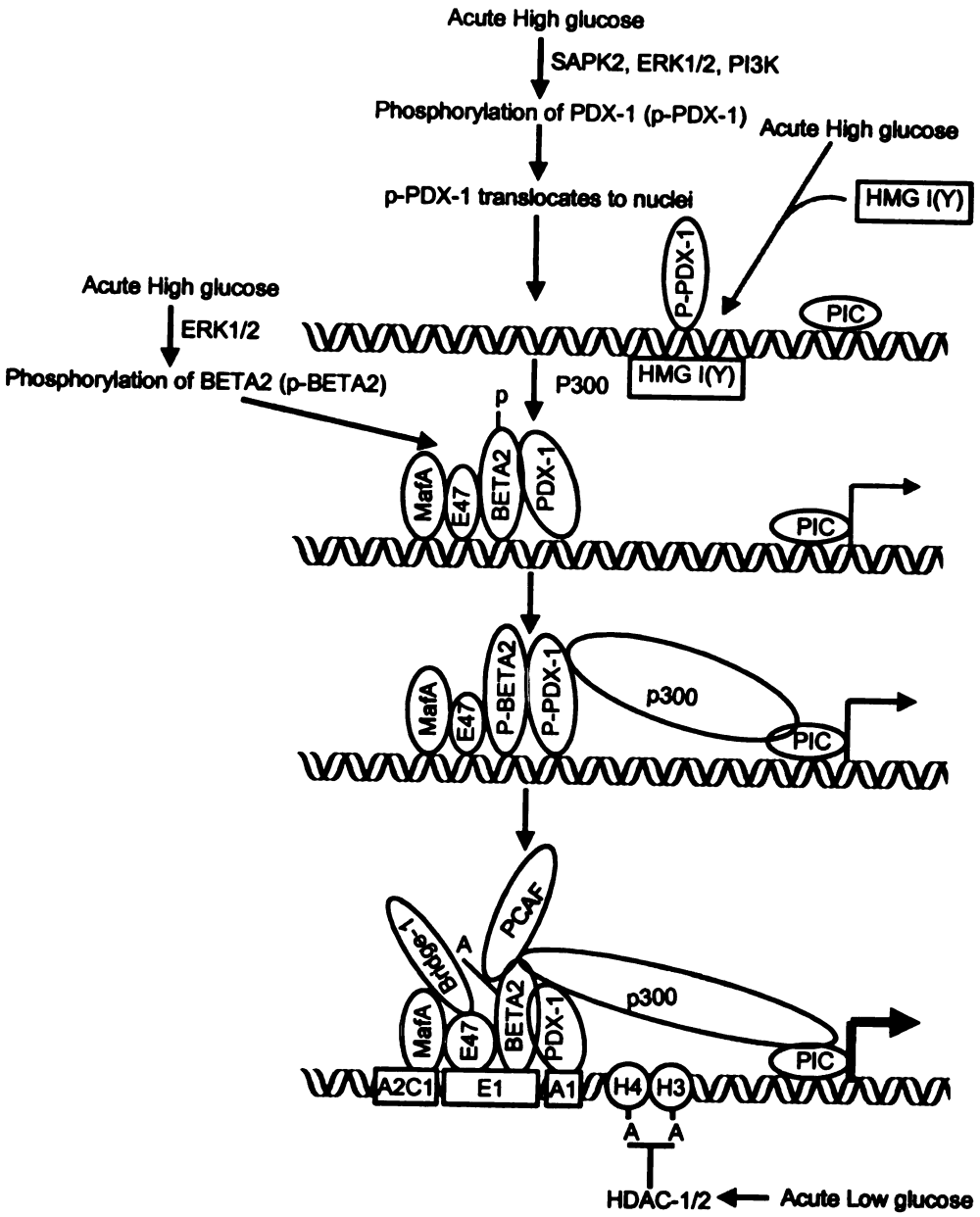
Figure 5. A model of integrated regulation of insulin gene transcription by transcription factors, chromatin modification proteins, and coactivators. When pancreatic β-cells are stimulated acutely with high glucose, PDX-1 and BETA2 are phosphorylated and bind to insulin promoter. HMG I(Y) also binds to the minor groove of AT-rich region of insulin promoter upon stimulation with high glucose. The binding of HMG I(Y) to DNA changes the chromatin conformation and facilitates PDX-1/E47/BEAT2 binding complexes formation. The interaction of PDX-1 with BETA2/E47 recruits p300 or CBP, which acetylates histone H4 or H3. Acetylation of histone H4 and H3 makes the chromatin more accessible for transcription binding complexes. p300/PDX-1/BEAT2/E47 interaction stimulates transcriptional elongation by RNA polymerase II. Binding of MafA further enhances PDX-1/BETA2/E47 stimulatory effects of insulin promoter. When cells are switched from high glucose to low glucose, HDAC-1 and HDAC-2 are rapidly recruited to the promoter by dephosphorylated PDX-1. HDAC-1 and HDAC-2 deacetylate histone H4 and decrease insulin gene transcription. Histone H3 is hypermethylated at insulin promoter when cells are stimulated with high glucose, facilitating gene transcription. PCAF is known to acetylate BETA2, which leads to stimulation of insulin promoter. Bridge-1, another coactivator, has been shown to enhance insulin promoter activity through its interaction with E47/12.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p300/PDX-1/BETA2/E47 interaction stimulates transcriptional elongation by RNA polymerase II. Binding of MafA further enhances PDX-1/BETA2/E47 stimulatory effects of insulin promoter, but the potentiation effect of MafA is not further affected by p300 (59, 60). When cells are switched from high glucose to low glucose, HDAC-1 and HDAC-2 are rapidly recruited to the promoter by dephosphorylated PDX-1. HDAC-1 and HDAC-2 deacetylate histone H4 and decrease insulin gene transcription. Histone H3 is also hypermethylated at Lys-4 (K-4) in β-cells treated with high glucose (80). Methylation of histone H3 modifies chromatin structure and facilitates gene transcription upon stimulation by elevated glucose concentration. PCAF can also acetylate BETA2, leading to stimulation of insulin promoter. Taken together, regulation of insulin promoter activity is a complex process involving the interaction of transcription factors and coactivators, chromatin modulation and posttranscriptional modification of transcription factors.

III. Type 2 diabetes mellitus

In a healthy individual, blood glucose is maintained at about 4.5 mM. The maintenance of normal glucose level depends on three major events: 1) stimulation of insulin secretion from pancreatic islets; 2) insulin-mediated suppression of hepatic gluconeogenesis; 3) insulin-mediated glucose uptake by peripheral tissues. High glucose itself can also suppress hepatic glucose production and enhance glucose uptake by peripheral tissues. The high glucose effects, however, are modest compared to insulin effects.

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Type 2 diabetes is associated with impaired insulin secretion and insulin resistance at peripheral tissues. Impaired GSIS is related to the adverse effects of hyperglycemia and hyperlipidemia. Both hyperglycemia and hyperlipidemia can lead to oxidative stress in β-cells, reduced insulin production, and increased β-cell apoptosis (81-88). Several mechanisms have been implicated in insulin resistance, and these include cellular defects in postreceptor signaling, glucose transport, and/or enzymes involved in glucose metabolism (1). In many obese people, insulin resistance induces a compensatory increase of β-cell mass, which leads to maintenance of normal blood glucose levels (89-91). Eventually, the β-cell compensatory mechanisms failed in some people, and hyperglycemia becomes apparent and type 2 diabetes occurs (89-91). Indeed, a net decrease of β-cell mass can be observed in most individuals with type 2 diabetes regardless they are obese or lean.

1. Five stages in the progression of type 2 diabetes

Weir et al have proposed that the progression of type 2 diabetes can be viewed of having five stages characterized by changes in various metabolic parameters and β-cell function (92).

Stage 1 is the compensatory stage, which is accompanied by increased insulin secretion in the face of insulin resistance. At this stage, there are increased β-cell mass and β-cell hypertrophy due to the increased demand of insulin production (89-91). The blood glucose level is kept at the normal level because of the increased insulin production. GSIS is normal at this stage.

Stage 2 is the stable adaptation stage, which is characterized with the loss of first phase of GSIS but preserved second phase of GSIS (93). Insulin secretion in response to non-glucose secretagogues such as arginine remains normal (94). In this stage, the fasting blood glucose level rises to 5 to 7.3 mM. The phenotype of β-cells has changed markedly with the downregulation of typically highly expressed genes including insulin, PDX-1, BETA2, glucokinase, and GLUT2 (95, 96). In contrast, genes that are usually suppressed are upregulated and these include genes important for lipid and glucose metabolism, apoptosis and inflammation (95-98). These alterations in gene expression profile are well correlated with the disrupted GSIS as demonstrated in animal models (96, 99). At this stage, chronic hyperglycemia and hyperlipidemia have been proposed to play important roles in the development of β-cell failure and diabetic complications. The adverse effects caused by hyperglycemia and hyperlipidemia are termed glucotoxicity and lipotoxicity, respectively.

Stage 3 is the early decompensation stage. Fasting blood glucose level rises rapidly in this stage to approximately 16 to 20 mM. β-cell mass decreases to the critical point that insulin production becomes insufficient to compensate for the hyperglycemia.

Stage 4 is the stable decompensation stage. At this stage, fasting blood glucose is as high as the stage 3. The patients are usually still able to produce enough insulin to remain in this stage, and for most cases, this stage can last life-long. β-cell mass is reduced by 50% at this stage (89, 91).

Stage 5 is the severe decompensation stage, which is characterized by ketoacidosis. The fasting blood glucose level rises above 22 mM at this stage. Patients

have lost so much of β-cell mass that they become ketotic and have to depend on insulin for survival.

2. Phenotypic changes of pancreatic β-cells in type 2 diabetes

Pancreatic β-cells undergo phenotypic changes in the progression of type 2 diabetes. Alteration of genes involved in lipid and carbohydrate metabolism has been demonstrated in several type 2 diabetic animal models. The phenotypic changes of pancreatic β-cells in type 2 diabetes are described below using the Zucker Diabetic Fatty (ZDF) rats as a model.

The Zucker Diabetic Fatty (ZDF) rat is a rodent model of type 2 diabetes with a predictable progression from prediabetic (1-6 weeks) to diabetic stage (7-10 weeks) (99, 100). ZDF rat has the mutation in the leptin receptor, which is required for the leptin-mediated metabolic pathway for energy expenditure. In the prediabetic stage, the ZDF rats are obese, insulin resistant and have impaired insulin secretion, but have normal blood glucose levels (99). Alterations in gene expression are present in prediabetic ZDF rat even before the onset of hyperglycemia (99). Reduced expression of genes involved in insulin secretion includes glucokinase, voltage-dependent Ca^{2+} channel, and K_{ATP} channel (99). Insulin gene expression is not changed in prediabetic ZDF rats (99). The diabetic ZDF rats have disrupted islet structure (99). They have many features in common with human type 2 diabetes including obesity, insulin resistance, high plasma fatty acid and triglyceride levels, hyperglycemia, and defects in insulin secretion (99). The islets of diabetic ZDF rats have high triglyceride content (101, 102). ZDF rats have decreased insulin gene expression after 6 to 7 weeks of age, which is associated with

decreased PDX-1 gene expression and increased expression of c-Myc and C/EBPβ insulin promoter repressors (103). Genes important for insulin secretion are further downregulated in diabetic ZDF rats. Lipogenic genes including ACC, FAS and sterol regulatory element binding protein-1c (SREBP-1c) are induced in islets of diabetic ZDF rats (101, 102). Increased triglyceride islet deposition is probably due to the increased lipogenic gene expression and elevated circulating lipids. In summary, diabetic ZDF rats show that the progression of diabetes is associated with elevated blood glucose and fatty acid levels, decreased insulin biosynthesis, increased islet lipogenesis and loss of β-cell function.

3. Glucotoxicity

The development of type 2 diabetes is associated with insulin resistance and β-cell dysfunction. Initially, β-cells can compensate for insulin resistance by increasing insulin secretion and β-cell mass. Once the β-cells compensation starts to decline, hyperglycemia becomes apparent. Chronic hyperglycemia causes the β-cell deterioration, which includes reduced insulin gene expression and impaired GSIS. If chronic hyperglycemia is left untreated, β-cell dysfunction and death will occur. Therefore, the adverse effects of chronic hyperglycemia are termed glucotoxicity. In patients with hyperglycemia, lowering the blood glucose level can slow down the progression of type 2 diabetes, implicating the important role of hyperglycemia in the transition of glucose intolerance to type 2 diabetes (104).

3.1. Effects of chronic hyperglycemia on insulin gene transcription

Chronic hyperglycemia-mediated decrease of insulin secretion can be partially due to reduced insulin gene transcription. In human islets and several cultured β-cell lines, incubating cells in high glucose for a long period of time reduced insulin gene transcription. The glucose-mediated reduction of insulin gene expression was associated with decreased binding of PDX-1 (17, 18, 21) and MafA (17-20, 105) to insulin promoter, and reduction in PDX-1 and MafA nuclear protein levels (16, 19, 21). *In vivo* studies showed that PDX-1 protein levels were reduced in 90% pancreactomy-induced diabetic animal models (103). Reduced BETA2 mRNA levels have also been shown in 90% pancreactomized diabetic rats (95). Decreased expression of these key transcription factors may be one of the reasons for the decreased insulin gene expression in chronic hyperglycemic condition.

Repressors are important in regulating gene transcription, and two known insulin gene transcriptional repressors C/EBPβ (97, 103) and c-myc (106, 107) are increased in pancreatic β-cells during chronic hyperglycemia. Increased expression of C/EBPβ and c-myc causes decreased transcription of the insulin gene by disrupting BETA2/E47 binding complex formation (106).

Chronic hyperglycemia mediated suppression of insulin gene transcription can be partially reversed (21, 108). When the islets were switched backed to normal glucose concentration, most of changes observed with high glucose were reversed (21). Lowering of hyperglycemia in diabetic animal models by using glucose lowering drug, phlorizin, preserved PDX-1 and insulin mRNA levels (108).

3.2. Effects of glucose-induced oxidative stress on insulin gene expression and β-cell function

Chronic exposure of pancreatic β-cells to hyperglycemia can increase generation of reactive oxygen species (ROS), which may be a primary cause of β-cell dysfunction and apoptosis (Fig. 6) (109). ROS such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and the concomitant nitric oxide production have been suggested in the development of both type 1 and type 2 diabetes (110). Pancreatic β-cells are vulnerable to glucose-induced oxidative stress due to its special physiological characters. They have high K_m GLUT2 (25 mM) and glucokinase (8 mM), and low amounts of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and thioredoxin (81, 111). These special features of β-cells make β-cells generate more ROS rapidly upon glucose stimulation but cannot eliminate ROS as fast. Several sources of ROS production exist, and these include nonenzymatic glycation reactions, hexosamine pathway, glyceraldehyde autoxidation and mitochondria electron transport chain (81). Free radicals may directly damage proteins, lipids, and DNA, causing mitochondrial and cell dysfunction. If the β-cell damage is severe, apoptosis can happen, leading to decreased β-cell mass.

3.2.1. Antioxidants protection against hyperglycemia-mediated β-cell dysfunction

In vivo studies have shown that antioxidants were able, in part, to prevent the hyperglycemia-mediated suppression of insulin gene expression and improve glucose intolerance. Treatment of ZDF prediabetic rats with N-acetyl-L-cysteine (NAC) or aminoguanidine (AG) partially prevented the development of hyperglycemia, glucose

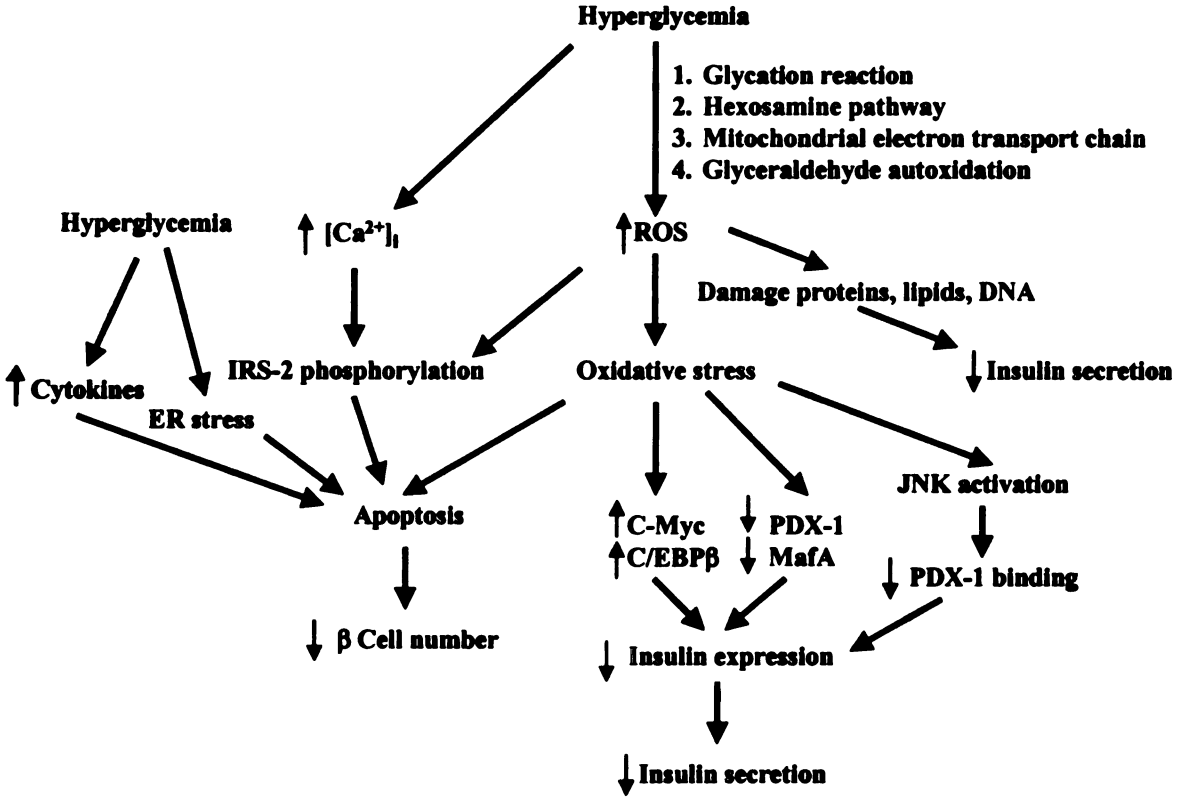

Figure 6. Mechanisms of hyperglycemia induced GSIS impairment and β-cell apoptosis. Several sources of ROS production exist, and these include nonenzymatic glycosylation reactions, hexosamine pathway, glyceraldehyde autoxidation and electron transport chain in mitochondria. Increased ROS can cause oxidative stress in β-cells, leading to reduced insulin gene expression, activation of stress response pathway (JNK) and apoptotic pathways. Activation of JNK pathway causes reduced insulin geen expression and secretion. Activation of apoptotic pathway leads to decreased β-cell mass. Free radicals can directly damage proteins, lipids, and DNA, causing mitochondrial and cell dysfunction. Increased cytokines, ER stress and intracellular calcium levels also contribute to apoptosis of β-cells. Reduction of β-cell mass will further lead to attenuation of insulin secretion.

intolerance, and impaired GSIS (112). Insulin gene expression and PDX-1 binding to insulin promoter were preserved in ZDF prediabetic rats treated with NAC or AG (112). The C57BL/KsJ-db/db mouse also has the mutation in the leptin receptor like ZDF rats (113). The db/db mice develop hyperglycemia due to decreased insulin production associated with β-cell failure (114). In diabetic db/db mice, NAC treatment leads to decreased blood glucose level, preserved PDX-1 and insulin gene expression, and improved GSIS compared to the controls (112, 114).

In HIT-T15 cells, chronic culturing in high glucose led to decreased insulin gene expression (112, 115). This defect in insulin gene expression has been shown to be associated with reduced PDX-1 and MafA gene expression and their bindings to insulin promoter. NAC has been demonstrated to prevent the loss of PDX-1 and MafA gene expression and bindings to insulin promoter (112, 115).

Since β-cells are low in antioxidant enzymes, these enzymes have been overexpressed to evaluate their effectiveness in protecting the β-cells against oxidative stress. Incubation of rat islets with high concentrations of ribose for a long-period of time (72 hr) has been shown to increase peroxide production (116). Glutathione peroxidase overexpression in islets protected them against ribose-mediated decreases in insulin mRNA, content, and secretion (116). Overexpression of other antioxidant enzymes such as superoxide dismutase and catalase have also been shown to provide protection against oxidative stress (117, 118). IL-1β has been shown to induce INS-1 cells death and human islets destruction, and these adverse effects were associated with low level of superoxide dismutase and increased production of NO in β-cells (117, 118). Overexpression of superoxide dismutase protected the cells against IL-β-induced

cytotoxicity (117, 118). In catalase transgenic mice, increased catalase gene expression protected the islets against H_2O_2-mediated decrease of insulin secretion (119). In STZ-induced mice, catalase overexpression lowered the blood glucose level compared to the controls (119).

Taken together, these studies indicate that antioxidants and increased levels of antioxidant enzymes can protect the β-cells against the adverse effects of chronic hyperglycemia.

3.2.2. Mechanisms of hyperglycemia-induced oxidative stress

ROS can be generated through four main processes, which include glycation reactions, hexosamine pathway, mitochondrial electron transport chain, and glyceraldehyde autoxidation (Fig. 6).

Under hyperglycemic conditions, reducing sugars such as glucose, glucose-6-phosphate and fructose react with various proteins to generate advanced glycosylation end products (AGE) and ROS. This reaction is called glycation reaction. Among all the reducing sugar, D-ribose has the most potent glycation activity. HIT-T15 cells treated with D-ribose led to reduction of PDX-1 binding to insulin promoter and suppression of insulin gene expression, insulin secretion and cellular insulin content (120). Antioxidants AG and NAC can preserve D-ribose-mediated loss of PDX-1 and thus insulin gene expression in HIT cells (120).

Excess glucose can also be converted to fructose-6-phosphate, which can further metabolized through glutamine:fructose-6-phosphate aminotransferase (GFAT) to generate glycosamine-6-phosphate. Glycosamine-6-phosphate is converted to UDP-N-

acetylglucosamine, which can interact with proteins and form O-linked glycoproteins. This pathway is called hexosamine pathway. In isolated rat islets, overexpression of GFAT led to increased H_2O_2 level, impaired GSIS and reduced expression of insulin, GLUT2, and glucokinase (121). The binding activity of PDX-1 to insulin promoter was also decreased. NAC can attenuate the adverse effects induced by overexpression of GFAT (121).

The mitochondrial electron transport chain is an important pathway for the generation of ROS, which is often the by-product of ATP production. It was suggested that chronic hyperglycemia led to increased ROS generation by mitochondria, and the ROS could not be eliminated fast enough by β-cell antioxidant defense system. This mismatch causes the toxic effects on β-cells. Another mitochondrial protein that has been implicated in impaired insulin secretion in type 2 diabetes is UCP2 (122). UCP2 diverts protons away from the ATP generation pathway, thus protons are used to produce heat instead of ATP. In mitochondria of β-cells, UCP2 expression is upregulated in both 90% pancreatectomized rats and ob/ob leptin deficient diabetic mice (96, 122). UCP2-deficient mice had higher islet ATP production, and increased GSIS (122). *In vivo* studies demonstrated that hyperglycemia-induced superoxide formation by the mitochondrial electron transport chain led to UCP2 activation (123). UCP2 activation leads to less ATP generation, and thus impaired GSIS (123).

Glyceraldehyde autoxidation is another pathway that has recently been considered in the hyperglycemia-induced ROS generation. D-glyceraldehyde is formed during glycolysis. Exposure of islets with D-glyceraldehyde for a short period of time (2 hr) stimulated insulin secretion, however, prolonged incubation of islets with D-

glyceraldehyde led to decreased GSIS, decreased insulin islet content, and increased intracellular peroxide levels (124). Pre-incubation islets with NAC attenuated the adverse effects induced by D-glyceraldehyde and reduced ROS generation (124). D-glyceraldehyde-induced oxidative stress in islets is through non-mitochondrial pathway since mitochondrial inhibitors failed to prevent D-glyceraldehyde-induced ROS generation (124). Glyceraldehyde-phosphate dehydrogenase (GAPDH) is the enzyme that facilitates D-glyceraldehyde entering the glycolytic pathway instead of glyceraldehyde autoxidation pathway. GAPDH activities were shown to be downregulated in islets exposed to high glucose concentration for a long period of time (125).

Several signal transduction pathways including c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), and protein kinase C (PKC) are activated in β-cells by oxidative stress (126). JNK pathway is the one that has been shown to involve in the oxidative stress-induced downregulation of insulin gene expression. Overexpression of dominant-negative JNK (DN-JNK) in STZ-induced diabetic animals preserved the insulin gene expression and lowered blood glucose level (126). Moreover, JNK activation in islets reduced PDX-1 binding to insulin promoter and insulin gene expression, and DN-JNK expression preserved PDX-1 nuclei translocation (126).

Taken together, chronic hyperglycemia can lead to elevated ROS in pancreatic β-cells, and ROS may play an important role in downregulation of insulin gene expression and impairment of GSIS.

3.3. Chronic hyperglycemia-induced pancreatic β-cell apoptosis

With the onset of diabetes, there is a progressive decrease of β-cell mass as demonstrated in both human and rodent models (92). This β-cell loss results from increased β-cell apoptosis, which outweighs β-cells replication and neogenesis (92). Many mechanisms can trigger β-cell apoptosis under chronic hyperglycemia, including endoplasmic reticulum (ER) stress, oxidative stress, increased intracellular calcium level, and cytokine production.

ER is responsible for posttranslational modification, folding, and assembly of newly synthesized secretory and membrane proteins. Its proper function is very important for the survival of a cell. Chronic hyperglycemia can induce ER stress in β-cells due to increased flux of proteins through the rough ER. Normally, flux of proteins is quite high in β-cells compared with other cell types, thus any further increase may tilt the balance and lead to ER stress-induced apoptosis (127). C/EBP homologous protein (CHOP) is a transcription factor that can be activated by physiological and pharmacological stress. CHOP is a mediator of ER stress induced apoptosis (128). Pancreatic islets from CHOP knock out mice showed resistance to NO-induced apoptosis (129). Araki et al proposed that hyperglycemia-induced increased NO production can deplete ER Ca^{2+} stores, induce CHOP levels and β cell apoptosis (130). Akita mouse is a spontaneous diabetic model with a mutation in insulin 2 gene (130). The progressive hyperglycemia of Akita mouse is accompanied by increased CHOP gene expression and β-cell apoptosis (129). In this model, misfolded insulin is linked to the induction of ER stress and β-cell apoptosis (129). The hypothesis that hyerglycemia can lead to increased protein flux to ER and ER stress needs to be further investigated.

Chronic hyperglycemia causes long-term increase of $[Ca^{2+}]_i$, which in turn could be pro-apoptotic. Continuous inflow of Ca^{2+} may activate Ca^{2+}-dependent intracellular proteases and stimulate apoptosis (131). Moreover, increased $[Ca^{2+}]_i$ has been shown to cause apoptosis by downregulation of insulin receptor substrate-2 (IRS-2), which has a function in regulating β-cell mass (132).

Chronic hyperglycemia can also activate apoptotic pathway in β-cells through increased cytokine synthesis. Production of IL-1β has been shown to be elevated in both type 2 diabetic patients and in the *gerbil psammomys obesus* during the development of diabetes (133). IL-1β inhibits β-cell function and induces Fas signaling pathway through the activation of NF-kB, and thus induce the apoptosis (133). Other cytokines TNFα, IL-6 and IFN-γ have also been shown to associate with hyperglycemia activation of apoptotic pathway (111).

3.4. Chronic hyperglycemia alters the expression of genes important for glucose and lipid metabolism

Chronic hyperglycemia leads to changes in the expression of genes important for glucose and lipid metabolism as demonstrated in the 90% partial pancreatized rats. The 90% partial pancreatized rat model is characterized by hyperglycemia, loss of β-cell differentiation associated with impaired insulin secretion, and β-cell hypertrophy (108). In this model, one week after partial pancreatectomy, the rats become mild to severe hyperglycemic that will be stable for the next three weeks (108). During the stable hyperglycemic period, the plasma nonesterified fatty acid level, plasma triglyceride level and islet triglyceride content are not altered (96, 108). Therefore, 90% partial

pancreatectomy rat model is considered as a chronic hyperglycemic model. Peroxisome proliferator-activated receptor α (PPARα) and acyl-CoA oxidase (ACO), which are involved in lipid catabolism are downregulated in the islets of 90% partial pancreatized rat model (96). In contrast, genes important for lipogenesis such as PPARγ, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) are increased in islets. Carnitine palmitoyl transferase-1 (CPT-1), an enzyme that transports fatty acids into mitochondria for lipid oxidation, is upregulated in islets of 90% partial pancreatized rat model (96). Expression of uncoupling protein 2 (UCP-2), an mitochondrial electron chain uncoupler, is also induced in this model (96). Increased UCP-2 leads to increased proton leak and thus reduced ATP synthesis. In contrast, other genes important for insulin secretion such as insulin, PDX-1, BETA2, glucokinase, K_{ATP} channel and GLUT2 expression are repressed in islets (95, 96). Expression of several enzymes involving gluconeogenesis, including glucose-6-phosphatase, fructose-1, 6-bisphosphate, are upregulated, and this may lead to increased glucose levels in islets (96). The alteration of gene expression profile by hyperglycemia was reversed by normalizing blood glucose only if the rats were in hyperglycemic condition for a short period of time (4-weeks) (95). In rats with 14-weeks hyperglycemia, normalizing blood glucose only partially reversed the changes (95). In conclusion, 90% partial pancreatectomized rat model demonstrates that chronic hyperglycemia leads to loss of β-cell phenotype by decreased expression of β-cell specific genes and induced expression of genes that are normally suppressed.

4. Lipotoxicity

Free fatty acids (FFAs) can be taken up and metabolized by pancreatic β-cells. FFAs and their metabolites can regulate protein kinase activity, ion channel activity, ROS production, protein acylation and gene expression (134). Acutely exposure (<6 hrs) of FFAs can enhance GSIS depending on the length and degree of saturation of the FFA chain (83, 135, 136). Long-chain fatty acyl-CoA is converted from FFAs and responsible for the K_{ATP}-channel-dependent insulin secretion (137). FFAs can also stimulate insulin secretion by augmenting L-type Ca^{2+} current, by directly inducing the secretory granule fusion, and by increasing the size of the readily releasable insulin granule pool (138). Chronic exposure of FFAs, however, is detrimental to β-cell function and survival, and this is termed lipotoxicity.

Lipotoxicity develops when the ectopic accumulation of lipids in non-adipose tissues causes dysfunction of these tissues. Triglycerides as the sources of fatty acids have been shown to accumulate in non-adipose tissues such as liver, skeletal muscle and pancreas β-cells *in vivo* in diabetic animal models and in diabetic patients (134). Therefore, lipotoxicity plays a central role in the pathogenesis of insulin resistance in muscle and liver, diabetic complications, and pancreatic β-cell dysfunction and apoptosis (134). Furthermore, several *in vitro* and *in vivo* studies suggested that under high glucose concentration, prolonged exposure to the high levels of fatty acids can inhibit insulin gene expression, reduce GSIS and induce β-cell apoptosis (82-88). The synergistic interplay of hyperglycemia and hyperlipidemia is termed glucolipotoxicity.

4.1. Effects of lipotoxicity on insulin biosynthesis

Chronic exposure of pancreatic β-cells to fatty acids leads to reduction of insulin biosynthesis (82, 84, 88). The decreased expression of several β-cell specific genes including insulin, PDX-1, GLUT2 and glucokinase has been shown with pancreatic islets exposed to fatty acids such as palmitate (84). Palmitate-suppression of glucose-stimulated insulin gene expression is due to decreased insulin promoter activity (139) that is mediated through increased ceramide synthesis (140). Decreased PDX-1 and MafA binding to the promoter (84, 141), decreased PDX-1 nuclear translocation, and reduced MafA gene expression (141) were also observed in islets treated with palmitate. In contrast, the binding activity of BETA2 and its expression were not affected by palmitate (141), demonstrating the critical roles of MafA and PDX-1 in palmitate-suppression of insulin gene expression. Another mechanism that FFAs affects insulin gene expression is through delaying the posttranslational processing of proinsulin (142). The posttranslational processing of insulin converting enzymes PC2 and PC3 were also delayed by FFAs (142).

4.2. Mechanisms underlying lipotoxicity-mediated impairment of GSIS.

Multiple mechanisms have been proposed for the lipotoxicity-mediated impairment of GSIS. These mechanisms include increased ROS production (143), enhanced activities of hexokinase and phosphofructokinase (144, 145), decreased activities of GLUT2 and glucokinase (84), activation of the K_{ATP}-channels (137, 146), and hyperglycemia (147). The following review will focus on the effects of hyperglycemia on lipotoxicity.

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Prentki and others believe that hyperglycemia and hyperlipidemia interplay with each other and lead to glucolipotoxicity (Fig. 7). They suggest that hyperglycemia leads to increased generation of cytosolic citrate, which is the precursor of malonyl-CoA. Malonyl-CoA inhibits carnitine palmitoyltransferase-1 (CPT-1), the enzyme responsible for transferring fatty acid into mitochondria for β-oxidation. Therefore, in the presence of glucose, fatty acids cannot be efficiently oxidized, and instead they are shunted toward esterification to generate triglyceride (148). Stored triglyceride is the source of FFAs, which can affect insulin gene expression and insulin secretion. Consistent with Prentki's proposal, the suppression of insulin gene expression, reduced GSIS, palmitate-induced accumulation of triglyceride were only observed when the isolated islets were incubated with high concentration of glucose and palmitate (149). In ZDF diabetic rats, lipid-lowering drugs such as bezafibrate decreased the plasma triglyceride level but not islet triglyceride content, and it failed to prevent hyperglycemia suppression of insulin gene expression (150). In contrast, blood glucose-lowering drug phlorizin prevented hyperglycemia, lowered islet triglyceride content, preserved insulin gene expression, but did not lower plasma triglyceride level in ZDF diabetic rats (150). In INS-1 832/13 cells, saturated fatty acids (palmitate and stearate) potentiated the high glucose (20 mM)-induced pancreatic β-cell apoptosis (151). These data demonstrate that hyperglycemia synergizes lipotoxic effects on β-cells.

FFAs are normally oxidized in mitochondria when the glucose level is in normal physiological range. Chronic hyperglycemia leads to increased lipogenesis in non-adipose tissues and excess energy can be stored as triglyceride. AMP-activated protein kinase (AMPK) is a metabolic sensor that detects changes in cellular energy status. It

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determines whether a cell should store excess energy or not. Under normal glucose concentration, AMPK negatively regulates the expression of ACC, FAS and other lipogenic enzymes through reduced expression of SREBP-1c (152). Therefore, under normoglycemia, lipogenesis is suppressed in β-cells. Chronic hyperglycemia suppresses AMPK activity in β-cells (153). SREBP-1c, ACC and FAS activities are upregulated under high glucose, leading to increased fatty acids synthesis and decreased FFAs β-oxidation (154, 155). The net result is increased lipogenesis and accumulation of triglyceride in β-cells under elevated glucose concentration. Triglyceride serves as a pool of endogenously released FFAs, which can stimulate expression of UCP2 through SREBP-1c binding to UCP2 promoter (156). UCP2 plays an important role in the impaired insulin secretion in type 2 diabetes (122). UCP2 is a mitochondrial electron chain uncoupler, and increased UCP2 leads to decreased ATP/ADP ratio and reduced GSIS. Small interfering RNA (siRNA) of UCP-2 were able to increase the ATP/ADP ratio and improve GSIS but not triglyceride accumulation in INS-1 cells with SREBP-1c overexpression, indicating UCP-2 plays a role in lipotoxicity-induced GSIS impairment (157). Another lipogenic gene that can be induced by FFAs is PPAR-γ (158). PPAR-γ has also been shown to stimulate UCP-2 promoter activity but it does not bind to the promoter directly (159). In summary, both hyperglycemia and elevated FFAs can cause increased triglyceride storage in β-cells, leading to β-cell dysfunction.

4.3. Lipotoxicity-induced pancreatic β-cell apoptosis

Lewis et al proposed that positive net energy balance would lead to increased storage of triglyceride in non-adipose tissues and adipose tissue (134). When the

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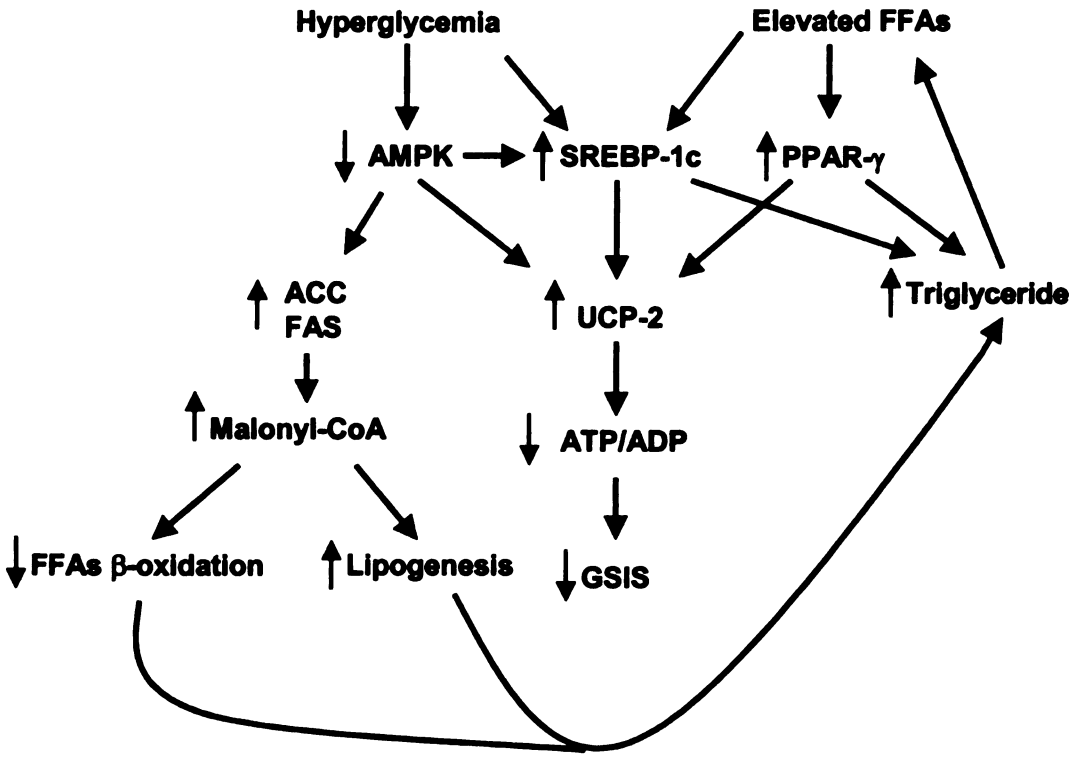

Figure 7. Potential mechanisms of lipotoxicity-induced GSIS impairment in pancreatic β-cells. Chronic hyperglycemia and elevated FFAs can stimulate lipogenesis and increase triglyceride storage in pancreatic β-cells. Hyperglycemia downregulates AMPK expression, leading to increased ACC, FAS and SREBP1c expression. Hyperglycemia also leads to increased malonyl-CoA level, leading to suppressed FFAs β-oxidation and enhanced lipogenesis. Triglyceride can serve as a pool of FFAs, which have been suggested to stimulate SREBP-1c and PPAR-γ binding to UCP-2 promoter. Increased UCP-2 expression leads to decreased ATP/ADP ratio and impaired GSIS. Figure is modified from *Beta-cell lipotoxicity: burning fat into heat?* (147).

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storage of triglyceride in adipose tissue exceeds its capacity, the FFAs will "spill over" to non-adipose tissue. Triglyceride will then accumulate in non-adipose tissues such as liver, skeletal muscle and islets. In islets, β-cell hyperfunction, dysfunction and apoptosis will then occur (134). Increased triglyceride storage in short-term actually protects non-adipocytes against fatty acids-induced lipotoxicity as demonstrated in mouse embryonic fibroblasts treated with oleic acid for 12 hrs (160). Long-term storage, however will lead to lipid-induced β-cells apoptosis by a number of pathways, including ceramide-induced mitochondrial apoptotic pathway (86, 161), and inhibition of protein kinase B (PKB) (162).

Unger et al have proposed that unoxidized fatty acids can be converted to fatty acyl CoA, which may provide substrate for *de novo* ceramide synthesis. Increased ceramide accumulation in β-cells upregulates iNOS and thus the generation of NO and peroxynitrite formation, causing β-cell apoptosis (86, 161). Both blocking ceramide generation and NO production led to prevention of fatty acid-induced β-cell apoptosis in pre-diabetic ZDF rats (86, 161). Fatty acyl CoA also promotes β-cell apoptosis via downregulation of antiapoptotic enzyme Bcl2 (85, 87).

PKB activity downregulation also plays an important role in FFA-induced apoptosis. It has been shown that chronic exposure of INS-1 cells with FFA reduced PKB activation and promoted β-cell apoptosis (162). Expression of constitutively active PKB protected the cells from FFA-induced apoptosis, possibly by inactivation of pro-apoptotic proteins such as glycogen synthase kinase-3α/β (GSK3α/β), foxhead protein FoxO1 and p53 (162).

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5. INS-1 cell model

INS-1 cell line was established from cells isolated from an x-ray-induced rat transplantable insulinoma (163). INS-1 cells depend on 2-mercaptoethanol for the continuous growth (163). INS-1 cells proliferate slowly and have many morphological and functional characteristics typical of native β-cells (163). These cells have numerous secretory granules, and some of these granules exhibit a central dense core and a peripheral clear halo as typical granules of normal insulin-producing cells. Immunofluorescence staining the INS-1 cells with a specific antibody against insulin, glucagon, somatostatin, or pancreatic polypeptide showed that INS-1 cells only contained insulin (163). INS-1 cells also have the cell surface antigens (R2D6 and A2B5) that are known to be present on the surface of pancreatic β-cells (163). The insulin cellular content of INS-1 cells is only 20% of the native β-cell content. INS-1 cells synthesize both rat proinsulin I and II and have the proinsulin to insulin conversion rates similar to those observed in rat islets (163). The glucose, however, does not stimulate the rate of proinsulin biosynthesis, indicating the abnormal insulin processing and packaging in INS-1 cells. Compared to RINm5F cell line that was also established from cells isolated from an x-ray-induced rat transplantable insulinoma, INS-1 cells are able to synthesize and store insulin, contain 30-40 times more insulin, and able to stimulate insulin in response to glucose (163).

In INS-1 cells, acute GSIS is maintained, and the GSIS is associated with the depolarization of plasma membrane and the increased intracellular Ca^{2+} levels (163). Like normal β-cells, INS-1 cells are also responsive to leucine, arginine and KCl stimulation and lead to insulin secretion (163). Although INS-1 cells stimulate insulin in

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response to acute glucose stimulation, the amount of insulin released is much less than the normal β-cells (163). INS-cells secrete insulin at the glucose concentration of 2 mM to 20 mM, which is the physiological range of glucose in a body (163).

Moreover, INS-1 cells maintain as functional β-cells with increased passage number up to about 80 passage (163). In contrast, HIT and β-TC cells lose their differentiated states as a function of tissue culture passage (164). Therefore, studies using HIT or β-TC cells have limitations in regard to cell stability and need close monitoring of the cell functions.

Like primary β-cells, the expression of β-cell specific genes can be suppressed by chronic hyperglycemia in INS-1 cells (17, 38, 165). Culturing INS-1 cells with glucose above 8 mM led to a decreased insulin gene expression (17). The glucose-suppression of insulin gene expression is associated with the reduced PDX-1 mRNA levels, and reduced PDX-1 and MafA bindings to insulin promoter in INS-1 cells (17, 38). High glucose also reduced GLUT2, glucokinase and IRS2 mRNA levels in INS-1 cells (165). Decreased expression of GLUT2 and glucokinase may lead to β-cell dysfunction since both GLUT2 and glucokinase are important for glucose metabolism and GSIS. IRS2 is important for β-cell replication, neogenesis and survival, and decreased IRS2 expression has been suggested to increase β-cell apoptosis (132).

The expression of genes involved in glucose and lipid metabolism is induced by chronic hyperglycemia during the development of type 2 diabetes. In INS-cells, several glycolytic enzymes have been shown to be induced by chronic hyperglycemia (166). These enzymes are phosphofructokinase-1 (PFK-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and liver type pyruvate kinase (L-PK) (166). Lipogenic genes

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including FAS, ACC and malic enzyme were also induced in INS-1 cells exposed to high glucose for a prolonged period (154). The altered expression of these metabolic genes led to chronically elevated citrate, malate and malonyl-CoA levels in INS-1 cells. Increased conversion of glucose to lipid, enhanced phospholipid and triglyceride synthesis, and increased glycogen deposition were observed in INS-1 cells treated with high glucose for a prolonged period (154). In contrast, fatty acid oxidation was suppressed (154). In addition, the active nuclear form SREBP-1c has been shown to be increased in INS-1 cells treated with high glucose (155). Overexpression of nuclear form of SREBP-1c led to increased triglyceride storage in β-cells and impaired GSIS (155, 157). The expression of several genes important for lipogenesis such as ATP citrate-lyase (ACL), acyl-CoA synthase (ACS), ACC, and FAS was upregulated by the overexpression of nuclear form of SREBP-1c (157). The expression of UCP-2 was also induced by SREBP-1c overexpression in INS-1 cells (157). These changes in gene expression and function of β-cells are typically observed in the β-cells of ZDF type 2 diabetic animal model and 90% pancreatecomized rat model (96, 98, 99, 101, 102).

Although INS-1 cells have many features in common of normal β-cells, they do have some characteristics different from the isolated islets. INS-1 cells have less insulin content and secret less insulin upon acute glucose stimulation (163). High glucose leads to adverse effects faster in INS-1 cells compared to isolated islets. The downregulation of insulin gene expression and reduced GSIS can be observed within 24 hr to 48 hr of high glucose treatment (17, 165). Similar effects can be observed in isolated islets within 4 days (21). The differences in response to chronic hyperglycemia could be because islets contain not only β-cells but also other types of pancreatic endocrine cells. The

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present of other pancreatic endocrine cells such as α-cell and γ-cells may help to keep the β-cells in differentiated state (167). It was demonstrated that maintaining human fetal pancreatic islets in aggregates prevented loss of β-cell phenotype (167). Beattie et al suggested that the cell-cell contact-mediated mechanisms regulated the transcription of islet-specific genes such as insulin (167). Therefore, one can speculate that cell-cell contact also attenuates the chronic hyperglycemia mediated changes in β-cell phenotype.

Another difference between the INS-1 cells and isolated islets is that INS-1 cells may have a lower glutathione level (163). This is because INS-1 cells require 2-mercaptoethanol for growth and replication. In contrast, isolated islets are normally cultured without 2-mercaptoethanol (163). Since INS-1 cells cultured with medium containing 2-mercaptoethanol led to an increased level of glutathione, it was suggested that INS-1 cells had a lower level of glutathione. The low level of glutathione could make INS-1 cells more susceptible to glucose-induced oxidative stress.

Overall, INS-1 cells have many morphological and functional characteristics that resemble normal β-cells. Chronic hyperglycemia causes changes in gene expression in INS-1 cells and alters the cell function. These effects of chronic hyperglycemia are similar to those observed with normal isolate islets. Therefore, INS-1 cell model is a useful cell model in studying the glucotoxicity of β-cells.

IV. Poly(ADP-ribose) Polymerases (PARPs) and their inhibitors

Nicotinamide (NAM) and its related compounds have been studied in diabetes and diabetic complications for years, but the mechanisms underlying these compounds are not well understood. These compounds are called poly(ADP-ribose) polymerase

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inhibitors (PARPi) due to their ability to inhibit poly(ADP-ribose) polymerase (PARP). The following sections will review PARP and the role of PARPi in diabetes.

1. Poly(ADP-ribosyl)ation

PARP is a family of enzymes present in all eukaryotic cells, but not in prokaryotic cells. It catalyzes the transfer of ADP-ribose (ADPr) from β-nicotinamide adenine dinucleotide (NAD^+) to proteins to form linear and/or branched poly(ADP-ribose) chains (168) (Fig. 8). Poly(ADP-ribose) attaches to proteins via the carboxyl groups of glutamic and aspartic residues (169-171). The chain length of polymers can reach about 200 units *in vitro*, and long polymers are usually branched (172, 173). The by-product of poly(ADP-ribosyl)ation is the generation of nicotinamide (NAM), which serves as an inhibitor of PARP and is generally recycled for use of β-NAD^+ synthesis.

PARP can bind to undamaged DNA, single-strand-break (SSB) DNA and double-strand-break (DSB) DNA, and the basal activity of PARP is independent of DNA strand breaks. Among undamaged DNA, PARP has higher affinity to supercoiled DNA than for relaxed DNA. PARP can also bind to DNA structures such as cruciform, curved, and some double stranded DNA sequences (174-176). Undamaged DNA, however, is inefficient in activating PARP enzymatic activity. PARP activation can be induced by its binding to SSB DNA and DSB DNA, and these DNA breaks can be generated by oxidation, alkylation, deamination, and depurination reactions, and ionizing radiation (177).

In vivo, poly(ADP-ribose) (pADPr) metabolism consists of anabolism and catabolism of the polymers (Fig. 8). Anabolism requires three distinct enzymatic

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activities, including initiation or mono(ADP-ribosyl)ation of the protein, elongation of the pADPr, and branching of the pADPr. All these activities are mediated mainly through PARP in living cells (168). Catabolism is carried out by three enzymes, which include pADPr glycohydrolase (PARG), ADP-ribosyl protein lyase and phosphodiesterase. PARG has exoglycosidase and endoglycosidase activities (178, 179). It hydrolyzes the glycosidic bonds between ADPr units located at the extremity (exoglycosidic activity) and within the polymer (endoglycosidic activity). ADPr-ribosyl protein lyase hydrolyzes the most proximal unit of ADPr on the nuclear proteins (180). Phosphodiesterase hydrolyzes pyrophosphate bonds (181). PARP activation leads to its auto-poly(ADP-ribosyl)ation, which makes PARP negatively charged and this cause PARP to fall off the DNA, thus terminating poly(ADP-ribosyl)ation of proteins (182).

2. Homologues of PARP

There are multiple genes encoding PARPs and a recent report suggest that there are at least 18 isoforms in PARP superfamily (183). Six isoforms that have been studied are PARP-1, PARP-2, PARP-3, vault-PARP (PARP-4, VPARP), and tankyrases (TANK-1, and 2). The other forms of PARP are only putative PARP homologues. Despite the differences in their structures, all PARP isoforms contain a catalytic domain that is highly conserved.

PARP-1 has been most extensively studied. Human PARP-1 is the classical PARP, and has molecular weight of 116 Kda. It has multiple functions including DNA repair (177, 184, 185), gene expression (186), cell proliferation and differentiation (183, 187-192), cell death (193-199), and protein degradation (200-204). Over the years,

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PARP-1 has been studied mainly for its role in DNA repair, maintenance of genomic stability and cell death. Basal PARP-1 activity in a cell is also very important in regulating gene expression during development and in response to specific cellular signals. PARP-1 regulates gene expression through several mechanisms including modulating chromatin structure (205-207), poly(ADP-ribosyl)ation of nuclear factors (208-211), binding to the DNA directly (176, 212-216), and forming binding complexes with transcription factors and coactivators (213, 214, 217-226).

PARP-2 is a nuclear protein. It is smaller than PARP-1, and has a molecular weight of 62 kDa. Among all the PARP isoforms, it bears the most homology with PARP-1 (227). It participates in DNA repair (228), maintains telomere length (229), and has a role in chromatin organization and checkpoint control during cell cycle (230). Double null mutant PARP-$1^{-/-}$/PARP-$2^{-/-}$ died early in the development at the onset of gastrulation, suggesting the important roles of PARP-1 and PARP-2 or poly(ADP-ribosyl)ation in the embryonic development (231).

PARP-3 is also a nuclear protein and a core component of the centromere with a molecular weight of 60 kDa. PARP-3 is not involved in DNA strand break signaling. PARP-3 acts at the G1/S cell cycle transition since overexpression of PARP-3 interferes with the G1/S transition (232). It is also the first known marker of the daughter centriole due to its preferential localization at the daughter centriole (232). PARP-1 interacts with PARP-3 at the centriole, and this interaction may play a role in regulating cell cycle after DNA damage (232).

VPARP can be found in nuclei, cytoplasm and mitotic spindle. It is a 193-kD vault protein. Vaults are ribonucleoprotein (RNP) complexes, and are composed of a

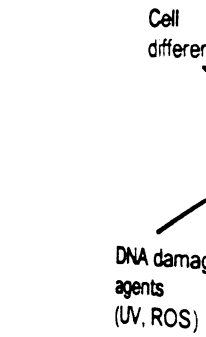

Figure 8.

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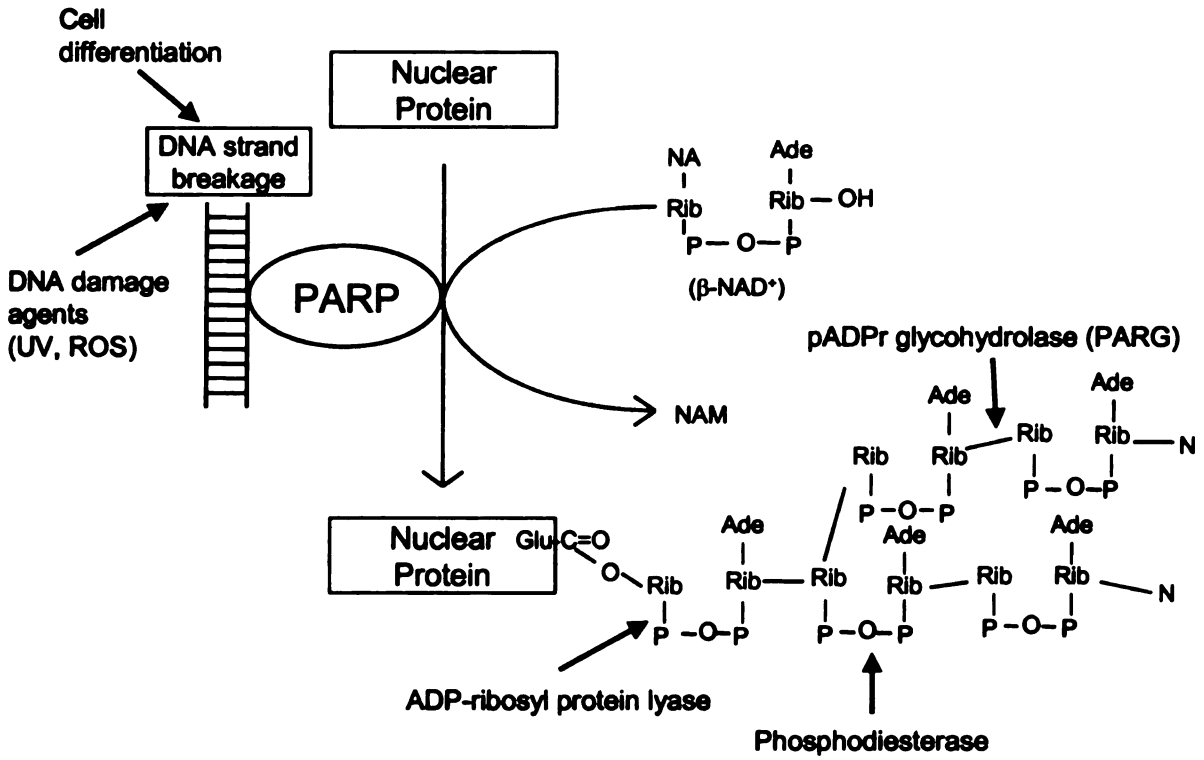

Figure 8. Mechanism of PARP and enzymes that are involved in degradation of poly(ADP-ribose). Upon activation, for example by binding to SSB DNA, PARP can catalyze the transfer of ADPr from β-NAD+ to nuclear proteins until there is a long chain of pADPr. Poly(ADP-ribosyl)ation is terminated by the automodification of PARP. Poly(ADP-ribosyl)ated PARP is very negatively charged, therefore, it falls off the DNA and loses its catalytic activity. The function of nuclear proteins can be modulated via poly(ADP-ribosyl)ation. Long-chains of polymers are degraded by pADPr glycohydrolase (PARG), ADP-ribosyl protein lyase, and phosphodiesterase. This figure is modified from Figure 9.1 in *Cell Death-The Role of PARP (233).*

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small untranslated RNA, major vault protein (MVP), VPARP, and telomerase-associated protein-1 (TFP1). VPARP is not exclusively associated with the vault particle, suggesting that it may interact with other proteins (234). Vault has been shown to be upregulated in cancer cells with multidrug resistance during chemotherapy (235). VPARP can modify MVP and itself, but VPARP activity is not required for the assembly of vaults (236). VPARP is associated with telomerase, however, VPARP-deficiency demonstrated that it is dispensable for telomerase function and vault structure *in vivo* (237).

Tankyrases (TANKs) are components of the telomeric complex. They are PARPs, but they also share certain structure similarities with signaling and cytoskeletal proteins (238). They can be found in the Golgi or in nuclear pore complexes. TANK-1 is also called PARP-5a and has a molecular weight of 142 kDa. TANK-1 is present at telomeres and is a positive regulator of telomere length (239). TANK-1 has also been implicated in the modulation of the apoptosis pathway (240). TANK-2 is also called PARP-5b with a molecular weight of 130 kDa. Both TANK-1 and TANK-2 have been shown to heterodimerize, indicating that they may function together as a complex (241). Overexpression of TANK-2 led to elongation of telomere, thus TANK-2 is also a positive regulator of telomere length (239, 242).

3. PARP inhibitors and their therapeutic potential in diabetes

3.1. PARP inhibitors

Currently, there are two types of PARP inhibitors available: low-potency PARP inhibitors (lp-PARPi) and potent PARP-1 inhibitors. Low-potency PARP inhibitors used

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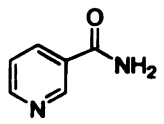
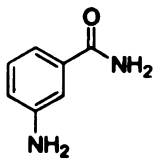
in this project include nicotinamide (NAM), 3-aminobenzamide (3-AB) and PD0128763 (PD). Potent PARP-1 inhibitors that will be reviewed are PJ34 and INO-1001.

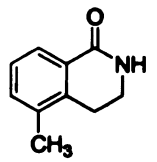
3.1.1. Low-potency PARP inhibitors (lp-PARPi)



Nicotinamide (NAM)



3-aminobenzamide (3-AB)



3,4-dihydro-5-methylisoquinolinone (PD128763 (PD))

NAM is a water-soluble vitamin B_3. It acts as a competitive inhibitor of PARP, however, most of tissues use NAM as the precursor for NAD^+ biosynthesis. The IC_{50} of NAM for purified PARP is 0.05 to 0.1 mM (243). Besides being an inhibitor for PARP, NAM is also an inhibitor for mono(ADP-ribosyl) transferase (mART), microsomal NADase, toxin ADP-ribosyltransferase, and cAMP phosphodiesterase. NAM is a substrate for other NAD-metabolizing enzymes, including Sir2, nicotinamide N-methyltransferase, deaminase, and phosphoribosyltransferase. NAM at concentration ≥1 mM can scavenge oxygen radical, but it is not a potent antioxidant and does not scavenge nitric oxide (NO) (243). Above 3 mM, NAM inhibits mono(ADP-ribosyl) transferase (mART) (244). Expression of a number of genes can be altered by NAM with concentration equal or higher than 10 mM (245-249).

3-AB is a much more potent and specific PARP inhibitor compared to NAM. The IC_{50} of 3-AB for purified PARP is 5.4 to 33 μM (244, 250). At high concentrations, it also inhibits mART, cAMP phosphodiesterase, chymotrypsin, and the esterase activity of carboxypeptidase. 3-AB competes with NAD^+ for the active site of PARP. In the presence of 25 nM NAD^+, both NAM and 3-AB can initiate poly(ADP-ribosyl)ation at

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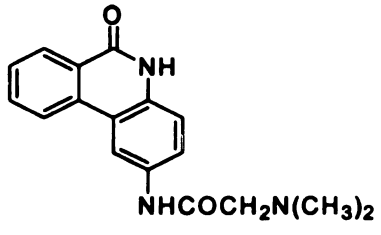
nanomolar concentrations to facilitate DNA repair (251). At high concentration such as 3 mM, 3-AB can inhibit PARP and lead to decrease of DNA repair (244). Similar to NAM, high concentration of 3-AB has also been shown to modulate expression of several genes (193, 249).

PD128763 is 3,4-dihydro-5-methyl-1(2H)-isoquinolinone. It is a potent PARP inhibitor with an IC_{50} of 0.1 to 0.5 μM against purified enzyme (243). Inhibition of PARP with 500 μM PD can lead to increased NAD^+ level as shown in Chinese hamster V79 cells (252). PD is a much more specific PARP inhibitor compared to 3-AB or NAM (253).

3.1.2. Potent PARP-1 inhibitors



N-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide (PJ34)

N-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide, designated as PJ34, is a much more specific and potent inhibitor of PARP-1 with IC_{50} of 20 nM in pure PARP enzymatic assay (254). Another potent PARP-1 inhibitor, INO-1001, is an isoindolinone derivative with an IC_{50} of 3 nM. Its chemical structure is not available to the public so far. Unlike NAM and 3-AB, PJ34 and INO-1001 have no antioxidant properties (254, 255).

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3.2. Beneficial effects of PARP inhibitors on diabetes

PARPs play multifunctional roles in a cell including DNA repair, maintenance of genomic stability, gene expression, and cell death. Therefore, PARP inhibitors (PARPi) have been studied in various pathophysiological states for their pharmacological use. PARPi have mainly been investigated for the treatment of inflammation, cancer, tissue injuries induced by oxidative stress, and diabetes. The effects of PARP inhibitors on diabetes and pancreatic β-cell differentiation and proliferation will be reviewed in this section.

3.2.1. Effects of PARP inhibitors on type 1 diabetes

Type 1 diabetes is characterized by islet-infiltrating leukocytes and autoimmune destruction of pancreatic β cells. Low potency PARP inhibitors such as nicotinamide have been shown to protect β cell from necrosis, but not cytokine-induced apoptosis (177).

PARPi have been shown to attenuate diabetes in streptozotocin (STZ) and alloxan-induced diabetes by increasing proinsulin synthesis and preventing DNA damage (254, 256-259). STZ is an antibiotic produced by *Streptomyces Achromogenes*, and is a glucose analogue (2-deoxy-2-[3-methyl-e-nitrosourido-D] glucopyranose). It is selectively taken up by GLUT2 glucose transporter (260). When STZ is metabolized within β cells, it rapidly causes large amount of DNA damage via alkylation and NO formation (261, 262). DNA damage caused by STZ has been proposed to activate PARP and lead to β cells toxicity and cell death (262, 263). Alloxan is an oxidation product of uric acid, and reduction of alloxan to dialuric acid leads to generation of ROS (264).

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Alloxan-induced ROS production has also been shown to cause DNA damage and PARP activation in islets (262, 263). Both STZ and alloxan have been shown to inhibit proinsulin synthesis (258, 259). NAM or 3-AB pretreatment protected the islets from STZ- and alloxan-mediated decrease of proinsulin synthesis and DNA damage (258, 259). In STZ-induced diabetic animals, PJ34 delayed the onset of type 1 diabetes (254, 256, 257).

The non-obese diabetic (NOD) mice have a high incidence of insulitis, which leads to development of type 1 diabetes. NOD mice appear normal up to about 3 to 4 weeks of age, and then pancreatic inflammation becomes evident (265). Following insulitis, destruction of pancreatic β-cells ensues in many mice with insulin depletion appearing between 3 and 7 months of age (265). In NOD mice, NAM treatment led to normal glucose tolerance, mild insulitis, and marked decrease of glucosuria (5). PJ34 treatment delayed the onset of diabetes, significantly increased pancreatic insulin content, and decreased β cell apoptosis (266). Moreover, leukocyte infiltration of islet grafts was significantly decreased due to increased apoptosis of leucocytes in PJ34 treated NOD mice (266). The expression of the T helper 1-type cytokine interferon (IFN)-γ was also reduced in PJ34 treated islet grafts (266). In NOD mice, treatment of PJ34 after the onset of diabetes did not ameliorate hyperglycemia (254, 256, 257).

As an enzyme important for DNA repair, PARP-1 is activated upon exposure to ROS, reactive nitrogen species or other DNA damage agents. Activation of PARP can lead to depletion of NAD^+ and ATP, ultimately, causing cell death. Pancreatic β cells are susceptible to oxidative stress due to their low levels of intrinsic antioxidant enzymes. Addition of 50 μM to 250 μM PD protected the rat islets from cell lysis when they were

incubated with the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP), the superoxide producing enzyme xanthine oxidase, or streptozotocin for 18 hrs (267). Pretreatment of rat islets with PD significantly improved survival of islets compared with islets pretreated with NAM or control (267).

In clinical trials, NAM has been demonstrated to improve insulin secretion in newly diagnosed type 1 diabetic patients (9), and decrease the rate of diabetes in children tested positively with islet cell antibodies (7, 8). More recent clinical studies, however, indicated that NAM failed to prevent the onset of type 1 diabetes (268-270).

Taken together, both lp-PARPi and potent PARP-1 inhibitor can delay the onset of type 1 diabetes through prevention of leukocytes islet infiltration, downregulation of cytokines and protection the islets against ROS and reactive nitrogen species.

3.2.2. Effects of PARP inhibitors on type 2 diabetic animal models

The beneficial effects of PARPi have been demonstrated in several diabetic animal models including 90% partial pancreatized rats and ZDF rats (3, 6). In partially pancreatized animals, NAM or 3-AB treatment caused a decreased blood glucose concentration, increased insulin content, and improved glucose tolerance (3, 6). Enlarged pancreas consisting largely of β-cells were observed, suggesting that PARP inhibition can lead to β-cell regeneration (3, 6). Long term (1 to 3 months) NAM therapy ameliorated diabetes in partial pancreatized rats (6).

In ZDF prediabetic rats, NAM pretreatment prevented islet disruption, and maintained blood glucose and FFA levels at the normal range (86). In diabetic ZDF rats, NAM has been shown to reduce plasma FFA levels, prevent triglyceride accumulation in

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islets and improve glucose-stimulated insulin secretion (GSIS) (4). The protective effects of NAM was due to attenuation of iNOS upregulation and NO production, thus preventing β-cell apoptosis (86, 161). The mechanisms underlying lp-PARPi protective effects in type 2 diabetes are not well understood.

3.2.3. Effects of NAM and 3-AB on primary pancreatic β-cells

PARP has been suggested to play roles in regulating cell differentiation (188, 190, 192). NAM promoted replication of islet cells both *in vivo* and *in vitro* (3, 6, 10-15, 271, 272). Chick dorsal pancreatic buds cultured with NAM showed increased proportion of insulin staining cells and promoted β-cell differentiation (272).

Porcine fetal pancreas can be considered to be a potential source of islet tissue for transplantation in diabetes. *In vitro*, fetal pancreas can outgrow to form islet-like cell clusters (ICCs) that can be used in transplantation. The generation of fetal porcine ICCs from fetal pig pancreas were enhanced after incubating in media with NAM (15, 271). The ICCs that were treated with NAM showed increased proinsulin biosynthesis, insulin content, and β-cell number (10, 12, 13, 15). The stimulatory effects of NAM on ICCs may due to the increased polyamine content in ICCs (15). Polyamines are low-molecular weight aliphatic cations such as spermidine, spermine, ornithine, and putrescine. They play important role in a number of cellular processes, including proper ion channel functioning, nucleic acid packaging, DNA replication, apoptosis, gene transcription and translation, and cell differentiation (273). Spermine has been shown to stimulate insulin production through prevention of insulin mRNA degradation (274). Fetal porcine ICCs treated with an inhibitor of polyamine synthesis prevented NAM stimulatory effects on

ICCs (15). Transplantation of porcine ICCs cultured in 10 mM NAM into diabetic mice doubled the number of insulin positive cells and shortened the time to achieve normoglycemia (10, 13).

In cultured human fetal pancreas, NAM stimulated islet-like cell clusters formation and increased the insulin content of ICCs (11, 14). The mRNA levels of endocrine hormones including insulin, glucagon, and somatostatin were increased significantly in human fetal ICCs cultured with NAM and high glucose concentration compared to ICCs incubated in control media (11). Similarly, 3-AB treated ICCs had increased GSIS, and increased insulin content (11). In adult islets, however, NAM neither increased GSIS nor insulin content, suggesting that NAM induces differentiation and maturation of fetal islets, not the adult islets (11, 275).

Lp-PARP inhibitors so far are the only PARP inhibitors that were demonstrated to induce islet β-cell differentiation and increase insulin biosynthesis. The mechanisms behind these properties still need to be defined.

Chapter 3. Materials and Methods

1. Materials

Cell culture media-RPMI-1640 was purchased from Invitrogen Corporation (Grand Island, NY). Lipofectamine, T4 DNA ligase, Taq DNA polymerase, and Random primers DNA labeling system were purchased from Invitrogen Corporation (Carlsbad, CA). SuperSignal® West Pico and SuperSignal® West Dura solution were obtained from Pierce (Rockford, IL). Trans-blot® transfer medium (0.45 μm nitrocellulose membrane) was purchased from Bio-Rad (Hercules, CA). Kodak BioMax MR film was obtained from Kodak Company (Rochester, NY). Plasmid Maxi Kits were obtained from Qiagen Inc. (Valencia, CA). Wizard® plus SV minipreps DNA purification system, and PCR Preps DNA purification system were purchased from Promega Corporation (Madison, WI). TurboBlotter™ rapid downward transfer system blotter pack, and Nytran® SuPerCharge were obtained from Schleicher & Schuell BioScience (Keene, NH). Mini Quick spin DNA columns were bought from Roche (Indianapolis, IN). Hydrogen peroxide (30%) was bought from J.T. Baker (Phillipsburg, NJ). Reactive oxygen species detection reagents (H_2DCFDA) were obtained from Molecular Probes (Eugene, OR). Cleaved caspase-9 (Asp353) antibody was purchase from Cell Signaling Technology® (Beverly, MA). p300 (N-15) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). MafA antibody was from Bethyl Laboratories, Inc. (Montgomery, TX). [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{14}C]chloramphenicol were from PerkinElmer Life Sciences (Boston, MA). Rat insulin radioimmunoassay (RIA) kits were obtained from Linco Research, Inc. (St. Charles, MO). Lowry reagent and Folin & Ciocalteu's phenol reagent and Brij® Polidocanol were from Sigma (St. Louis, MO).

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pTKCAT is a thymidine kinase promoter (-108 bp +56 bp) CAT reporter gene, and it is a gift from Dr. R. Mikcicek, Michigan State University, East Lansing, MI. Human insulin minienhancer CAT reporter genes pFox(rip1)CAT, pFox(X5rip1)CAT, and pFox(Y5rip1)CAT (62) were received from Dr. M. German, University of California, San Francisco, CA. pcDNA3 is a plasmid containing a cytomegalovirus (CMV) enhancer-promoter, and was purchased from Invitrogen. pCI-FLAG-p300, which is a plasmid containing a CMV promoter driving the expression of p300, was received from Dr. Y. Nakatani, Dana-Farber Cancer Institute, Boston, MA. pCDNA3-FLAG-Sir2 was obtained from Dr. L. Guarente, Massachusetts Institute of Technology, Cambridge, MA. pCDNA3-FLAG-CtBP was received from Dr. R.H. Goodman Oregon Health and Science University, Portland, OR. pCR3.1-BETA2 was received from Dr. M.J. Tsai MJ, Baylor College of Medicine, Houston, TX.

2. Cell culture

Rat insulinoma cells, INS-1 cells, were maintained at 37°C in INS-1 medium (RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 10 mM Hepes, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 mg/ml streptomycin) in 5% CO_2 incubator. Cells were passed weekly after detachment with 0.25% trypsin-EDTA. All experiments were performed with INS-1 cells between passages 70 and 80.

3. Polymerase chain reaction (PCR) and plasmid construction

INS(-327)CAT, the chloramphenical reporter under the transcriptional control of human insulin promoter containing sequences of –327 bp to +30 bp, was constructed as

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previously described (18). Truncated insulin CAT reporters (INS(-230)CAT, INS(-201)CAT, INS(-165)CAT, and INS(-114)CAT) were produced by using INS(-327)CAT as the template and oligonucleotide primers listed in Table 1. Mutated insulin CAT reporters INS(-230mA1A3)CAT, INS(-230mC1)CAT, and INS(-230mC1A1A3)CAT were generated by using INS(-230mA1)CAT, INS(-230)CAT, and INS(-230mA1A3)CAT as templates, respectively. The A1 element (-82 bp to –77 bp) was mutated from CCCTAATGGG to CCGCGCGCGG. The A3 element (-215 bp to –210 bp) was mutated from TAAT to TCCT. The C1 element was mutated from GCCTCA to GCTGCA. MafA was subcloned downstream of the CMV enhancer contained within the pCR3.1 expression vector. Human insulin CAT reporters were generated by PCR using primers containing Xba I and Xho I restriction sites. Primers for PCR were designed from human insulin promoter, and they were synthesized by Integrated DNA Technologies, Inc. In general, every PCR reaction contains 4 ng of template (INS(-327)CAT), 20 pmol of appropriate primer, and 0.2 mM dNTP, Vent or Taq DNA polymerase, and polymerase buffers according to the manufacturer's protocol (Invitrogen Corporation). The thermal cycle for PCR used a 2-5 min of denaturation at 94°C followed by 25 cycles of amplification, which included denaturation at 94°C for 30 sec, annealing at 55°C-59°C for 30 sec, and elongation at 72°C for 30 sec. An additional elongation step with 1 cycle of 5-7 min at 72°C was used after the amplification step. After PCR amplification, the product was fractionated on a low-melting-point (L.M.P.) gel and purified by use of Wizard® PCR Preps DNA purification system. The purified DNA insert was ligated into an opened CAT reporter, which was digested at XbaI and Xho I restriction sites. The ligation mixture contained 0.3 pmol, 0.6 pmol or 0.9 pmol

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Xba I/XhoI digested PCR product, 0.1 pmol digested CAT reporter, ligase, and ligation buffer according the manufacturer's protocol (Invitrogen Corporation). The ligation mixture was then incubated at 14°C overnight or at room temperature for 1 hr. Ligated plasmids were amplified in competent *E. Coli DH 5α*. Plasmid constructs were then purified by using Qiagen kit and stored at -20°C.

4. Cell transfections

INS-1 cells were seeded on 6-well plates at a density of $1.5x10^6$ cells per well and cultured for two days in INS-1 medium. Cells were then transfected for 5 hrs using medium containing 1 μg plasmid and 2 μL Lipofectamine per well at 37°C (17). For p300 and MafA overexpression studies, 0.5 μg pCI-FLAG-p300 or 0.25 μg pCR3.1-CMV-MafA was co-transfected with insulin promoter reporter genes. pCR3.1-CMV (0.25 or 0.5 μg) containing no insert was used as a control in overexpression studies. After transfection, cells were treated for 48 hrs with different conditions and harvested (see figure legends). To harvest the cells, cells were washed once with PBS, and detached with ice-cold TEN (40 mM Tris, pH7.5, 150 mM NaCl, and 1 mM EDTA, pH 8.0). The cells were then scraped, pelleted, and lysed in 250 mM Tris, pH 7.5. The lysate was spun down and the supernatant was stored at -20°C.

5. Chloramphenicol acetyl-transferase (CAT) Assay

CAT assay was performed to measure the promoter activity. Every assay contained 50 μL sample, and 100 μL CAT assay buffer (250 mM Tris, pH 7.5, 3.33 mM butyryl-Coenzyme A, and 2 μl/reaction of ^{14}C-chloramphenicol (54 mCi/mmol, 0.05

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INS(-201)(
INS(-165)(
INS(-114)(
Reverse Pr

Table 1.

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Name	5' end base pair	Sequence
INS(-230)CAT	-230	GCGTCTAGACCCCTGGTTAAGACT
INS(-201)CAT	-201	GCCTCTAGAGTCCTGAGGAAGAGGTG CTGA
INS(-165)CAT	-165	GCCTCTAGATCCCACAGACCCAGCACC AGG
INS(-114)CAT	-114	GCCTCTAGACAGCCATCTGCCGACCCC
Reverse Primer	+11/+30	GCGCTCGAGCTCTTCTGATGCAGCCTG TC

Table 1. Oligodeoxynucleotide sequence used for construction of human insulin CAT reporters. The underline is the sequence of restriction site.

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mCi/mL)). The reaction mixture was incubated at 37°C. The length of incubation was determined such that CAT activity was measured within the linear range of the reaction. ^{14}C-butyryl-chloramphenicol was extracted with xylene, mixed and extracted again with 250 mM Tris, pH7.5. The amount of ^{14}C-butyryl-chloramphenicol generated was quantitated by scintillation counting.

6. RNA Extraction

INS-1 cells were plated on 60 mm dishes at a density of $5x10^6$, cultured in INS-1 medium for two days, and then treated according to the experimental design (see figure legends). Trizol reagent (750 μL/dish) (Invitrogen) was used to isolate total RNA from cells according to the manufacturer's protocol. Total RNA was dissolved in 250 μL guanidinium thiocyanate/2-mercaptoethanol (GITC/BME) buffer. RNA was reprecipitated with isopropanol by freezing at –20°C for 1.0 hr, and washed with 70% ethanol. The RNA pellet was air-dried, and dissolved in autoclaved filtered distilled water. RNA was quantitated with the spectrophotometer using wavelengths 260 nm and 280 nm.

7. Northern blot analysis

Thirty micrograms of total RNA were separated by electrophoresis on 1% to 1.5% agarose-formaldehyde gel depending on the size of RNA, and then transferred to nylon membrane (Schleicher & Schuell), and cross-linked with UV light. [^{32}P]dCTP-radiolabeled cDNA probes were generated by Random Primers DNA Labeling System (Invitrogen Corporation). The membranes were prehybrydized at 42°C overnight with

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prehybrydization solution containing 50% formamide, 5x SSC (0.75 M NaCl, 0.075 M sodium citrate), 5x Dehhardts' solution (0.5 g Ficoll, 0.5 g polyvinylpyrroidone, 0.5 g bovine serum albumine), 0.05 M NaPO4, 0.1% salmon sperm DNA (boiled), and 0.1% SDS. The labeled probes (see figure legends) were then added to the membrane to hybridize with RNA at 42°C overnight. Membranes were washed twice with cold washing buffer (10% 20x SSC and 0.1% SDS, room temperature), and twice with hot washing buffer (1% 20x SSC and 0.1% SDS, 54°C). Northern blots were quantified using a phosphoimager (Molecular Dynamics).

8. Nuclear extract preparation

INS-1 cells were seeded on 100 mm plates at a density of $9x10^6$, cultured in INS-1 medium for two days, and then treated according to the experimental design (see figure legends). Nuclear extracts were made according to the method of Schreiber et al (276). Cells were washed once with PBS, scraped and pelleted. Cell pellets were resuspended in 800 µL Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 0.2 µL/mL leupeptin, 0.2 µL/mL aprotinin, 10 mM NaF, 1 mM Na_4PPi, 100 µM β-glycerolphosphate, 1 mM Na_3VO_4). Cells were incubated on ice for 15 min, and then 50 µL 10% NP-40 was added. The sample was then vortexed and pelleted at 16,000 g. The pellet was resuspended in Buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.2 µL/mL leupeptin, 0.2 µL/mL aprotinin, 10 mM NaF, 1 mM Na_4PPi, 100 µM β-glycerolphosphate, 1 mM Na_3VO_4). After rocking for 15 min on a shaking platform, the

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nuclear debris was removed by centrifugation. The supernatant, which contained nuclear proteins, was stored at -80°C.

9. Whole cell lysate preparation

INS-1 cells were seeded on 100 mm plates at a density of $9x10^6$, cultured in INS-1 medium for two days, and then treated according to the experimental design (see figure legends). Cells were washed once with PBS, scraped and pelleted. Cells were resuspended in 150 to 200 μL cell lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, and 10% glycerol, 1 mM PMSF, 0.2 μL/mL leupeptin, 0.2 μL/mL aprotinin, 10 mM NaF, 1 mM Na_4PPi, 100 μM β-glycerolphosphate, 1 mM Na_3VO_4). Cells were incubated in lysis buffer on ice for at least 30 min, and then spun down at 16,000 g. The supernatant, which contained total cell proteins, was stored at -80°C.

10. Western blot analysis

Thirty to 120 μg proteins of each sample were used for Western blot analysis. The proteins were diluted in the sample buffer (0.25 M Tris, pH 6.8, 5% SDS, 10% glycerol, 0.05% BME), boiled for 5 min, and separated on a 5-10% polyacrylamide gel by electrophoresis at 100 V in the running buffer (0.25 M glycine, 0.025 M Tris, 0.1% SDS). The proteins were then transferred to nitrocellulose membrane with transferring buffer (19.2 M glycine, 0.025 M Tris, 20% methanol) for 1 to 2 hr at 100 V in cold room, or 30 V overnight. Membranes were blocked with Blotto (5% non-fat dry milk, 0.05% Tween 20, 50 mM Tris, pH 8.0, 80 M NaCl, 2 mM $CaCl_2$) for 1 hr at room temperature. MafA, Pdx-1, p300, CtBP and cleaved caspase-9 immunoreactivity were detected with its

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respective antibody. The immunoreactivity was visualized with SuperSignal® West Pico chemiluminescent kit (Pierce).

11. Electrophoretic mobility shift assay (EMSA)

EMSA was used to determine the binding activities of proteins present in nuclear extracts to specific insulin promoter elements. Double-stranded oligodeoxynucleotide probes to rat insulin 2 promoter A2C1 elements (-129 AGCTTGGAAACTGCAGCTTCAGCCCCTCTGAGCT –96) and to human A3 elements (-230 CCCCTGGTTAAGACTCTAATGACCCGCTGG -201) were labeled with [α-^{32}P]-dCTP by filling in overhanging 5'-ends with the large fragment of DNA polymerase 1. Double-stranded oligodeoxynucleotide probe to human insulin gene A5/core elements (-323 CTGGTCTAATGTGGAAAGTG –304) was end labeled with [γ-^{32}P]-dATP using T4 polynucleotide kinase. Specific DNA probes (30,000 cpm per reaction) were incubated with nuclear extract (20 μg protein per lane) at room temperature with or without antibody for 30 min. MafA supershift analyses were performed by using 4 μg of anti-MafA antibodies as recommended by manufacturer. Pdx-1 supershift analyses were performed by using 1 μL of anti-Pdx-1 antibodies. The reaction mixture was then resolved through nondenaturing polyacrylamide gel (40 mM Tris, 0.384 M glycine, 2 mM EDTA, pH 8.3, 5.1% acrylamide, 0.076% ammonium persulfate, and 0.038% TEMED). After the reaction mixtures were fractionated, the gel was dried at 80°C. EMSA gels were quantified using a phosphoimager (Molecular Dynamics).

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12. Insulin secretion study

INS-1 cells were plated in 12-well plates at a density of $0.65x10^6$ cells per well, and cultured in INS-1 medium for two days. They were then treated as indicated in figure legends. Culture medium was collected after the treatment. Cells were then incubated with Krebs-Ringer-bicarbonate (KRB) buffer (0.12 M NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 3.4 mM $CaCl_2 \cdot 2H_2O$, 5 mM $NaHCO_3$, 10 mM HEPES), 0.1% BSA and 2 mM glucose at 37°C for 1 hr. After washing cells with KRB buffer containing 2 mM glucose, they were incubated at 37°C with KRB buffer containing 2 mM glucose, 8 mM glucose or 16.7 mM glucose for 30 min. Supernatants were collected. Cells were then washed once with PBS, total protein or total cellular insulin content were collected. For total protein analyses, cells were solubilized in 1 N NaOH. Protein assay was performed using Lowry assay. For total insulin content, cell insulin was extracted in acidified-ethanol (1.5% concentrated HCl, 75% ethanol).

Rat ^{125}I-insulin radioimmunoassay (RIA) (Linco research, Inc.) was used to quantitate the total cellular insulin, insulin released upon glucose stimulation and insulin accumulated in culture media. RIA was set up according to the manufacturer's protocol. After adding 100 μL of hydrated ^{125}I-insulin (0.19 μCi) and 100 μL rat insulin antibody to each sample, samples were incubated at 4°C overnight. ^{125}I insulin/antibody complexes were precipitated with Precipitating Reagent (goat anti-guinea pig IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05 M phosphaline, 0.025 MEDTA, 0.08% sodium azide). ^{125}I insulin/antibody complexes were quantitated by gamma counting.

13. Dihydrodichlorofluorescein (H_2DCF) oxidation assay

Dihydrodichlorofluorescein (H_2DCF) oxidation assay was modified from the method of Boulares-HA et al (277) and a Molecular Probes protocol. INS-1 cells were plated on a 96-well plate at the density of $0.0784x10^6$, and the cells were cultured in INS-1 medium for two days. Cells were then incubated for 1.5 hr in INS-1 medium containing 16.7 mM glucose in the absence or presence of 10 mM NAC, 10 μM quercetin, 1 mM lipoic acid, 10 mM NAM, 10 mM 3-AB or 500 μM PD. Cells were then incubated with 50 μM H_2DCFDA for 1 hr at 37°C, after which, cells were washed once with PBS. Cells were then challenged with 1 mM H_2O_2 in the presence or absence of antioxidants or lp-PAPRi. In the presence of ROS, the nonfluorescent H_2DCFDA is oxidized to fluorescent DCF, which then can be quantified on a microplate reader with the excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Chapter 4. High Glucose Attenuates Activity of Human Insulin Promoter in INS-1 Pancreatic Cells through Reduced MafA Binding to C1 and A5/core Elements

Abstract

Chronic hyperglycemia leads to reduction of insulin gene expression, in part, through decreased insulin promoter activity. Glucose-suppression of insulin promoter activity is associated with decreased PDX-1 nuclear protein levels and binding to insulin promoter. In addition, binding of the C1-activator to the insulin promoter is also reduced under chronic hyperglycemia. MafA has been recently identified to be a C1 element binding factor. The importance of MafA in glucose-suppression of insulin promoter was unknown. Therefore, the hypothesis of this study was that loss of MafA binding and protein played an important role in chronic hyperglycemia suppression of insulin promoter activity.

Electrophoretic mobility shift assays showed that MafA/C1 binding complex was significantly reduced in INS-1 cells cultured in high (16.7 mM) glucose. In contrast, PDX-1/A3 binding complex was only marginally reduced. Overexpression of MafA, but not PDX-1, stimulated insulin promoter activity irrespective of glucose concentration. These data indicate that MafA can override or possibly reverse glucose-suppression of insulin promoter activity. Binding complex formation at A5/core elements, previously shown to bind MafA and PDX-1, was also significantly reduced by elevated glucose. Overexpression of MafA, but not PDX-1, attenuated glucose-reduction of distal insulin promoter reporter genes containing the A5/core elements. Overall, chronic

hyperglycemia attenuates the activity of insulin promoter through reduced MafA binding to the C1 and A5/core elements in INS-1 cells.

Introduction

Chronic hyperglycemia plays an important role in the progression of type 2 diabetes. It causes changes in gene expression profile in pancreatic β-cells, ultimately leading to β-cell dysfunction (95, 96). Chronic hyperglycemia leads to reduced insulin gene expression, in part, through the decreased insulin promoter activity (16-20). The human insulin promoter contains several cis-elements, which mediate glucose regulation of insulin promoter activity. Changes in transcription factors binding to the A and C1 elements are associated with glucose-suppression of insulin promoter activity (17). The A elements involved include A1 and A3 elements on human insulin promoter (17-19, 21). Pancreatic duodenal homeobox factor 1 (PDX-1) binds to the consensus sequence TAAT located in the A elements. Chronic hyperglycemia reduces PDX-1 binding to the A1 and A3 elements in several β-cell lines and human pancreatic islets (17-19, 21). Glucose-mediated reduction of PDX-1 binding is associated with downregulation of PDX-1 nuclear protein levels (19). Glucose-suppression of insulin promoter activity is also mediated by reduced binding to the C1 element (17, 20). The identity of the C1-activator lost in β-cells exposed to elevated glucose was unknown. Recently, MafA has been shown to bind to the C1 element and induce insulin promoter activity upon acute glucose stimulation (25-28). The present study was designed to determine whether MafA was the C1-activator whose binding was reduced in response to chronic hyparglycemia. The relative importance of PDX-1 and MafA in the glucose-suppression of insulin promoter activity was also examined.

It has been shown that the distal insulin promoter (-327/-231) plays an important role in glucose-suppression of insulin promoter activity in INS-1 cells (38). Within this

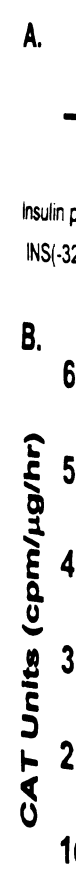
region, the A5/core elements were suggested to be involved in glucose-suppression of distal insulin promoter activity (38). The A5/core binding complex contains MafA and PDX-1 (38), suggesting that high glucose may also affect MafA and PDX-1 binding to the A5/core elements. The effects of chronic hyperglycemia on A5/core binding complex formation were also examined in this sutdy.

Overall, this study indicates that chronic hyperglycemia attenuates human insulin promoter activity in INS-1 cells through reduced MafA protein and binding at C1 and A5/core elements. These data also support the conclusion that loss of MafA plays a more significant role in loss of insulin promoter activity in β-cells exposed to elevated glucose.

Results

1. Removal of sequences between –327 bp to –231 bp leads to a partial loss of glucose suppression of insulin promoter activity.

Previous studies showed that exposure of INS-1 cells to 16.7 mM (high) glucose led to a marked suppression of insulin promoter activity within 24 hrs (17). To further study the glucose suppression of insulin promoter, effects of glucose on a simplified insulin promoter, –230 insulin promoter (Fig. 9A), were studied. –230 insulin promoter contains several cis-elements, including A1, A3 and C1, which are involved in mediating glucose-suppression of insulin promoter activity (17, 18, 20, 21). INS-1 cells were transfected with –230 insulin promoter reporter gene (INS(-230)CAT) in which the human insulin promoter sequence between –230 bp to +30 bp controls the expression of a chloramphenical acetyltransferase (CAT) reporter gene. Deletion of insulin promoter sequences from –327 bp to –231 bp led to a significant reduction (68.4%) of insulin promoter activity in INS-1 cells cultured with 4.0 mM (low) glucose (Fig. 9B). The expression of INS(-230)CAT under high glucose, however, was not significantly reduced compare to the expression of INS(-327)CAT, which is a CAT reporter gene driven by the human insulin promoter containing the sequences between –327 bp to +30 bp (Fig. 9A and B). These data suggest that sequences between –327 bp to –231 bp mediate the majority of glucose-induction of insulin promoter activity. Moreover, high glucose suppressed the expression of INS(-327)CAT and INS(-230)CAT by 84.6% and 62%, respectively, compared to cells incubated with low glucose (Fig. 9B). These data indicate that removal of sequences between –327 bp to –231 bp leads to reduced glucose-suppression of insulin promoter activity.



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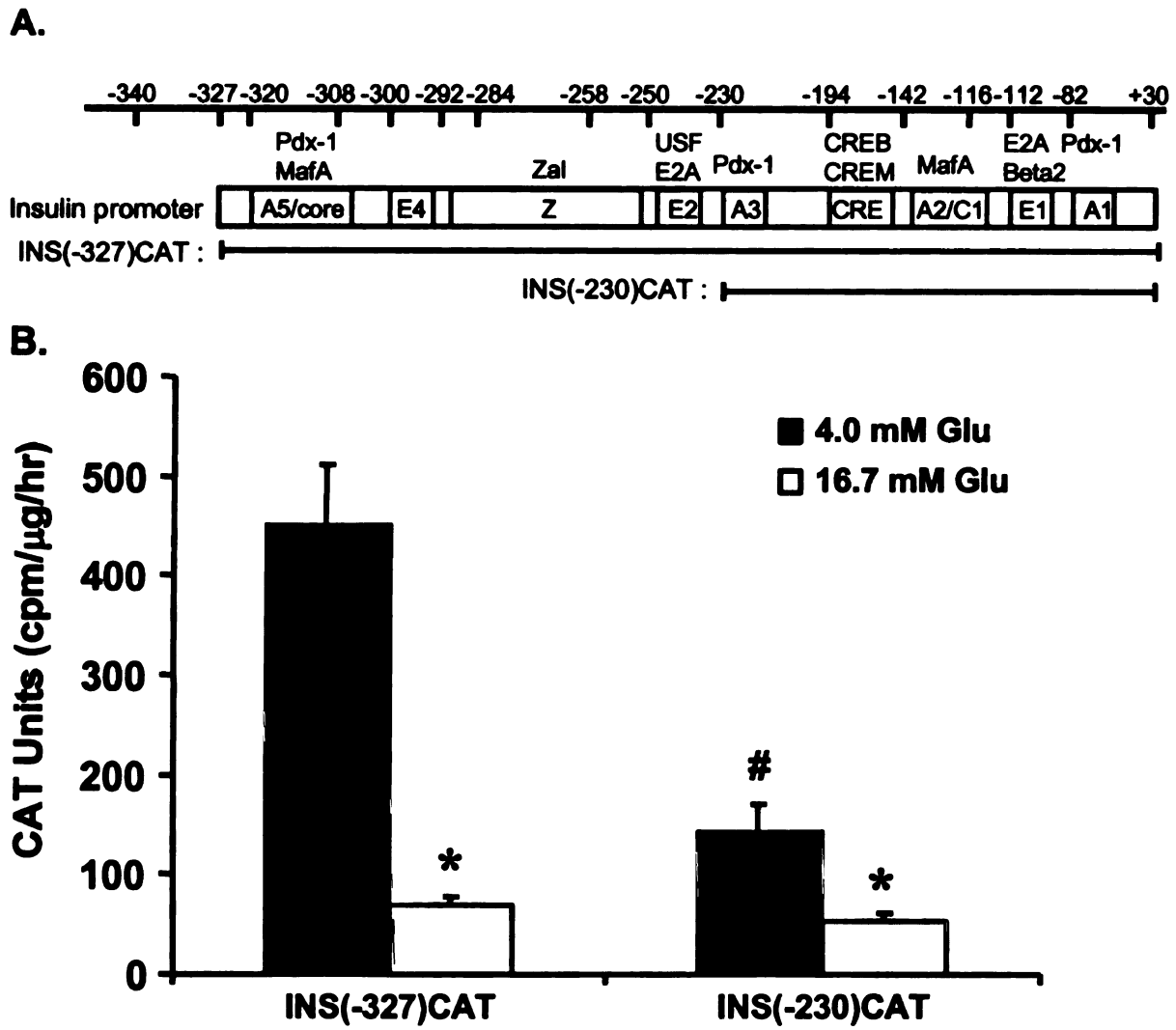

Figure 9. Removal of sequences between –327 bp to –231 bp leads to a partial loss of glucose-suppression of insulin promoter activity. A) Schematic representation of human insulin promoter. –327 insulin promoter and –230 insulin promoter used in this study are shown. B) INS-1 cells were transfected transiently with INS(-327)CAT or INS(-230)CAT and then cultured for 48 hrs with medium containing 4.0 mM glucose or 16.7 mM glucose. Cells were then harvested and CAT assays performed. Values are mean ± SEM. (n=6). * 4.0 mM Glu versus 16.7 mM Glu for respective CAT reporter gene, and # INS(-327)CAT versus INS(-230)CAT, $p<0.05$.

2. MafA binding to the C1 element is significantly reduced by high glucose.

Chronic exposure of β-cells to elevated glucose reduces insulin promoter activity, in part, by reducing binding to the C1 element (17, 20). Recently, MafA has been shown to bind to the C1 element and induce insulin promoter activity upon acute glucose elevation (25-28). To determine whether chronic hyperglycemia decreased MafA binding to the C1 element, EMSA were performed with the nuclear extracts derived from INS-1 cells cultured in low or high glucose. A ^{32}P labeled A2C1 probe was used in this study because the A2 element has been shown to stabilize the C1 binding complex (39). Nuclear extracts from cells cultured in 4.0 mM glucose formed a single complex to the A2C1 probe (Fig. 10). This A2C1 binding complex was reduced in nuclear extracts derived from the INS-1 cells cultured in 16.7 mM glucose (Fig. 10, lanes 2 and 3). Antibodies against MafA supershifted the A2C1 binding complex, indicating that MafA is present in the complex (Fig. 10, lanes 4 and 5). High glucose also reduced binding to the A3 probe, however, the A3 binding complex was only marginally reduced compared to the A2C1 binding complex (Fig. 10, lanes 2, 3, 7 and 8). PDX-1 antibodies were able to supershift A3 binding complex, demonstrating the presence of PDX-1 in this complex (Fig. 10, lanes 9 and 10). These data indicate that chronic hyperglycemia diminishes MafA binding to the C1 element, and marginally reduces PDX-1 binding to the A3 element.

3. Chronic hyperglycemia reduces MafA nuclear protein level.

To determine whether the reduced MafA binding to the C1 element was due to reduced MafA nuclear protein level, INS-1 cells were treated with 4.0 or 16.7 mM

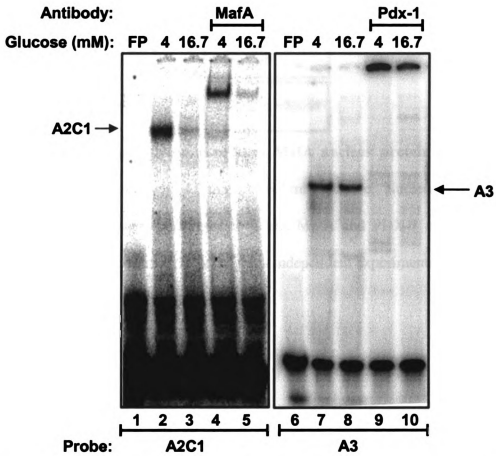

Figure 10. MafA binding to the C1 element is significantly reduced by high glucose. INS-1 cells were treated for two days with 4.0 mM or 16.7 mM glucose. Cells were then harvested and nuclear extracts were prepared. EMSA using ^{32}P-labeled A2C1 or A3 were performed. Antibodies against MafA or PDX-1 were added to lanes 4 and 5, or 9 and 10, respectively. Shown here is a representative gel of three independent experiments. (FP=free probe)

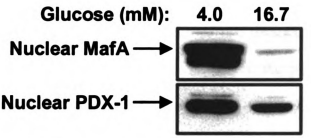

Figure 11. Chronic hyperglycemia reduces MafA nuclear protein level. INS-1 cells were treated for two days with 4.0 mM or 16.7 mM glucose. Nuclear extracts were prepared. Western blots were performed to detect MafA and PDX-1 nuclear protein levels. Shown here is a representative gel of three independent experiments.

glucose for 48 hrs and MafA nuclear protein levels were determined by Western blot analysis. Antibodies against MafA detected two distinct bands under 4.0 mM glucose (Fig. 11). The two different sizes in MafA are the phosphorylated (top band) and dephosphorylated (bottom band) form of MafA (278). High glucose markedly reduced MafA nuclear protein level (Fig. 11). PDX-1 nuclear protein level was also reduced by high glucose, however, the reduction was small compared to MafA (Fig. 11). These data show that high glucose significantly reduces MafA nuclear protein level, and marginally decreases PDX-1 nuclear protein level. These data also suggest that the loss of MafA binding to the C1 element is most likely due to reduced MafA nuclear protein level.

4. Overexpression of MafA increases insulin promoter activity.

Since chronic hyperglycemia reduced both MafA and PDX-1 nuclear protein levels, PDX-1 or MafA was overexpressed in INS-1 cells to determine their relative abilities to restore insulin promoter activity. High glucose significantly reduced the expression of INS(-327)CAT (Fig. 12A and B). Overexpression of MafA in INS-1 cells markedly induced the expression of INS(-327)CAT (Fig. 12A). In contrast, PDX-1 overexpression did not stimulate the expression of INS(-327)CAT (Fig. 12B). These data suggest that increased MafA expression, but not PDX-1, can restore insulin promoter activity in INS-1 cells cultured under chronic hyperglycemia.

A1 and A3 elements, and C1 element are located within –230 insulin promoter, and serve as binding sites for PDX-1 and MafA, respectively (Fig. 9A). Moreover, these elements are involved in mediating glucose-suppression of insulin promoter activity (17, 18, 20, 21). Therefore, the effects of MafA or PDX-1 overexpression on -230

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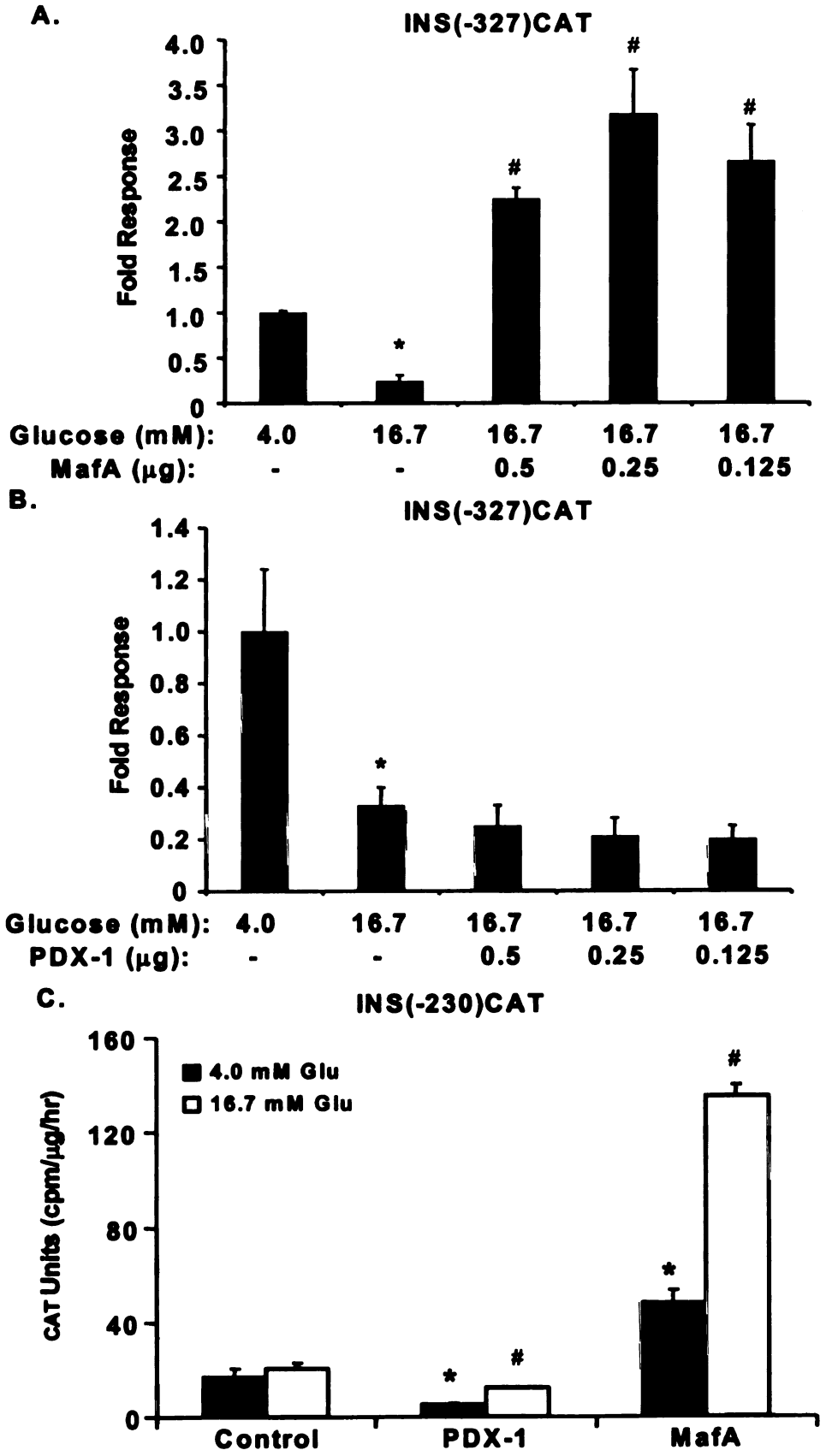
Figure 12. Overexpression of MafA increases insulin promoter activity. A) INS-1 cells were cotransfected with INS(-327)CAT (1 μg) and increased amount of MafA expression vector (pCR3.1-CMV-MafA) (n=4). * 4 mM Glu versus 16.7 mM Glu, # 16.7 mM Glu versus 16.7 mM Glu plus expression vector, $p<0.05$. B) INS-1 cells were cotransfected with INS(-327)CAT (1 μg) and increased amount of PDX-1 expression vector (pCR3.1-CMV-PDX-1) (n=4). * 4 mM Glu versus 16.7 mM Glu, $p<0.05$. C) INS-1 cells were cotransfected with INS(-230)CAT (1 μg) and 0.25 μg pCR3.1-CMV-MafA or pCR3.1-CMV-PDX-1 (n=4). * 4 mM Glu control versus expression vector, # 16.7 mM Glu control versus expression vector, $p<0.05$. For all three experiments, equivalent amounts of expression plasmid (0.5 μg) was transfected per well by the addition of pCR3.1-CMV containing no insert. Cells were treated with 4 mM or 16.7 mM glucose for 48 hrs after transfection. Cells were then harvested and CAT assays performed. Values are mean ± SEM.


A.
INS(-327)CAT
Fold Response
4.0
3.5
3.0
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2.0
1.5
1.0
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Glucose (mM): 4.0 16.7 16.7 16.7 16.7
MafA (μg): - - 0.5 0.25 0.125
B.
INS(-327)CAT
Fold Response
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Glucose (mM): 4.0 16.7 16.7 16.7 16.7
PDX-1 (μg): - - 0.5 0.25 0.125
C.
INS(-230)CAT
4.0 mM Glu
16.7 mM Glu
CAT Units (cpm/μg/hr)
160
120
80
40
0
Control
PDX-1
MafA

insulin promoter activity were examined. MafA overexpression strongly induced -230 insulin promoter activity in cells treated with 4.0 mM or 16.7 mM glucose (Fig. 12C). In contrast, overexpression of PDX-1 significantly suppressed –230 insulin promoter activity in INS-1 cells cultured in medium containing 4.0 mM or 16.7 mM glucose (Fig. 12C). These data suggest that MafA increases the insulin promoter activity in such a way that it can override glucose-suppression of insulin promoter activity.

5. MafA binding to the A5/core element is significantly reduced by high glucose.

A previous study showed that high glucose suppressed two distal insulin promoter reporter genes containing the A5/core element, suggesting that A5/core element mediate part of glucose-suppression at the distal insulin promoter (38). MafA and PDX-1 were identified to be the nuclear factors bound to the A5/core element (38). To determine whether high glucose also reduced MafA binding to A5/core, EMSA were performed with the nuclear extracts derived from INS-1 cells cultured in low or high glucose using ^{32}P labeled A5/core probe. Nuclear extracts from cells cultured in 4.0 mM glucose formed a slow migrating complex to the A5/core probe (Fig. 13, lanes 2). This A5/core binding complex was reduced in nuclear extracts derived from the INS-1 cells cultured in 16.7 mM glucose (Fig. 13, lanes 2 and 3). Antibodies against MafA attenuated A5/core binding complexes formation, indicating the presence of MafA in this binding complex (Fig.13A, lanes 4 and 5).

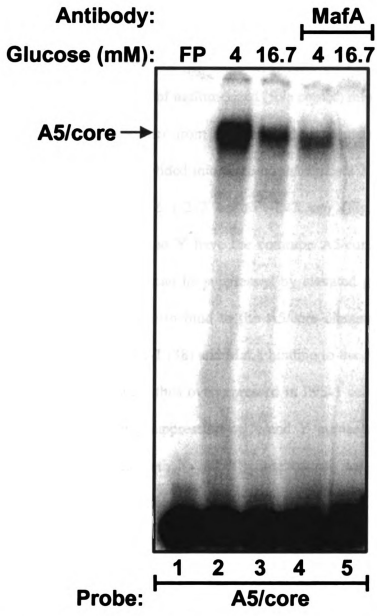


Figure 13. MafA binding to the A5/core element is significantly reduced by high glucose. INS-1 cells were treated for two days with 4 mM or 16.7 mM glucose. Cells were then harvested and nuclear extracts were prepared. EMSA was performed using ^{32}P-labeled A5/core. Antibodies against MafA were added to lanes 4 and 5. Shown here is a representative gel of three independent experiments. (FP=free probe)

6. Overexpression of MafA stimulates X and Y minienhancer activities.

Previously, the distal region of human insulin promoter (-341 bp to –243 bp) was characterized by the use of multimerized (five copies) minienhancers linked to the –85 bp minimal insulin promoter from rat insulin 1 gene promoter (62). The distal human insulin promoter was divided into three minienhancers X (-342 bp to –293 bp), Y (-317 bp to –268 bp) and Z (-292 bp to –243 bp) (Fig. 14A). Among these three minienhancers, the X and Y have the common A5/core elements, and both X and Y minienhancer activities can be suppressed by elevated glucose (38). Both PDX-1 and MafA have been shown to bind to the A5/core elements, and chronic hyperglycemia leads to decreased PDX-1 (38) and MafA binding to the A5/core in INS-1 cells (Fig. 13). PDX-1 and MafA were thus overexpressed in INS-1 cells to determine their abilities in attenuation of glucose-suppression of X and Y minienhancer activities. High glucose significantly reduced both X and Y minienhancer activities (Fig. 14B). Under high glucose, MafA overexpression gave a 10.5-fold or 35.7-fold stimulation of X or Y minienhancer activity, respectively (Fig. 14B). Under low glucose, MafA overexpression led to a 3.2-fold or 4.5-fold induction of X or Y minienhancer activity, respectively (Fig. 14B). Overexpression of PDX-1 suppressed X or Y minienhancer activity in cells cultured with 4.0 mM glucose (Fig.14B). In cells incubated with 16.7 mM glucose, overexpression of PDX-1 led to small but significantly increases of X and Y minienhancer activities (Fig. 14B). These data show that loss of MafA also plays an important role in mediating glucose-suppression of distal insulin promoter activity.

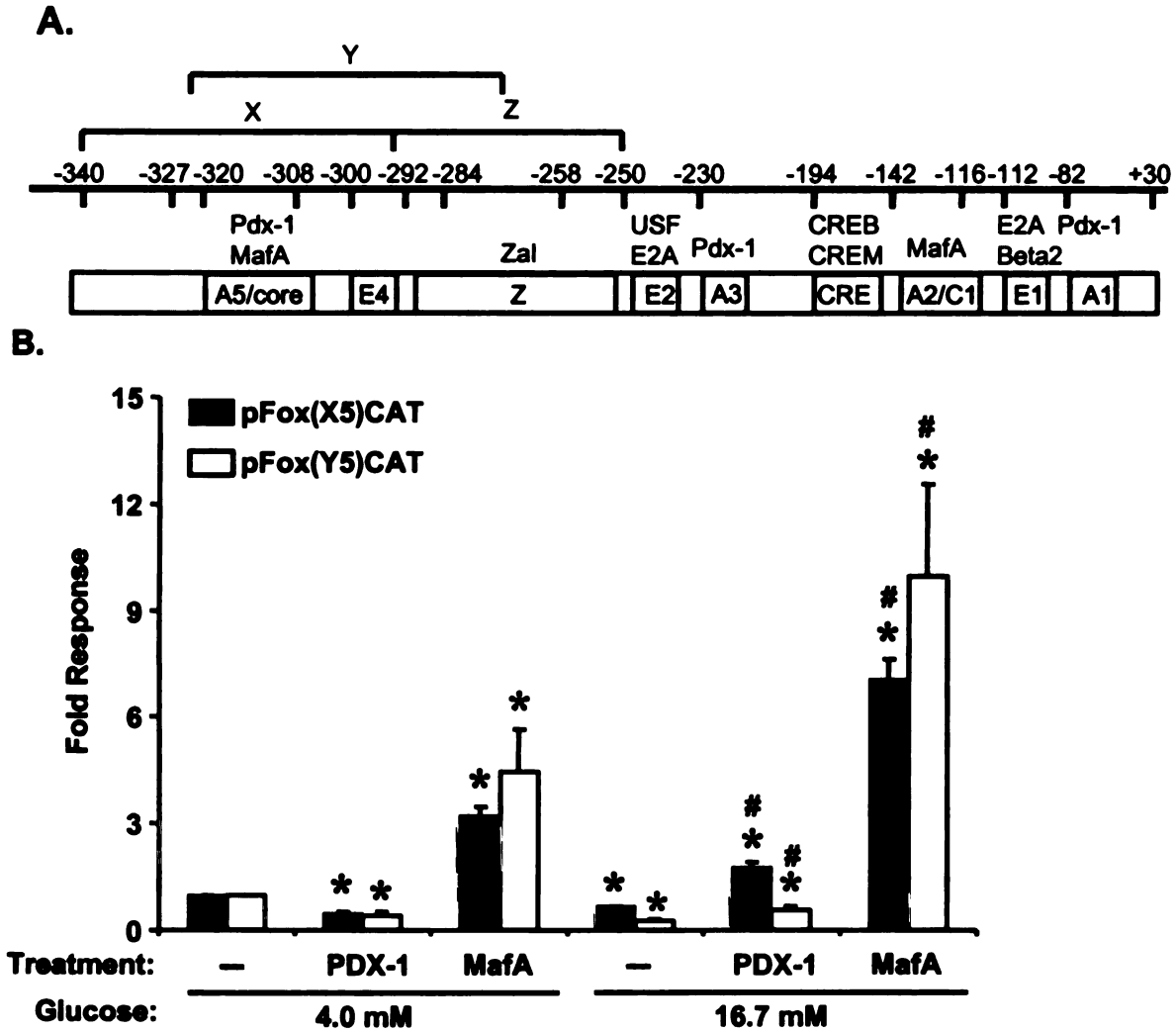


Figure 14. Overexpression of MafA stimulates X and Y minienhancer activities.

A) Schematic representation of human insulin promoter. Minienahncers used in multimerized CAT reporter gene by Sander et al and the present study are indicated (62). B) INS-1 cells were transfected with pFox(X5)CAT or pFox(Y5)CAT, with or without overexpression of PDX-1 or MafA. The cells were then incubated in medium containing 4.0 mM glucose or 16.7 mM glucose for 48 hrs. After which, cells were harvested and CAT assays performed. Values are mean ± SEM. For pFox(X5)CAT or pFox(Y5)CAT: * 4.0 mM Glu control versus the other conditions, and # 16.7 mM Glu versus 16.7 mM Glu plus PDX-1 or MafA, $p<0.05$. (n=4).

Discussion

The present study showed that chronic hyperglycemia reduced MafA binding to the C1 element in INS-1 cells. Recently, studies from Harmon et al also showed that MafA binding to the C1 element was decreased in HIT-T15 cells chronically passed in elevated glucose (16). Importantly, glucose-reduction of PDX-1 binding to the A3 element was much less compared to the reduction of MafA binding to the C1 element in INS-1 cells (Fig. 10). These data indicate that MafA binding activity may be affected more by chronic hyperglycemia than PDX-1 in INS-1 cells. Previously, our laboratory has shown that C1 binding complex formation was completely lost while A3 binding complex formation was only partially lost in INS-1 cells (17). In βTC-6 cells, chronic hyperglycemia also markedly diminished formation of the C1 binding complex but not the A3 binding complex (20). Chronic culturing human islets with elevated glucose, however, reduced PDX-1 binding to the A3 element more than C1-activator binding to the C1 element (21). These different responses to chronic hyperglycemia between β-cells lines and human islets could be due to the species differences or phenotypic differences between x-ray-induced tumor cell line (INS-1), T-antigen immortalized cell lines (βTC-6) and primary β-cells. Nevertheless, all these data support the hypothesis that chronic hyperglycemia can decrease MafA binding to the C1 element. Decreased MafA binding is consistent with the large reduction in MafA nuclear protein level in INS-1 cells cultured in high glucose (Fig. 11). In agreement with this study, Harmon et al recently demonstrated that MafA nuclear protein levels were also diminished in HIT-T15 cells chronically exposed to high glucose (16). Although the binding affinity of MafA could be affected by MafA posttranslational modification such as phosphorylation (278), it is

unlikely that posttranscriptional modification plays a role in glucose-reduction of MafA binding to insulin promoter. Taken together, these data suggest that reduced MafA nuclear protein level is responsible for the reduced C1 binding complex formation under high glucose.

Overexpression of MafA stimulated insulin promoter activity in INS-1 cells cultured with high glucose (Fig. 12A). These data indicate that either loss of MafA binding accounts for loss of insulin promoter activity or that MafA can act as a strong activator to override glucose-suppression of insulin promoter activity. The latter possibility was supported by the observation that overexpression of MafA significantly stimulated the activity –230 insulin promoter which was only modestly suppressed by high glucose (Fig. 12C). Removal of -327 bp to -231 bp of insulin promoter usually leads to a large loss of glucose-suppression of insulin promoter activity (Fig. 9B) (38). High glucose, however, can still suppress –230 insulin promoter (Fig. 9B), and this is most likely due to altered binding at the A or C1 elements. In MafA overexpression study, however, high glucose did not consistently suppress –230 insulin promoter activity (Fig. 12C). This is because transfecting control expression vectors markedly reduced INS(-230)CAT expression in cells cultured in 4.0 mM glucose (data not shown). We believe this occurs by squelching or limiting general coactivators or transcription factors necessary for activation of the –230 insulin promoter. Nevertheless, these data indicate that MafA can act as a strong transcriptional activator capable of overriding glucose-suppression of insulin promoter activity.

This study also showed that chronic hyperglycemia reduced PDX-1 binding to the A3 element (Fig. 10). PDX-1 nuclear protein levels were significantly reduced in INS-1

cells cultured in high glucose, suggesting that decreased A3 binding complex formation was due to reduced PDX-1 nuclear protein levels (Fig. 11). In agreement with our findings, chronic hyperglycemia has been shown to decrease PDX-1 protein levels in both β-cell line and animal studies (16, 19, 103). Overexpression of PDX-1 in INS-1 cells, however, failed to induce either the –327 or –230 insulin promoter (Fig. 12B and C). More so, PDX-1 overexpression tended to reduce insulin promoter activity. Overexpression of PDX-1, however, has been shown to increase insulin promoter activity in HIT-T15 cell chronically cultured in high glucose for about 48 weeks (279). The discrepancy could be because of the differences in cell lines and length of culturing. HIT-T15 cells cultured with high glucose for 48 weeks led to a complete loss of PDX-1 binding (279) while INS-1 cells cultured with high glucose for 2 days only led to a small reduction of PDX-1 binding (Fig. 10). Indeed, overexpression of PDX-1 did not significantly induce insulin promoter activity in HIT-T15 cells cultured in high glucose for 30 weeks, which only had a partial reduction of PDX-1 binding (279). In chronically passed RIN-38 cells, Seijffers et al showed that PDX-1 nuclear protein levels were elevated with increased passage number, whereas the insulin promoter activity was decreased (22). These data suggested that decreased promoter activity was not due to decreased PDX-1 levels (22). Moreover, overexpression of PDX-1 in RIN-38 cells did not restore insulin promoter activity, but actually suppressed its activity (22). The inability to stimulate insulin promoter activity with enhanced PDX-1 expression may suggest that reduced PDX-1 protein levels do not account for glucose-suppression of insulin promoter activity. This hypothesis is further supported by the observation that antisense PDX-1 RNA can lower PDX-1 protein levels in MIN6 or βTC1 cells, but does

not affect insulin mRNA levels (24). Consistent with antisense study, mice containing one functional allele for PDX-1 have reduced PDX-1 protein levels but normal pancreatic insulin content (23). Taken together, the current literature and our study suggest that the loss of PDX-1 in β-cells cultured in elevated glucose does not account for the loss of insulin promoter activity.

The different effects of overexpression of PDX-1 and MafA on insulin promoter activity may be due to their different ability in interacting of coactivators. PDX-1 is known to recruit p300/CBP to insulin promoter upon acute high glucose stimulation (43, 47). Excess PDX-1 may sequester these coactivators, thus limiting the amount of coactivators available to stimulate insulin promoter activity. In contrast, MafA does not interact with p300 (59), thus excess MafA will not sequester coactivators, and overexpression of MafA increases insulin promoter activities (Fig. 12A and C). Indeed, a mutation of PDX-1 that leads to increased PDX-1 binding affinity to p300 has been shown to be associated with maturity-onset diabetes of the young 4 (MODY4), which is a rare form of diabetes caused by heterozygous mutations in the PDX-1 gene (280).

Posttranscriptional modification of PDX-1 may play a role in the loss of PDX-1 binding to insulin promoter during chronic hyperglycemia. PDX-1 phosphorylation is important for its translocation to nucleus and binding to the insulin promoter (43, 45-47, 74). Dephosphorylated PDX-1 has been shown to recruit histone deacetylases HDAC-1 and HDAC-2 to insulin promoter, causing decreased insulin gene expression in cells treated with low glucose (48). Therefore, it is possible that dephosphorylated PDX-1 protein levels are increased in INS-1 cells chronically exposed to high glucose.

Removal of the human insulin promoter sequences between –327 bp to –231 bp diminished glucose suppression of insulin promoter activity. These data indicated that the distal end of insulin promoter was also involved in glucose-suppression of insulin promoter activity (Fig. 9B). Consistent with this study, removal of the sequences between –340 bp to –260 bp causes a significant decrease of insulin promoter activity in rat islets cultures (51). Contained within –327 bp to –261 bp region is a previously described glucose-responsive element, the Z element, which is very active in primary rat islets but serves as negative regulatory element (NRE) of insulin promoter in SV40 or T-antigen immortalized β-cell lines (62, 78, 281). Deletion studies have shown that removal of Z element reduced insulin promoter activity in primary rat islets but stimulated insulin promoter activity in HIT cells (51, 78). Suppression of the Z element activity in HIT cells may be related to elevated cell proliferation because the Z element activity was also very low in proliferating fibroblasts (78). Pino et al have shown in INS-1 cells cultured in 4.0 mM glucose, that the Z element is active and deletion of it markedly reduces insulin promoter activity (38). Culturing INS-1 cells in elevated glucose suppressed the Z element activity and this was associated with reduced binding to a palindromic region and an E-box located within the Z element (38). When cultured in elevated glucose, INS-1 cells have increased proliferation (282). We currently hypothesized that glucose suppression of the Z element activity in INS-1 cells is mostly due to increased proliferation as observed in HIT cells and fibroblasts (51, 78).

Pino et al has also shown that the A5/core elements located upstream of the Z element bind to a complex that contains MafA and PDX-1 (38). Our study showed that the A5/core binding complex was decreased in INS-1 cells cultured with high glucose

(Fig 13). This finding is consistent with the general reduction in MafA protein level by elevated glucose. Addition of MafA antibodies did not appear to supershift A5/core binding complex but diminished it (Fig. 13). The A5/core binding complex, however, is a large binding complex and contains two or more nuclear proteins. Addition of antibodies against MafA likely increased the size of this large binding complex and prevented its entry into the EMSA gel. Addition of PDX-1 antibodies also prevented the A5/core binding complex from consistently entering the EMSA gel (data not shown). A lower percentage acrylamide gel may have helped to resolve the supershifted binding complexes. Nevertheless, these data suggest that high glucose reduces MafA binding to the A5/core elements.

Since both MafA and PDX-1 are present in the A5/core binding complex, the relative importance of PDX-1 and MafA in attenuation of glucose-suppression of distal insulin promoter activity was estimated by overexpressing PDX-1 or MafA. Our data showed that chronic hyperglycemia suppressed X and Y minienhancer activities (Fig. 14). This is consistent with our previous study, which showed that prolonged incubating INS-1 cells with medium containing glucose concentration higher than 8.0 mM reduced X and Y minienhancer activities (38). Overexpression of MafA, but not PDX-1, attenuated glucose-suppression of X and Y minienhancer activities (Fig. 14). These data suggest that reduced MafA protein level is also responsible for the decreased A5/core binding complex formation in INS-1 cells cultured with high glucose. Moreover, as observed with overexpression of MafA in cells transfected with INS(-230)CAT, overexpression of MafA stimulated X and Y minienhancer activities in cells cultured

with low or high glucose, indicating again that MafA can override glucose-suppression of insulin promoter activity.

Interestingly, INS-1 cells treated with low glucose, overexpression of MafA also induced expression of INS(-230)CAT, pFox(X5)CAT or pFox(Y5)CAT (Fig. 12C and 14B). The induced expression of INS(-230)CAT, pFox(X5)CAT or pFox(Y5)CAT, however, was smaller in cells cultured in low glucose compared to the cells cultured in high glucose. The binding affinity of MafA can affected by MafA posttranslational modification such as phosphorylation (278). Recently, MafA phosphorylation by ERK1/2 has been suggested to be important for insulin promoter activity in response to acute glucose elevation (283). This indicates that high glucose may lead to more MafA phosphorylation, thus enhanced MafA binding and insulin promoter activation. Therefore, although equal amount of MafA overexpressed, less amount of MafA was phosphorylated and bound to insulin promoter under low glucose, resulting less insulin promoter activity.

In conclusion, MafA is the major transcription factor that its binding activity and nuclear protein levels are important in attenuation of glucose-suppression of insulin promoter activity. MafA is also a strong transcription factor that can override glucose-suppression of insulin promoter activity. This study shows that A5/core elements are additional cis-elements mediating glucose-suppression at the distal insulin promoter.

Chapter 5. Low-potency Poly(ADP-ribose) Polymerase Inhibitors (lp-PARPi) Induce Insulin Gene Expression through Enhanced MafA Gene Expression

Abstract

Nicotinamide (NAM), a low-potency poly(ADP-ribose) polymerase inhibitor (lp-PARPi), has been shown to increase insulin biosynthesis in porcine and human fetal islets. The mechanisms whereby these compounds regulate insulin biosynthesis were not known. The goal of the present study was to determine whether lp-PARPi increased insulin biosynthesis through increased insulin promoter activity. Since loss of MafA binding to insulin promoter plays an important role in mediating glucose-suppression of insulin promoter activity, the effects of lp-PARPi on MafA gene expression and binding to insulin promoter have also been examined in INS-1 cells.

Culturing INS-1 cells in high glucose (16.7 mM) for 48 hrs led to a significant reduction of cellular insulin content, basal insulin secretion and glucose stimulated insulin secretion (GSIS) compared to cells incubated with low glucose (4.0 mM glucose). Lp-PARPi, NAM, 3-aminobenzamide (3-AB) and PD128673 (PD), increased cellular insulin content and improved GSIS in cells prolonged cultured in high glucose. The increase in cellular insulin content is, in part, due to increased insulin gene expression. INS-1 cells cultured in high glucose, there was a 64% reduction in insulin mRNA levels compared to cells cultured in low glucose. Under high glucose, lp-PARPi led to an average of 3.5-fold increase in insulin mRNA levels. Lp-PARPi-induction of insulin mRNA is partially due to enhanced insulin promoter activity. Glucose-mediated reduction of MafA binding to the C1 element and A5/core elements was attenuated by lp-

PARPi. In contrast, lp-PARPi did not enhance PDX-1 binding to the A3 element. The increased nuclear MafA protein levels accounted for the increased MafA binding to insulin promoter by lp-PARPi. Consistent with the binding studies, lp-PARPi did not increase nuclear PDX-1 protein levels in INS-1 cells cultured in high glucose. Increased MafA protein levels by lp-PARPi were due to their ability to restore MafA mRNA levels in INS-1 cells. Further studies with the inhibitor of protein synthesis indicated that lp-PARPi did not increase MafA protein levels through enhanced MafA protein stability.

Taken together, this study shows that lp-PAPRi induce insulin promoter activity through a mechanism involved in enhanced MafA gene expression. Lp-PARPi-induction of insulin gene expression leads to partial improvement of INS-1 cells function under high glucose.

Introduction

The insulin gene is specifically expressed in pancreatic β-cells. Acute increases in glucose can stimulate insulin gene transcription and translation, and secretion (284). Chronic exposure of pancreatic β-cells to elevated glucose, however, can lead to β-cell dysfunction and ultimately cell death (21, 111, 130-133, 285). This phenomenon is called glucotoxicity. Chronic hyperglycemia leads to reduced insulin gene expression, in part, through decreased insulin promoter activity (16-20). The human insulin promoter contains several cis-elements, which mediate glucose regulation of insulin promoter activity. Changes in transcription factor binding to the A and C1 elements are associated with glucose-suppression of insulin promoter activity (17). The A elements involved include A1 and A3 elements on human insulin promoter (17-19, 21). Pancreatic duodenal homeobox factor 1 (PDX-1) binds to the consensus sequence TAAT located in the A elements. Chronic hyperglycemia reduces PDX-1 binding to A1 and A3 elements in several β-cell lines and human pancreatic islets (17-19, 21). Glucose-mediated reduction of PDX-1 binding is associated with downregulation of PDX-1 nuclear protein levels (19). Glucose-suppression of insulin promoter activity is also mediated by reduced binding to the C1 element (17, 20). Recently, MafA has been shown to bind to the C1 element and induce insulin promoter activity upon acute glucose stimulation (25-28). In chapter 4, I showed that the A5/core, another PDX-1 and MafA binding element, mediated the glucose-suppression of distal insulin promoter activity in INS-1 cells. Compared to PDX-1, loss of MafA nuclear proteins and binding to C1 and A5/core elements plays an important role in glucose-suppression of insulin promoter activity in INS-1 cells.

Culturing of human and porcine fetal islets with low-potency-PARP inhibitors (lp-PARPi), nicotinamide (NAM) and 3-aminobenzamide (3-AB), increased insulin biosynthesis, insulin content, stimulated β-cell differentiation, and improved GSIS (10, 12, 13, 15). The beneficial effects of lp-PARPi in diabetes have also been demonstrated in several diabetic animal models (3, 6). In partially pancreatized animals, NAM or 3-AB treatment caused decreased blood glucose levels, increased insulin content, and improved glucose tolerance (3, 6). The effects of lp-PARPi in partially pancreatized animals were suggested to be associated with the ability of lp-PARPi in stimulating β-cell differentiation. In streptozotocin (STZ)-induced diabetes, destruction of islets induces hyperglycemia, which was proposed to increase production of reactive oxygen and nitrogen species, causing DNA strand breaks and PARP activation (254). PARP activation and the glucose-induction of pro-inflammatory mediators have been shown to lead to endothelial dysfunction in STZ-induced diabetes (254). NAM or 3-AB pretreatment protected islets from STZ-mediated decrease of proinsulin synthesis and DNA damage, suggesting that PARP activation may be involved in the oxidant induced β-cell damage in STZ-induced diabetes (258, 259). Although NAM and 3-AB have been shown to increase insulin biosynthesis, insulin content, stimulate β-cell differentiation and improve GSIS (10, 12, 13, 15), the mechanisms whereby these compounds mediate beneficial effects in β-cells were unknown. The goal of the present study was to determine whether lp-PARPi increased insulin biosynthesis through increased insulin promoter activity. Since loss of MafA binding to insulin promoter plays an important role in mediating glucose-suppression of insulin promoter activity, the effects of lp-

PARPi on MafA gene expression and binding to insulin promoter were also examined in INS-1 cells.

INS-1 cells have many morphological and functional characteristics that resemble normal β-cells. Like primary β-cells, the expression of β-cell specific genes such as insulin, GLUT2 and glucokinase can be suppressed by chronic hyperglycemia in INS-1 cells (17, 38, 165, 166). The glucose-suppression of insulin gene expression is associated with the reduced PDX-1 mRNA levels, and reduced PDX-1 and C1 binding to insulin promoter in INS-1 cells (17, 38). Similar results were observed with human pancreatic islets chronically exposed to high glucose (21). Chronic hyperglycemia leads to reduced GSIS in both INS-1 cells and human islets, and this is associated with reduced expression of β-cell specific genes (21, 166). Using INS-1 cells as a model of glucotoxicity, the present study showed that lp-PARPi increased insulin gene expression through the increased MafA binding to C1 and A5/core elements of the insulin promoter. Increased MafA binding to insulin promoter was due to enhanced MafA gene expression. Lp-PARPi also improved GSIS partially through the increased insulin gene expression.

Results

1. Lp-PARPi increase insulin accumulation in medium in INS-1 cells exposed to high glucose over a prolonged period.

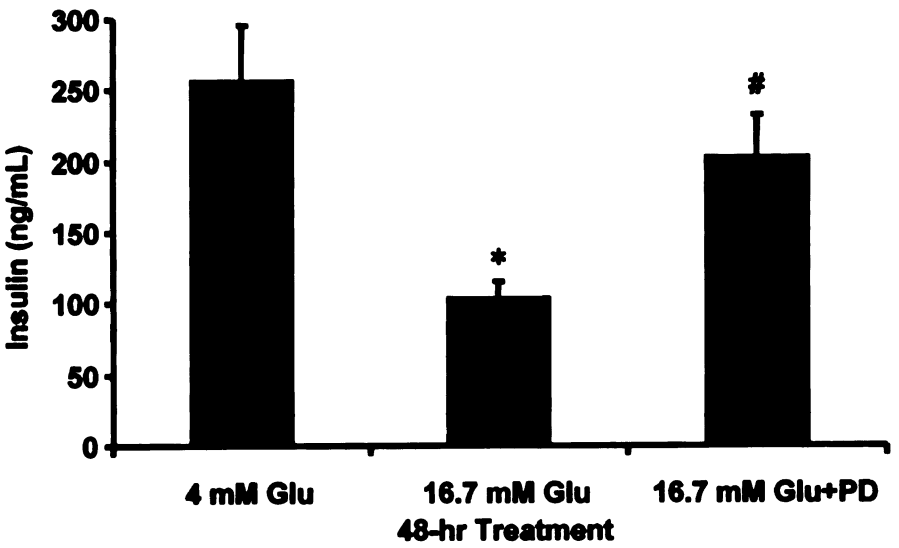
INS-1 cells were incubated for 48 hrs in medium containing 4.0 mM (low), 16.7 mM (high) or 16.7 mM glucose plus 10 mM NAM, 10 mM 3-AB or 500 μM PD. INS-1 cells treated with 16.7 mM glucose had lower amounts of insulin accumulation in the medium within 24 hr (59%, Fig. 15A) and 48 hr (71%, Fig. 15B) compared to the cells treated with 4.0 mM glucose. The amount of insulin present in medium was higher in cells treated with NAM (2.9-fold), 3-AB (3.2-fold) and PD (1.9-fold) compared to cells treated with high glucose alone (Fig. 15A and B). Since both 4.0 mM glucose and 16.7 mM glucose treatments are stimulatory glucose concentrations, insulin was constantly secreted and accumulated in the culture medium. These data show that lp-PARPi increase insulin secreted in medium in INS-1 cells exposed to high glucose over a prolonged period.

2. Lp-PARPi improve basal and acute glucose-stimulated insulin secretion in INS-1 cells exposed to high glucose.

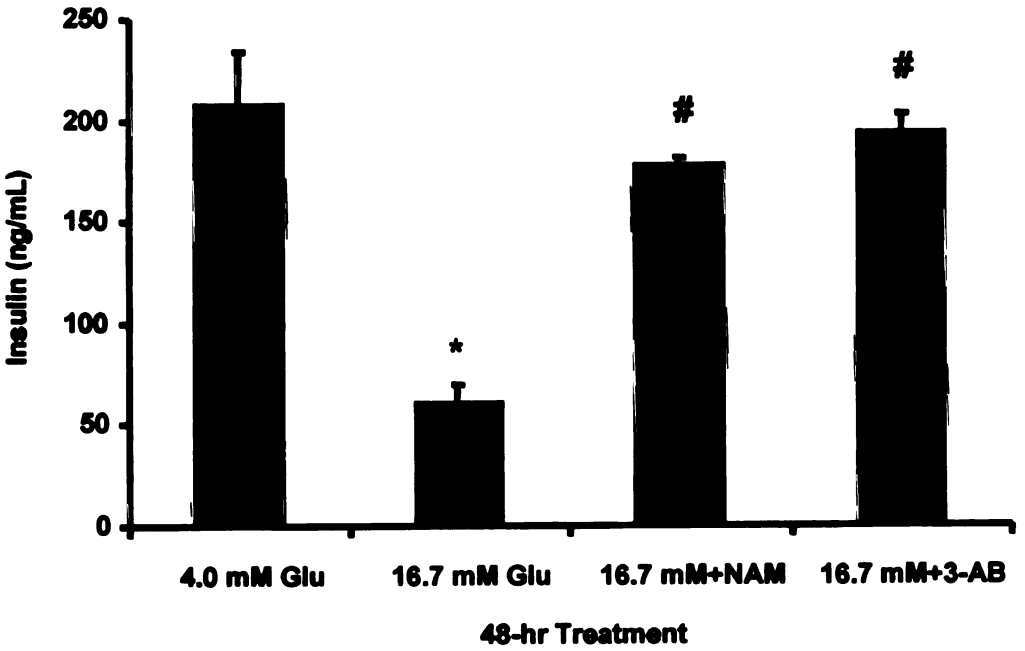
INS-1 cells were incubated for 48 hrs in medium containing low glucose, high glucose or high glucose plus lp-PARPi. Glucose stimulated insulin secretion studies were performed. Basal insulin secretion (cells challenged at 2.0 mM glucose) was significantly (~80%) reduced in cells cultured in high glucose compared to INS-1 cells cultured in low glucose. Insulin release in response to 8.0 mM or 16.7 mM glucose showed that prolonged incubation of INS-1 cells in high glucose led to a significant

Figure 15. Lp-PARPi increase insulin accumulation in medium in INS-1 cells exposed to high glucose. INS-1 cells were treated with 4.0 mM glucose, 16.7 mM glucose, 16.7 mM glucose plus 500 μM PD (A), 10 mM NAM or 10 mM 3-AB (B) for 48 hrs. For PD, fresh medium was added every 24 hr. Insulin release into the culture medium was measured after 24 hr (PD) or 48 hr (NAM, 3-AB). Insulin levels were determined by ^{125}I-insulin radioimmunoassay (RIA). Values are mean±SEM. Each value is the mean of four independent experiments with each sample measured in duplicate. * 4 mM Glu versus 16.7 mM Glu, # 16.7 mM Glu+PD versus 16.7 mM Glu, $p<0.05$.

A.


300
250
200
150
100
50
0
Insulin (ng/mL)
*
#
4 mM Glu
16.7 mM Glu
16.7 mM Glu+PD
48-hr Treatment

B.


250
200
150
100
50
0
Insulin (ng/mL)
*
#
#
4.0 mM Glu
16.7 mM Glu
16.7 mM+NAM
16.7 mM+3-AB
48-hr Treatment

decrease in acute GSIS (Fig. 16A and B). Lp-PARPi treatment caused a significant increase in basal insulin secretion as well as acute GSIS in cells cultured in high glucose, but did not lead to a complete restoration of GSIS (Fig. 16A and B). These data show that lp-PARPi improve basal and acute GSIS in INS-1 cells exposed to high glucose.

Insulin content in INS-1 cells was also measured after the secretion study. Cellular insulin content was significantly (~70.8%) decreased in cells cultured in high glucose (Fig. 17A and B). Addition of lp-PARPi significantly increased (1.4-fold) the cellular insulin content (Fig. 17A and B). These data indicate that the increase of acute GSIS is, in part, due to the increased intracellular insulin level.

3. Lp-PARPi induce insulin mRNA level in INS-1 cells.

Since lp-PARPi increased cellular insulin content, insulin mRNA levels in INS-1 cells treated with lp-PARPi under low or high glucose were measured. In cells cultured in high glucose, there was a significant decrease (~64%) in insulin mRNA level compared to the cells cultured in low glucose (Fig. 18A and B). Under high glucose, NAM, 3-AB and PD led to a 4-, 3.8-, and 2.6-fold increase in insulin mRNA level, respectively (Fig. 18B). NAM, 3-AB and PD also caused a 1.6-, 2.2-, and 1.6-fold increase in insulin mRNA level in cells cultured in low glucose (Fig. 18B). These data indicate that lp-PARPi can upregulate insulin gene expression.

4. Lp-PARPi stimulate insulin promoter activity.

To determine whether lp-PARPi can affect insulin gene transcription, INS-1 cells were transfected transiently with INS(-327)CAT, and then cultured for 48 hrs in medium

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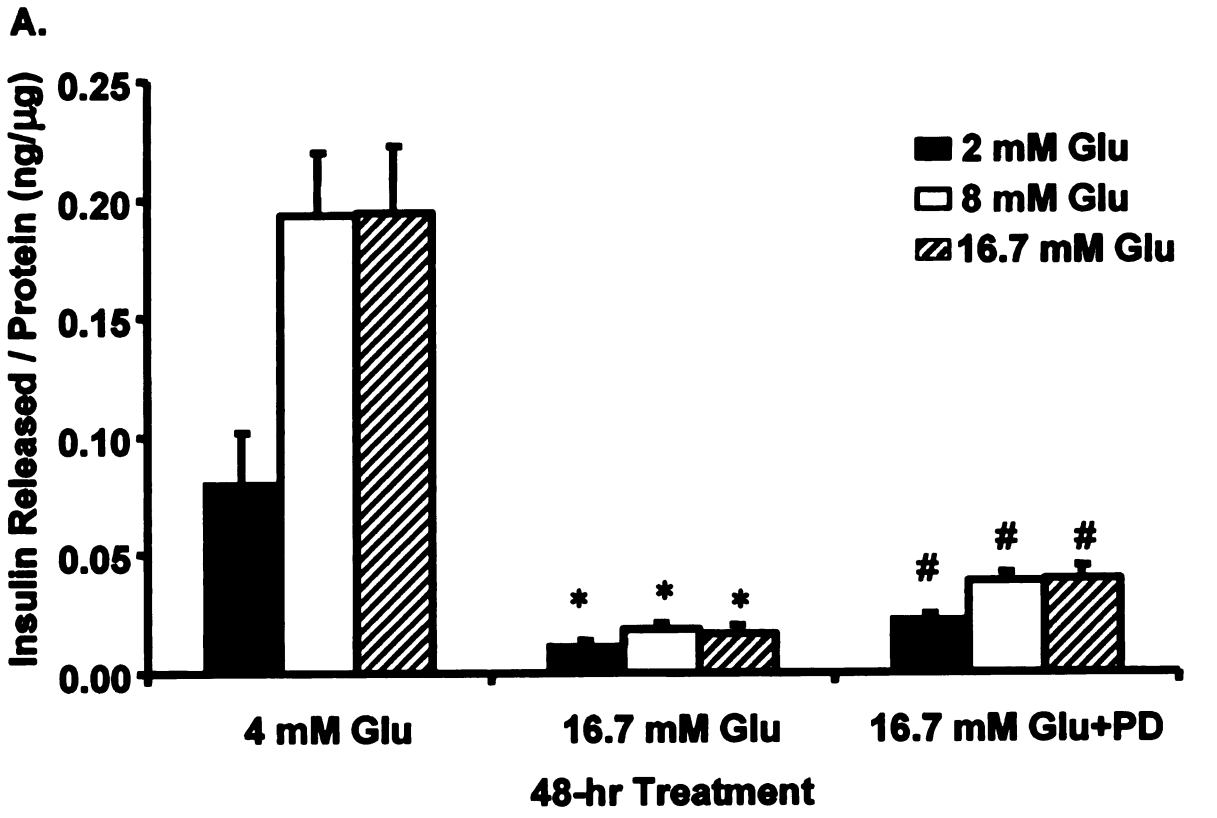
measured

16.7 mM G

Figure 16. Lp-PARPi improve basal and glucose-stimulated insulin secretion (GSIS) in INS-1 cells exposed to high glucose. INS-1 cells were treated with 4.0 mM glucose, 16.7 mM glucose, 16.7 mM glucose plus 500 μM PD (A), 10 mM NAM or 10 mM 3-AB (B) for 48 hrs. The culture medium was removed and cells were incubated for 1 hr with KRB buffer containing 2.0 mM glucose to make the cells quiescent. Cells were then challenged with KRB buffer containing 2.0 mM, 8.0 mM or 16.7 mM glucose for 30 min. The amount of insulin secreted was measured by ^{125}I-insulin RIA. Values are mean±SEM. Each value is the mean of four independent experiments with each sample measured in duplicates. * 4 mM Glu versus 16.7 mM Glu, # 16.7 mM Glu+PD versus 16.7 mM Glu, $p<0.05$.


Insulin Released (


A.
Insulin Released / Protein (ng/µg)
0.25
0.20
0.15
0.10
0.05
0.00
2 mM Glu
8 mM Glu
16.7 mM Glu
*
#
4 mM Glu
16.7 mM Glu
16.7 mM Glu+PD
48-hr Treatment

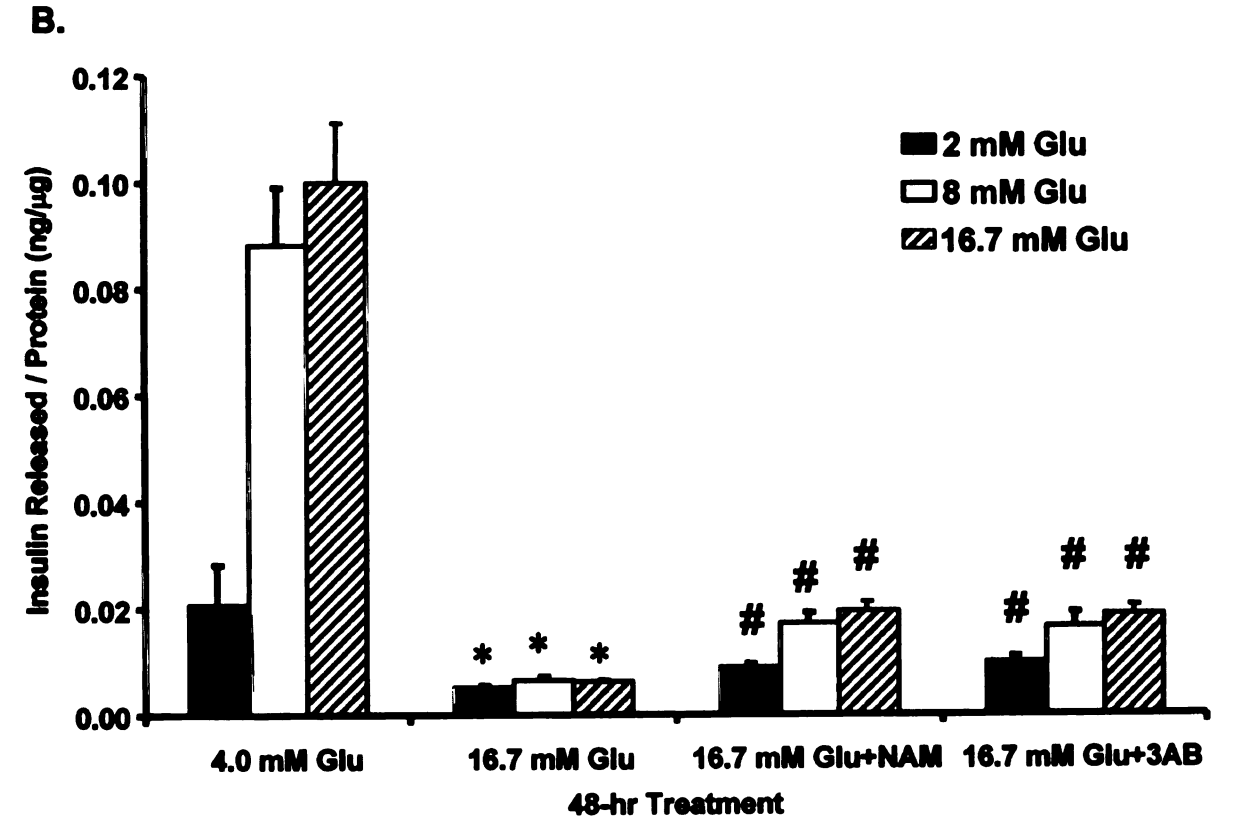

B.
Insulin Released / Protein (ng/µg)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
2 mM Glu
8 mM Glu
16.7 mM Glu
*
#
4.0 mM Glu
16.7 mM Glu
16.7 mM Glu+NAM
16.7 mM Glu+3AB
48-hr Treatment

Figure 17.

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glucose, 16.7 m

48 hrs. The c

buffer contain

challenged wit

Cellular insuli

by ^{125}I-insulin

insulin secret

value is the

duplicates. *

$p<0.05$.

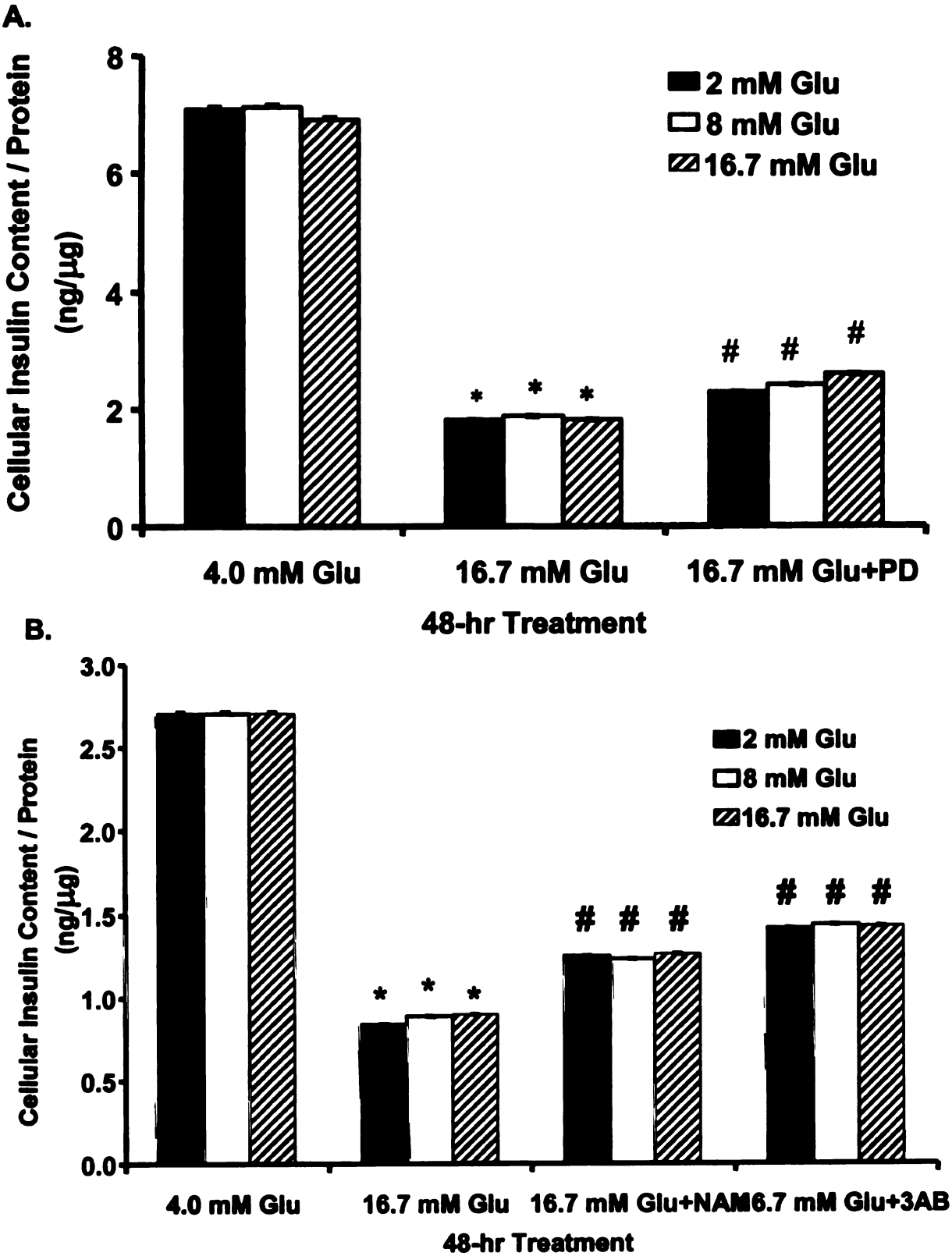
Figure 17. Lp-PARPi increase total cellular insulin content in INS-1 cells exposed to high glucose. INS-1 cells were treated with 4.0 mM glucose, 16.7 mM glucose, 16.7 mM glucose plus 500 μM PD (A), 10 mM NAM or 10 mM 3-AB (B) for 48 hrs. The culture medium was removed and cells were incubated for 1 hr with KRB buffer containing 2.0 mM glucose to make the cells quiescent. The cells were then challenged with KRB buffer containing 2.0 mM, 8.0 mM or 16.7 mM glucose for 30 min. Cellular insulin content was extracted in acidified-ethanol after GSIS and was measured by ^{125}I-insulin RIA. The total cellular insulin content was calculated by adding the insulin secreted and the leftover cellular insulin content. Values are mean±SEM. Each value is the mean of two independent experiments with each sample measured in duplicates. * 4 mM Glu versus 16.7 mM Glu, # 16.7 mM Glu+PD versus 16.7 mM Glu, $p<0.05$.

A.

Cellular Insulin Content / Protein

B.

Cellular Insulin Content / Protein


A.
Cellular Insulin Content / Protein (ng/μg)
8
6
4
2
0
2 mM Glu
8 mM Glu
16.7 mM Glu
*
*
*
#
#
#
4.0 mM Glu
16.7 mM Glu
16.7 mM Glu+PD
48-hr Treatment
B.
Cellular Insulin Content / Protein (ng/μg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2 mM Glu
8 mM Glu
16.7 mM Glu
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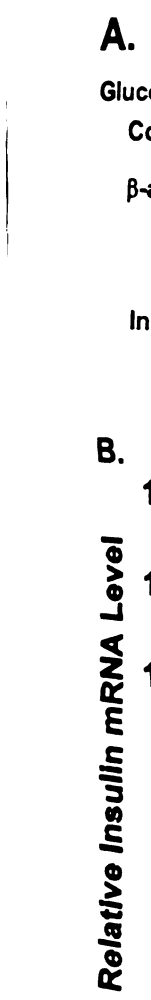
48-hr Treatment



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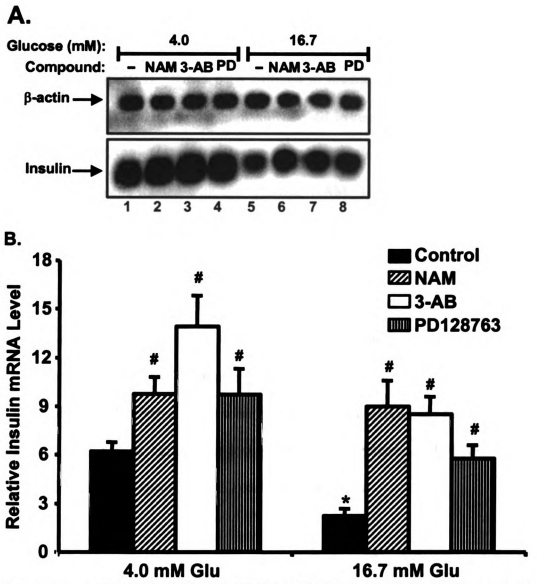

Figure 18. Lp-PARPi induce insulin mRNA levels. INS-1 cells were cultured with 4.0 mM or 16.7 mM glucose in the absence or presence of 10 mM NAM, 10 mM 3-AB, or 500 μM PD128763 for 48 hrs. Insulin and β-actin mRNA levels were measured by Northern blot analyses. A) A representative Northern blot. B) Summary of quantified data for insulin mRNA level (n=8). Values are mean ± SEM. * 4.0 mM Glu versus 16.7 mM Glu, and # control versus lp-PARPi, $p<0.05$.

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containing different lp-PARPi under low or high glucose. High glucose caused a significant decrease (~80%) in INS(-327)CAT expression compared to cells cultured in low glucose (Fig. 19A). INS-1 cells cultured in medium containing 10 mM NAM, 10 mM 3-AB or 500 μM PD under high glucose led to respectively 7.0-, 8.7-, or 7.1-fold increase in INS(-327)CAT expression compared to cells cultured in high glucose alone (Fig. 19A). In cells treated with low glucose, NAM, 3-AB or PD caused 2.7-, 2.0-, or 1.5-fold increase of promoter activity, respectively (Fig. 19A). These data demonstrated that under high glucose, lp-PARPi were able to stimulate the insulin promoter activity back to the level as observed for cells cultured in low glucose.

To test whether the lp-PARPi effect was specific to insulin promoter, control experiments with pTKCAT reporter gene (thymidine kinase promoter controlling the expression of CAT reporter gene) were performed. The expression of pTKCAT was not altered in INS-1 cells cultured in low glucose, high glucose or lp-PARPi (Fig. 19B). Thus, lp-PARPi have specific effects on insulin promoter.

5. Potent PARP-1 inhibitors do not stimulate insulin promoter activity.

PARP-1 participates in a number of cellular functions including DNA repair, cell cycle regulation, cell death, chromosomal stability, and gene expression (186). The *ability* of lp-PARPi to induce insulin gene expression suggested the possible involvement of PARP-1 in regulation of insulin promoter activity. Thus, two potent PARP-1 inhibitors-PJ34 and INO-1001 were tested for their abilities to stimulate insulin promoter activity. PJ34 is a phenanthridinone with IC_{50} of 20 nM for inhibition of PARP-1 *in vitro*

Figure

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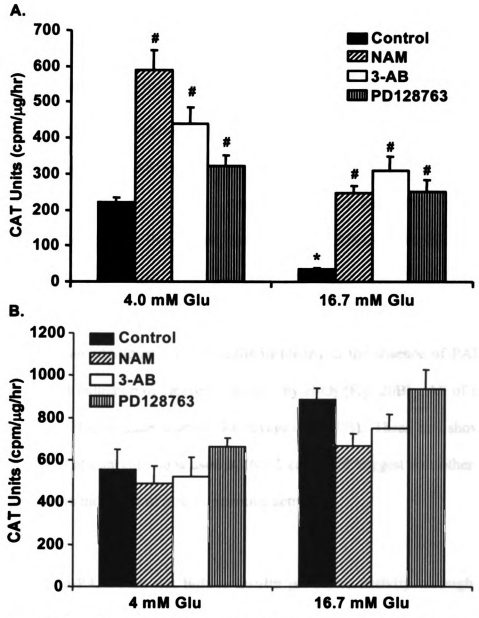

Figure 19. Lp-PARPi stimulate insulin promoter activity. INS-1 cells were transfected with INS(-327)CAT (A) (n=6) or pTKCAT (B) (n=3) and then treated with 4.0 mM or 16.7 mM glucose in the absence or presence of 10 mM NAM, 10 mM 3-AB, or 500 μM PD128763 for 48 hrs. Cells were harvested, and CAT assays performed. Values are mean ± SEM. * 4.0 mM Glu versus 16.7 mM Glu, and # control versus lp-PARPi, $p<0.05$.

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(254). INO-1001 is an isoindolinone derivative with an IC_{50} of 3 nM for inhibition of purified PARP-1 *in vitro* (255). PJ34 or INO-1001 did not stimulate insulin promoter activity (Fig. 20A). These data indicate that lp-PARPi may induce insulin promoter activity in a PARP-1 independent manner.

PARP-1 activation has been implicated in the initiation of apoptosis and caspases activation, and lp-PARPi and potent PARP-1 inhibitors (PARPi) are known to attenuate apoptosis (198). To determine whether PJ34 and INO-1001 actually functioned as PARP-1 inhibitors in INS-1 cells, the ability of PARPi to inhibit H_2O_2-induced caspase-9 cleavage was measured. INS-1 cells were pre-treated with 10 mM NAM, 10 mM 3-AB, 500 μM PD, 5 μM PJ34 or 3 μM INO-1001 for 1.5 hr, and then challenged with 1 mM H_2O_2 for an additional 1 hr. In cells incubated in the absence of PARPi, there was a significant induction of cleaved caspase-9 by H_2O_2 (Fig. 20B). All of the PARPi tested prevented H_2O_2-induced caspase-9 cleavage (Fig. 20B). These data show that PARPi are functional at concentrations used in INS-1 cells, and suggest that other mechanisms are involved in induction of insulin promoter activity.

6. Lp-PARPi do not induce insulin promoter activity through an antioxidant mechanism.

Chronic hyperglycemia mediated oxidative stress has been shown to suppress insulin gene expression (112, 115). Glucose-mediated loss of PDX-1 and MafA protein levels and binding activities to insulin promoter can be prevented by antioxidants such as N-acetyl-L-cysteine (NAC) (112, 115). NAM and 3-AB have been shown to act as

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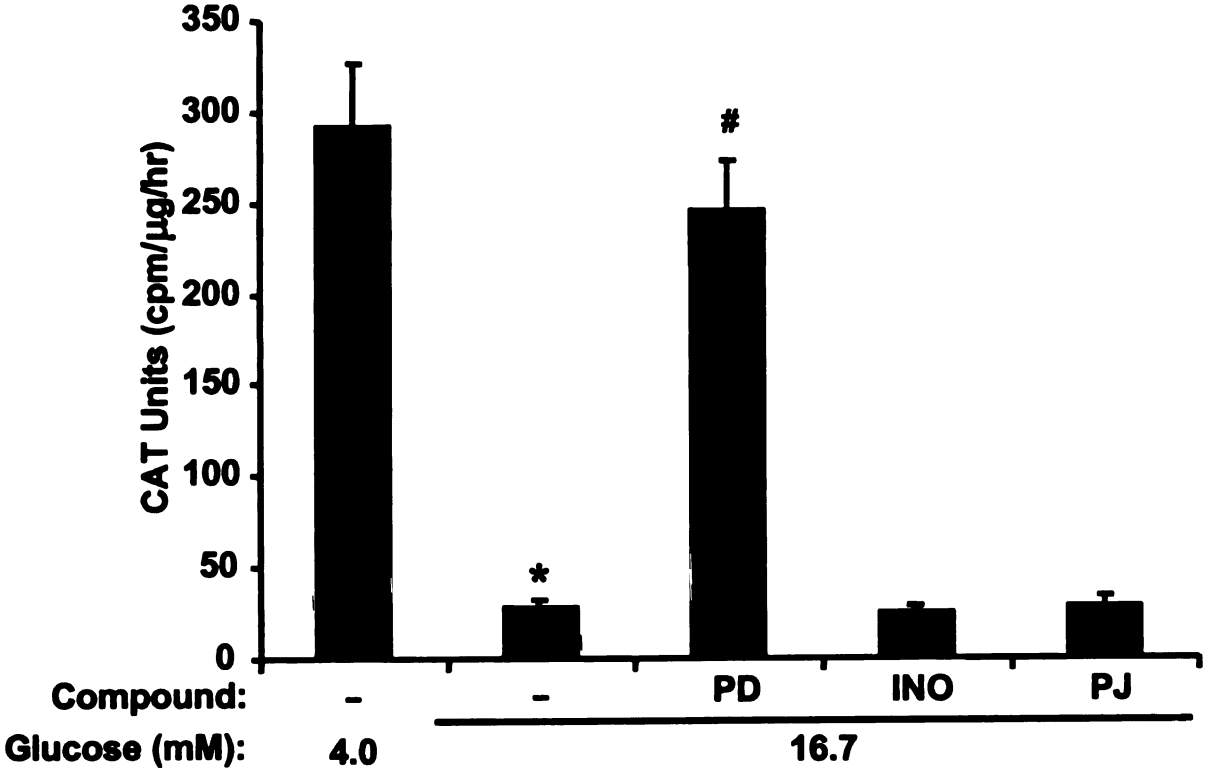
Figure 20. Potent PARP-1 inhibitors do not affect insulin promoter activity. A) INS-1 cells were transfected with INS(-327)CAT, and then treated with 4.0 mM or 16.7 mM glucose in the presence or absence of 500 μM PD, 3 μM INO-1001 (INO), or 5 μM PJ34 (PJ). After two days treatment, cells were harvested and CAT assays performed. Values are mean ± SEM. (n=5-6). * 4.0 mM Glu versus 16.7 mM Glu, and # 16.7 mM Glu versus 16.7 mM Glu plus compound, $p<0.05$. B) INS-1 cells were pre-treated with 10 mM NAM, 10 mM 3-AB, 500 μM PD, 5 μM PJ34, or 3 μM INO-1001 for 1.5 hr. After the cells were challenged with 1 mM H_2O_2 for an additional 1 hr, the cells were harvested and caspase-9 cleavage was measured by Western blot analysis. Shown here is a representative blot of three independent experiments.

A.


350
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CAT Units (cpm/µg/hr)
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Compound:
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PD
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Glucose (mM):
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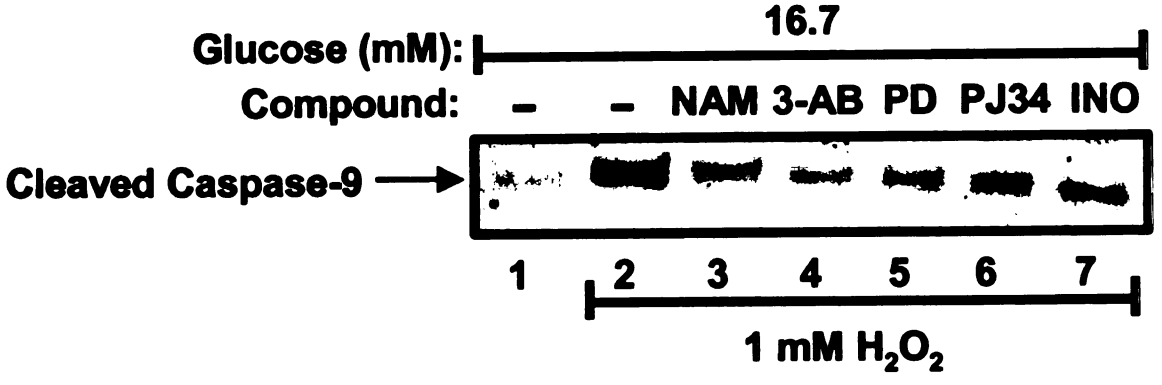
B.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antioxidants and prevent against oxidant-induced tissue injury (286, 287). Thus, several antioxidants were tested for their ability to stimulate insulin promoter activity. INS-1 cells cultured with high glucose medium containing 10 μM quercetin (Q), 1 mM α-lipoic acid (LA) or 10 mM NAC stimulated insulin promoter by 2.1-, 1.4-, and 2.4- fold, respectively (Fig. 21A). None of these antioxidants induced insulin promoter activity to the level observed for PD (10.2-fold) (Fig. 21A). These data suggest that lp-PARPi may induce insulin promoter through pathways independent of an antioxidant mechanism.

To confirm that the concentrations of antioxidants used were sufficient to prevent oxidative stress induced by oxidants, ROS generation was measured in cells challenged with H_2O_2. INS-1 cells were pre-treated with 10 mM NAC, 1 mM LA, 10 μM Q, 10 mM NAM, 10 mM 3-AB or 500 μM PD for 1.5 hr, and then loaded with dihydrodichlorofluorecein (H_2DCF). Cells were subsequently challenged with 1 mM H_2O_2. ROS production was significantly increased in cells treated without lp-PARPi or antioxidants (Fig. 21B). NAC and Q effectively prevented ROS accumulation in the cells (Fig. 21B). LA, NAM, 3-AB and PD did not significantly decrease ROS levels (Fig. 21B). These data demonstrate that lp-PARPi are not efficient antioxidants in INS-1 cells, and lp-PARPi-induction of insulin promoter is independent of antioxidant mechanisms.

7. Insulin promoter sequences between -201 bp to -114 bp mediate the majority of lp-PARPi-induction of insulin promoter activity.

Insulin promoter activity is regulated by several cis-regulatory elements. Deletion or site-directed mutation of these elements can cause reduced insulin promoter activity

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Figure 21. Lp-PARPi do not induce insulin promoter activity through an antioxidant mechanism. A) INS-1 cells were transfected with INS(-327)CAT, and then treated with 4.0 mM or 16.7 mM glucose in the presence or absence of 500 μM PD, 10 μM quercetin (Q), 1 mM α-lipoic acid (LA), or 10 mM N-acetyl cysteine (NAC). After two days treatment, cells were harvested and CAT assays performed. Values are mean ± SEM. (n=4-12). * 4.0 mM Glu versus 16.7 mM Glu, and # 16.7 mM Glu versus 16.7 mM Glu plus compound, $p<0.05$. B) INS-1 cells were pre-treated with 10 mM NAM, 10 mM 3-AB, 500 μM PD, 10 mM NAC, 10 μM quercetin, or 1 mM LA for 1.5 hr. Cells were then loaded with 50 μM dihydrodichlorofluorecein (H_2DCF), and challenged with 1 mM H_2O_2 in the presence or absence of antioxidants or PARPi. Fluorescent DCF, an indicator of ROS, was detected with the excitation and emission wavelengths of 485 mm and 530 nm 40 min after H_2O_2 treatment. Values are mean ± SEM. (n=2-4). * 16.7 mM Glu versus 16.7 mM Glu plus H_2O_2, and # 16.7 mM Glu plus H_2O_2 versus 16.7 mM Glu plus compound and H_2O_2, $p<0.05$.

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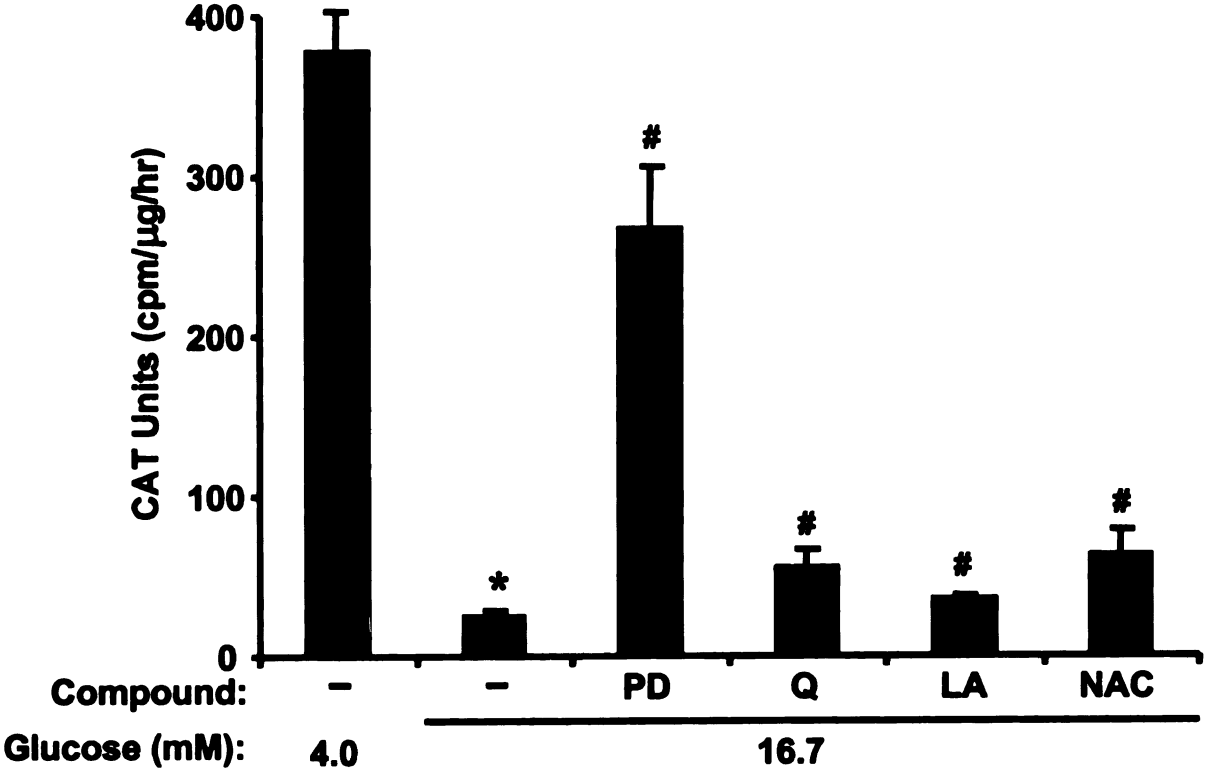
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CAT Units (cpm/µg/hr)
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Compound:
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NAC
Glucose (mM):
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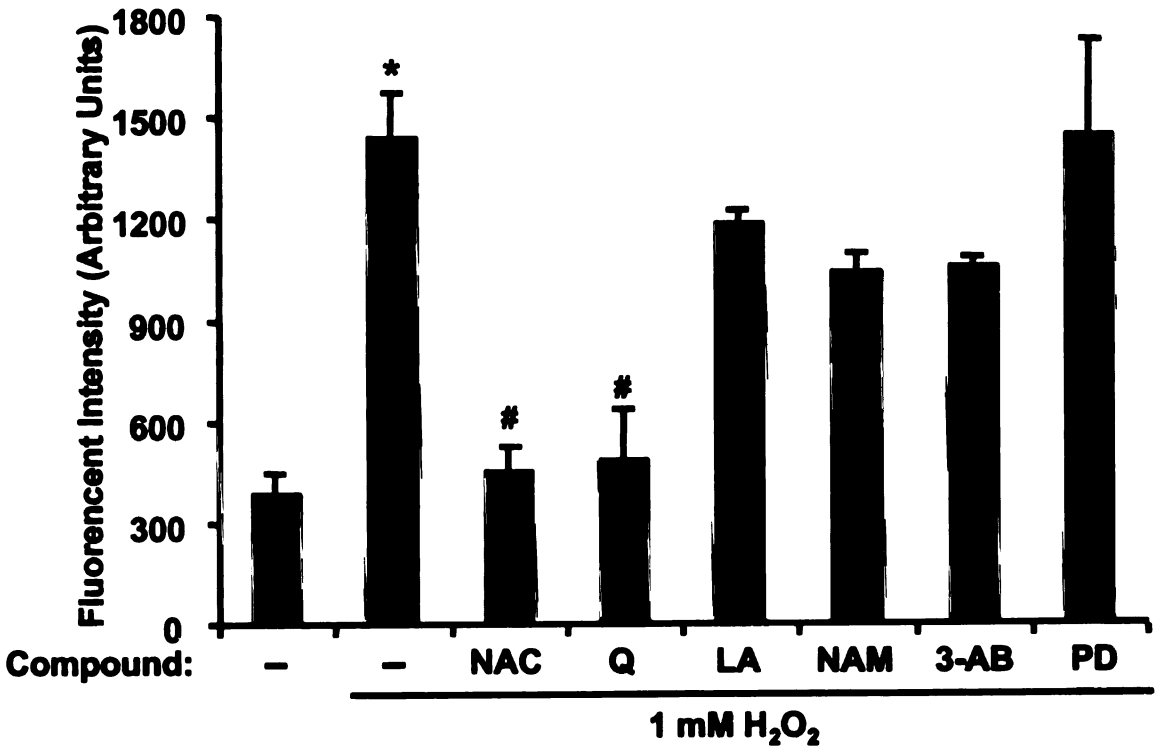
1800
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Fluorencent Intensity (Arbitrary Units)
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1 mM H_2O_2

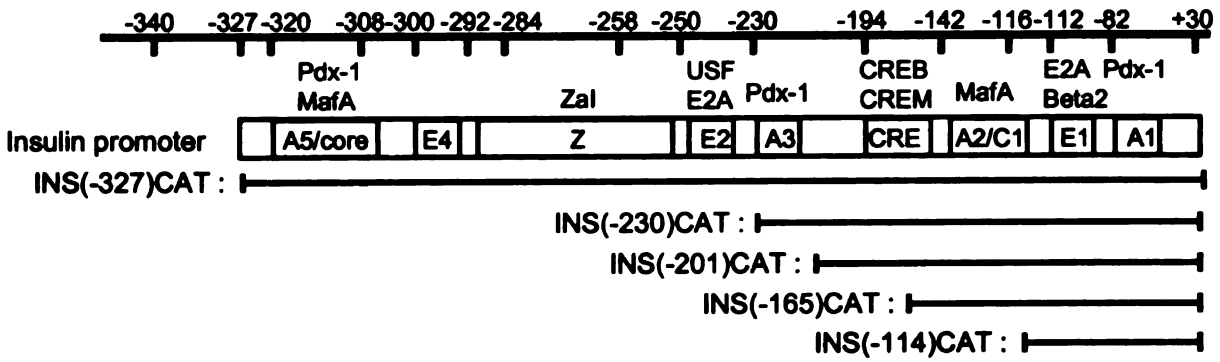
(288). To identify the cis-element(s) regulated by lp-PARPi, a series of reporter genes driven by truncated insulin promoters, INS(-230)CAT, INS(-201)CAT, INS(-165)CAT, and INS(-114)CAT were generated (Fig. 22A). Serial deletion showed a gradual loss of lp-PARPi stimulatory effect on the insulin promoter (Fig. 22B). Removal of insulin promoter sequences between –327 bp to –201 bp did not significantly affect the ability of 3-AB to stimulate insulin promoter activity but significantly reduced PD induction of insulin promoter activity (Fig. 22B). These data indicate that the sequences between –327 bp to –201 bp may mediate some of PD induction of insulin promoter activity. Removal of insulin promoter sequences from –201 bp to –165 bp led to an average of 58.4% decrease in the ability of lp-PARPi to stimulate promoter activity compared to cells transfected with INS(-327)CAT (Fig. 22B). These findings suggest that elements between –201 and –165 bp also mediate lp-PARPi-induction of insulin promoter activity. Further deletion down to –144 bp, which removed the A2C1 elements, led to an average of 70.6% loss of the lp-PARPi-induction of the insulin promoter. In cells treated with 3-AB, the expression of INS(-114)CAT is significantly lower than the expression of INS(-165)CAT. Overall, these data suggest that the cis-elements between –201 bp to –114 bp mediate the majority of lp-PARPi stimulatory effect on the insulin promoter.

8. C1 and CRE elements are involved in lp-PARPi-induction of insulin promoter activity.

Serial deletion analyses of the insulin promoter suggest that lp-PARPi affect regulatory elements located between –201 bp to –114 bp, which contain several potential targets including the CRE and C1 elements. Since the A1 and A3 are also strong

Figure 22. Insulin promoter sequences between –201 bp to -114 bp mediate the majority of lp-PARPi induction of insulin promoter activity. A) Schematic representation of human insulin promoter and serial deletions of human insulin promoter used. B) INS-1 cells were transfected with INS(-327)CAT, INS(-230)CAT, INS(-201)CAT, INS(-165)CAT, or INS(-114)CAT (n=4). The cells were then treated with 16.7 mM Glu, 16.7 mM Glu plus 10 mM 3-AB, or 16.7 mM Glu plus 500 μM PD for 48 hrs. Cells were harvested, and CAT assays performed. Values are mean ± SEM. * 16.7 mM Glu plus PD of truncated insulin promoter CAT reporter gene versus INS(-327)CAT control, # 16.7 mM Glu plus 3-AB of truncated insulin promoter CAT reporter gene versus INS(-327)CAT control, + 16.7 mM Glu plus 3-AB: INS(-114)CAT versus INS(-165)CAT, $p<0.05$.

A.


-340 -327 -320 -308 -300 -292 -284 -258 -250 -230 -194 -142 -116 -112 -82 +30
Pdx-1
MafA
Zal
USF
E2A
Pdx-1
CREB
CREM
MafA
E2A
Beta2
Pdx-1
Insulin promoter
A5/core
E4
Z
E2
A3
CRE
A2/C1
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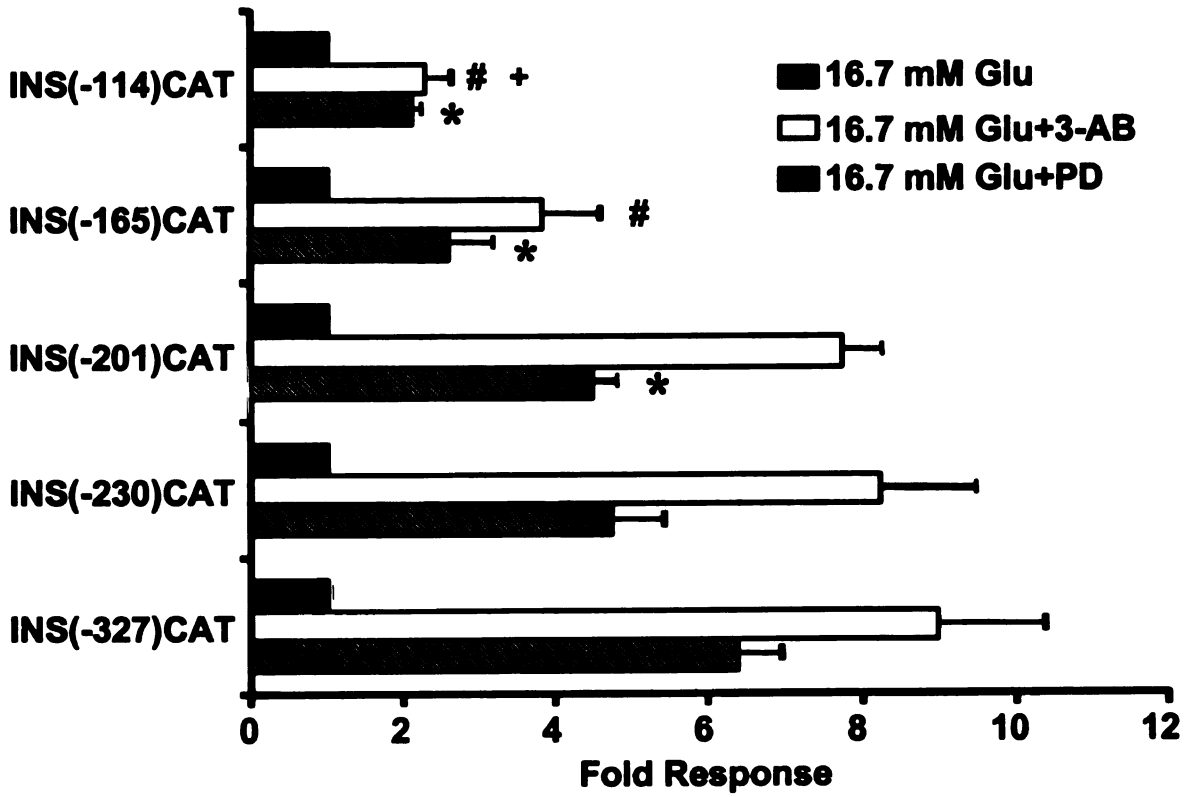
INS(-327)CAT :
INS(-230)CAT :
INS(-201)CAT :
INS(-165)CAT :
INS(-114)CAT :

B.


INS(-114)CAT
INS(-165)CAT
INS(-201)CAT
INS(-230)CAT
INS(-327)CAT
16.7 mM Glu
16.7 mM Glu+3-AB
16.7 mM Glu+PD
0
2
4
6
8
10
12
Fold Response

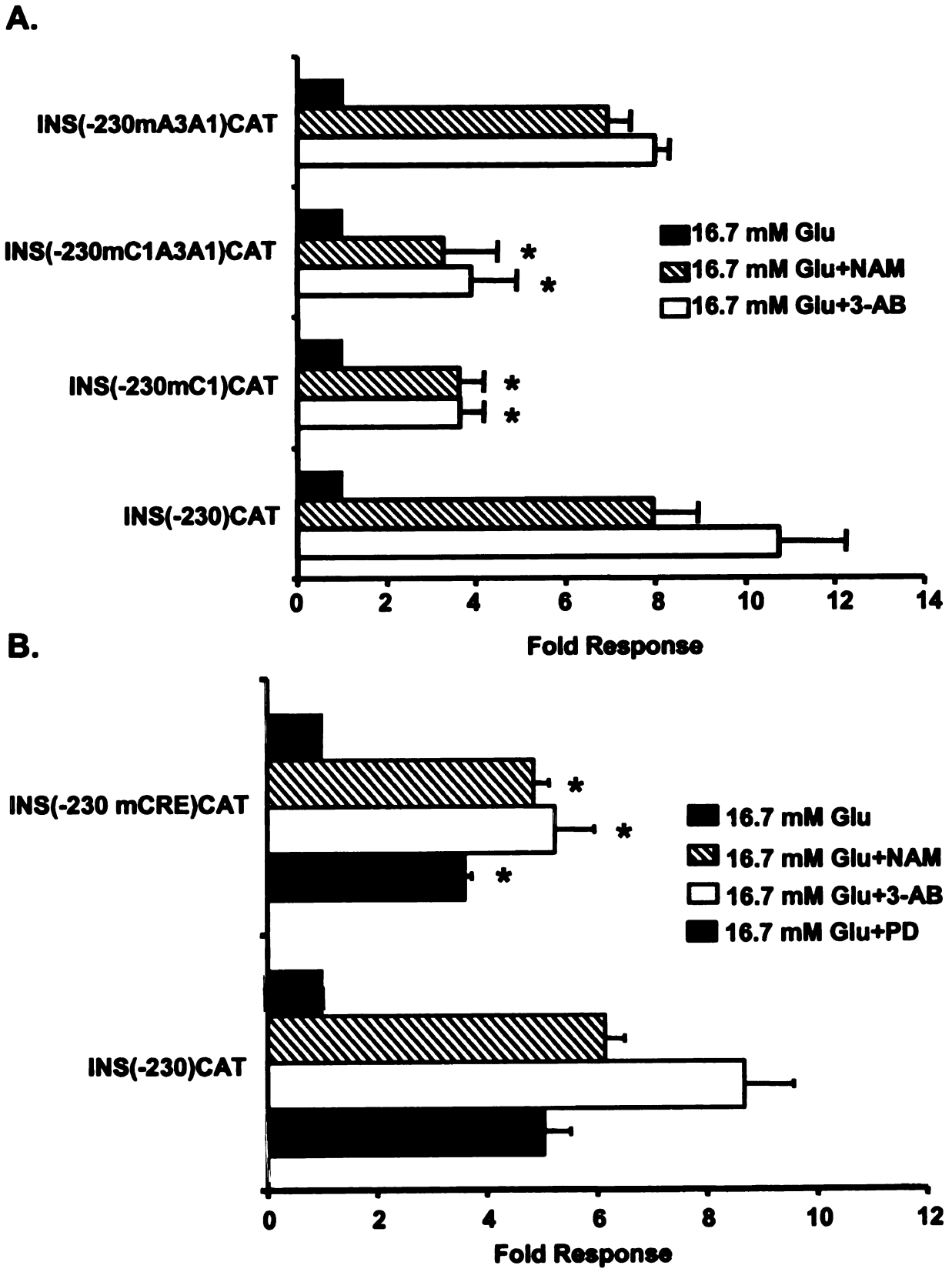
regulatory elements of insulin promoter, the possible roles of them in mediating lp-PARPi induction of insulin promoter were also examined. Reporter genes containing site-specific mutations of the A3, CRE, C1 and A1 elements were tested for lp-PARPi regulation in INS-1 cells. Mutations of these elements were done in the context of -230 insulin promoter since this region contained A3, C1 and A1, which have been known to mediate glucose-suppression of insulin promoter activity (17-21). Mutations of the A1 and A3 elements did not significantly decrease insulin promoter activity when INS-1 cells were treated with NAM or 3-AB (Fig. 23A). In contrast, mutations of the C1 element showed a 54.3% and 66% decrease of NAM- and 3-AB-induced insulin promoter activity, respectively (Fig. 23A). Combined mutation of the A1, A3, and C1 showed a similar reduction in NAM- or 3-AB-stimulated insulin promoter activity (Fig. 23A). These data suggest that the C1 element is involved in lp-PARPi induction of insulin promoter activity.

To determine whether CRE located between –201 bp to –165 bp also plays a role in lp-PARPi induction, a reporter gene containing a mutated CRE was examined. Mutation of the CRE reduced NAM, 3-AB and PD induction of promoter activity by 20.7%, 39.5%, or 28.8%, respectively (Fig. 23B). Overall, these data suggest that CRE may also mediate part of lp-PARPi-induction of insulin promoter activity.

9. Lp-PARPi enhance the C1 binding complex formation.

In human islets and several cultured β-cell lines, incubation at high glucose for a long period of time reduced insulin gene transcription, and this was associated with decreased binding of PDX-1 (17, 18, 21) and MafA (Fig. 10 and 13) to insulin promoter.

Figure 23. Mutational analyses show that the C1 and CRE elements are involved in lp-PARPi-induction of insulin promoter activity. A) INS-1 cells were transfected with INS(-230)CAT, INS(-230mC1)CAT, INS(-230mA3A1)CAT, or INS(-230mC1A3A1)CAT (n=4). Cells were then treated with 16.7 mM glucose in the presence or absence of 10 mM NAM or 10 mM 3-AB. B) INS-1 cells were transfected with INS(-230)CAT, or INS(-230mCRE)CAT (n=8). Cells were then treated with 16.7 mM glucose in the presence or absence of 10 mM NAM, 10 mM 3-AB or 500 μM PD. For both experiments, cells were harvested after 48 hrs and CAT assays performed. Fold response was calculated between cells treated with lp-PARPi to 16.7 mM for each vector. Values are mean ± SEM. * 16.7 mM Glu plus lp-PARPi of mutated insulin promoter versus their respective INS(-230)CAT control, $p<0.05$.


A.
INS(-230mA3A1)CAT
INS(-230mC1A3A1)CAT
INS(-230mC1)CAT
INS(-230)CAT
16.7 mM Glu
16.7 mM Glu+NAM
16.7 mM Glu+3-AB
*
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2
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6
8
10
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14
Fold Response
B.
INS(-230 mCRE)CAT
INS(-230)CAT
16.7 mM Glu
16.7 mM Glu+NAM
16.7 mM Glu+3-AB
16.7 mM Glu+PD
0
2
4
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8
10
12
Fold Response

Both PDX-1 and MafA play essential roles in stimulating insulin promoter activity (26, 27, 35, 37). To test whether lp-PARPi stimulate insulin promoter by enhancing MafA or PDX-1 binding to insulin promoter, EMSA were performed with nuclear extracts isolated from INS-1 cells cultured in low or high glucose in the presence or absence of 10 mM 3-AB or 5 μM PJ34. Chronic hyperglycemia led to decreased formation of the C1 binding complex in INS-1 cells cultured in high glucose (Fig. 24A lanes 3 and 8). Incubating cells with 3-AB enhanced the C1 complex formation under elevated glucose (Fig. 24A lane 10). PJ34 did not increase the C1 binding complex formation under low or high glucose treatment (Fig. 24A lanes 11 and 12). Antibodies against MafA caused a supershift of the C1 binding complex, indicating that this complex contained MafA (Fig. 24A lanes 4 and 5). The A3 binding complex formation was not modulated by lp-PARPi or PJ34 (Fig. 24B lanes 4-7). Antibodies against PDX-1 caused a supershift of the A3 binding complex, indicating that this complex contained PDX-1 (Fig. 24B lane 8). These data clearly indicate that lp-PARPi increase MafA/C1 binding complex formation at the insulin promoter in cells cultured with elevated glucose.

10. Lp-PARPi stimulate the distal insulin promoter activity through the increased MafA binding to the A5/core elements.

Since the A5/core elements are also binding sites for MafA (Fig. 14), the effects of lp-PARPi on the A5/core binding complex formation was tested by EMSA. High glucose decreased the A5/core binding complex formation compared to nuclear extracts from cells cultured in low glucose (Fig. 25A, lanes 2 and 3). In nuclear extracts derived from INS-1 cells treated with 3-AB, the binding complex formation was restored under

Figure 24. Lp-PARPi enhance the C1 binding complex formation. INS-1 cells were treated with 4.0 mM or 16.7 mM glucose in the presence or absence of 10 mM 3-AB or 5 μM PJ34 for two days. Nuclear extracts were prepared and EMSA were performed. [α-^{32}P]-dCTP-labeled oligodeoxynucleotide probes to rat insulin 2 promoter A2C1 elements (A) or human A3 element (B) were incubated with 20 μg nuclear extracts at room temperature with or without anti-MafA (A) or PDX-1 (B) antibodies for 30 min. Shown here is a representative gel of three independent experiments. (FP=free probe)

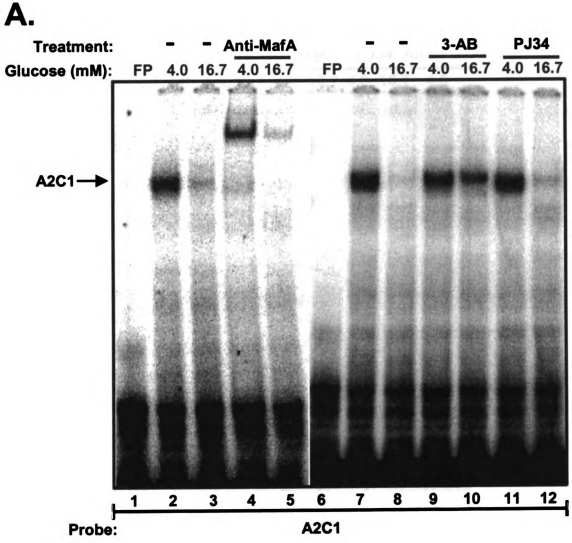
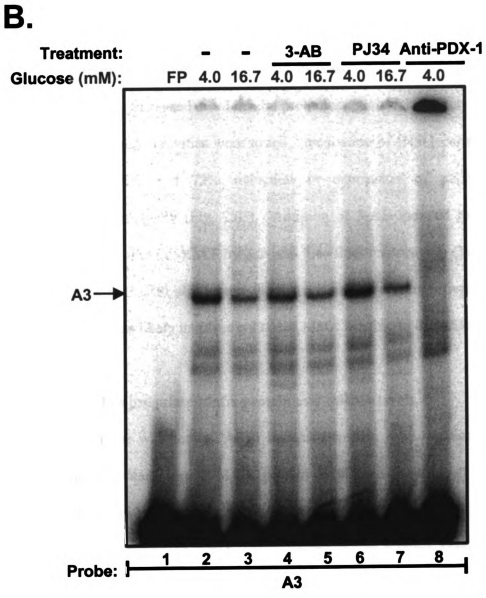

A.
Treatment: - - Anti-MafA - - 3-AB PJ34
Glucose (mM): FP 4.0 16.7 4.0 16.7 FP 4.0 16.7 4.0 16.7 4.0 16.7
A2C1
1 2 3 4 5 6 7 8 9 10 11 12
Probe: A2C1

Figure 24 (cont'd)



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high glucose (Fig. 25A, lane 5). In contrast, PJ34 was unable to increase the binding to the A5/core (Fig. 25A, lane 7). These data demonstrate that lp-PARPi restore MafA binding to the A5/core elements.

To determine whether the lp-PARPi-increased MafA binding to the A5/core elements will lead to increased distal insulin promoter activity, the effects of lp-PARPi on X and Y minienhancer activities were tested. Incubation of INS-1 cells with 16.7 mM glucose led to 32% and 72% reduction in expression of pFox(X5)CAT and pFox(Y5)CAT, respectively (Fig. 25B). Addition of 3-AB induced the expression of pFox(X5)CAT and pFox(Y5)CAT by 3.5- and 5.4- fold, respectively (Fig. 25B). These data suggest that lp-PARPi also restore the distal insulin promoter activity suppressed by high glucose, and this likely involves enhanced MafA binding to the A5/core elements.

11. Lp-PARPi upregulate MafA protein and mRNA levels.

To determine whether lp-PARPi increased insulin gene expression through the increased MafA protein levels, MafA protein levels were measured in INS-1 cells cultured for 48 hrs in low or high glucose in the presence or absence of 10 mM 3-AB or 5 μM PJ34. Elevated glucose significantly downregulated nuclear MafA protein levels (Fig. 26A, lanes 1 and 4). Addition of 3-AB significantly increased nuclear MafA protein levels (Fig. 26A, lane 5). In contrast, PJ34 failed to increase nuclear MafA protein levels (Fig. 26A, lane 6). Similar results were observed for the whole cell lysates (Fig. 26A). In INS-1 cells incubated for 48 hrs in low glucose, 3-AB or PJ34 did not affect MafA protein levels (Fig. 26A, lanes 1-3). Elevated glucose led to a reduction of

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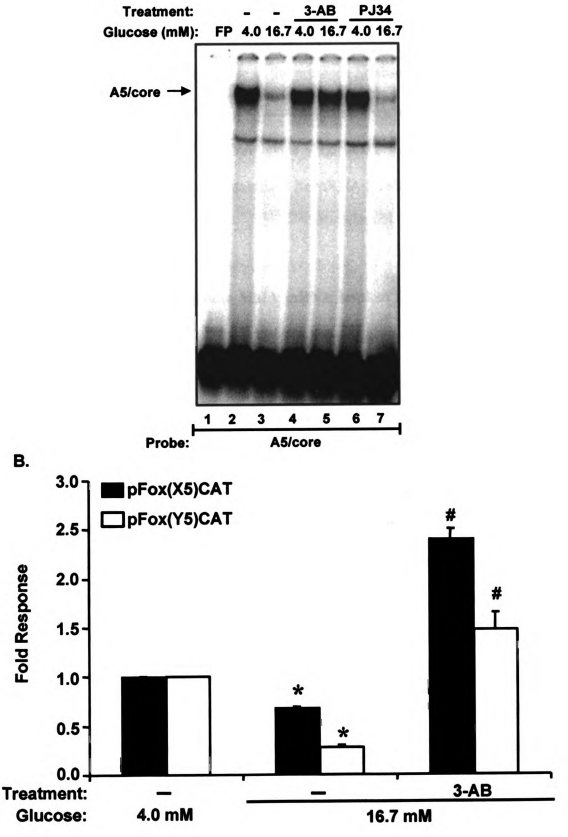
Figure 25. Lp-PARPi enhance the A5/core/MafA binding complex formation at the upstream of insulin promoter. A) INS-1 cells were treated with 4.0 mM or 16.7 mM glucose with or without 10 mM 3-AB or 5 μM PJ34 for 48 hrs. The cells were then harvested and nuclear proteins were isolated for EMSA using ^{32}P-labeled A5/core. Shown here is a representative gel of three independent experiments. (FP=free probe) B) INS-1 cells were transfected with pFox(X5)CAT or pFox(Y5)CAT. The cells were then incubated in medium containing 4.0 mM glucose, or 16.7 mM glucose in the presence or absence of 10 mM 3-AB. After 48-hr treatment, the cells were harvested and CAT assays performed. Values are mean ± SEM. * 4.0 mM Glu versus 16.7 mM Glu, and # 16.7 mM Glu versus 16.7 mM Glu plus 3-AB, $p<0.05$. (N=4).


A.
Treatment: - - 3-AB PJ34
Glucose (mM): FP 4.0 16.7 4.0 16.7 4.0 16.7
A5/core
1 2 3 4 5 6 7
Probe: A5/core
B.
pFox(X5)CAT
pFox(Y5)CAT
Fold Response
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
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#
Treatment: - - 3-AB
Glucose: 4.0 mM 16.7 mM

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nuclear PDX-1 protein levels, and 3-AB or PJ34 did not affect nuclear PDX-1 protein levels (Fig. 26A, lanes 1, 4-6).

Next, effects of lp-PARPi on MafA mRNA levels were measured in INS-1 cells cultured for 48 hrs in low or high glucose in the presence or absence of 10 mM 3-AB or 500 μM PD. High glucose significantly reduced MafA mRNA levels (Fig. 26B). 3-AB and PD markedly induced MafA mRNA levels (Fig. 26B), indicating that lp-PARPi stimulated MafA gene transcription or stabilized its mRNA levels in INS-1 cells cultured with elevated glucose.

12. Lp-PARPi rapidly upregulate MafA mRNA level in INS-1 cells cultured in high glucose.

Time course analyses were performed to determine the kinetic relationship between the increased MafA protein levels and mRNA levels. INS-1 cells are normally cultured for two days in INS-1 cell medium containing 11.1 mM glucose to promote cell proliferation. When the cell density reached about 80% confluence, INS-1 cells were switched from medium containing 11.1 mM glucose to 4 mM glucose, 16.7 mM glucose, or 16.7 mM glucose plus 10 mM 3-AB. Nuclear MafA protein and MafA mRNA levels were then measured at 4, 8, 12, and 24 hr. The transition from 11.1 mM glucose to 4.0 mM glucose led to a gradual increase of MafA mRNA level (Fig. 27A and B). Nuclear MafA protein levels were slightly increased by 24-hr in cells cultured in 4.0 mM glucose (Fig. 27A). Nuclear MafA protein levels were reduced significantly after incubating INS-1 cells in high glucose for 24 hr (Fig. 27A, lanes 5 and 8). MafA mRNA levels were only decreased marginally (Fig. 27B). Addition of 3-AB to cells cultured in high glucose

Figure 26.

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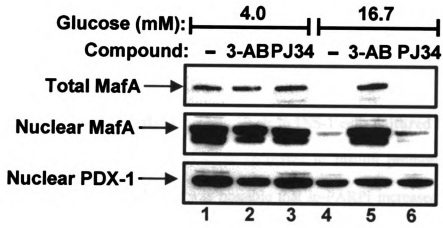
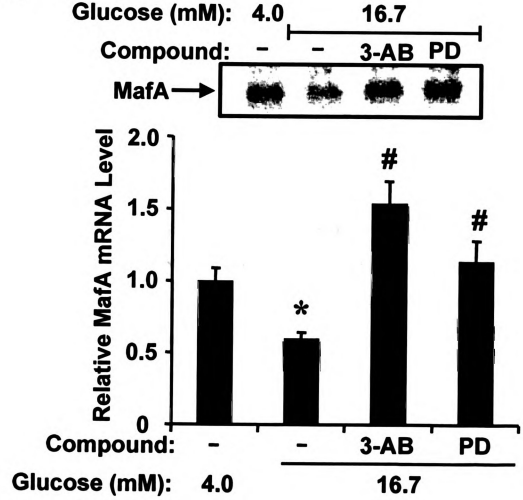
Figure 26. Lp-PARPi upregulate MafA protein and mRNA levels. A) INS-1 cells were cultured in 4.0 mM or 16.7 mM glucose in the presence or absence of 10 mM 3-AB or 5 μM PJ34 for two days. Nuclear extracts or whole cell lysates were prepared. Western blots were performed to detect MafA total protein and nuclear protein levels, or PDX-1 nuclear protein levels. Shown here is a representative blot of three independent experiments. B) INS-1 cells were incubated in 4.0 mM glucose or 16.7 mM glucose in the presence or absence of 10 mM 3-AB or 500 μM PD for two days. MafA and β-actin mRNA levels were measured by Northern blot analysis. Top panel is a representative blot of four separate experiments. Bottom panel is the summary of quantified data for MafA mRNA levels (n=4). Data were plotted as the fold change in MafA mRNA relative to β-actin mRNA and normalized to 4.0 mM glucose. Values are mean ± SEM. * 16.7 mM Glu versus 4.0 mM Glu, and # 16.7 mM Glu plus lp-PARPi versus 16.7 mM Glu, $p<0.05$.

A.


Glucose (mM): 4.0 16.7
Compound: – 3-AB PJ34 – 3-AB PJ34
Total MafA
Nuclear MafA
Nuclear PDX-1
1 2 3 4 5 6

B.


Glucose (mM): 4.0 16.7
Compound: – – 3-AB PD
MafA
Relative MafA mRNA Level
2.0
1.5
1.0
0.5
0
*
#
#
Compound: – – 3-AB PD
Glucose (mM): 4.0 16.7

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caused an increase of MafA mRNA levels by 4 hrs. This increase in MafA mRNA precedes an increase in MafA protein (8-hr) under high glucose (Fig. 27A and B, lanes 9 and 10). These data show that lp-PARPi upregulate MafA mRNA rapidly and this occurs prior to the increase of MafA protein in cells treated with high glucose.

13. Lp-PARPi increase MafA protein levels in INS-1 cells cultured in low glucose.

Since lp-PARPi also led to small increases in insulin promoter activity and mRNA levels (Fig. 18 and 19), it was possible that lp-PARPi increased MafA protein levels in INS-1 cells cultured in low glucose. Cells switched from 11.1 mM glucose to 4.0 mM glucose, there was a slight increase of MafA protein levels by 20-hr, and then a slight decrease by 36-hr (Fig. 27A and 28). Addition of 3-AB significantly increased MafA protein levels by 12-hr, and the MafA protein level again decreased slightly by 36-hr (Fig. 28). Nevertheless, these data indicate that lp-PARPi can stimulate MafA gene expression at low glucose concentration.

14. Lp-PARPi do not affect MafA protein stability.

Although MafA mRNA levels were induced rapidly by lp-PARPi, the increase seemed small compared to their stimulatory effects on MafA protein levels. Therefore, it is possible that lp-PARPi also increased MafA protein stability. MafA protein stability was examined in INS-1 cells treated with an inhibitor of protein synthesis, cycloheximide (CHX). MafA protein levels started to decrease at 4 hr after 40 μM CHX treatment, and addition of 3-AB did not prevent the CHX-induced loss of MafA (Fig. 29). Overall, these data show that lp-PARPi do not affect MafA protein stability.

Figure 27. **Lp-**

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analysis (n=3) an

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to 4.0 mM glucos

points, # 16.7 mM

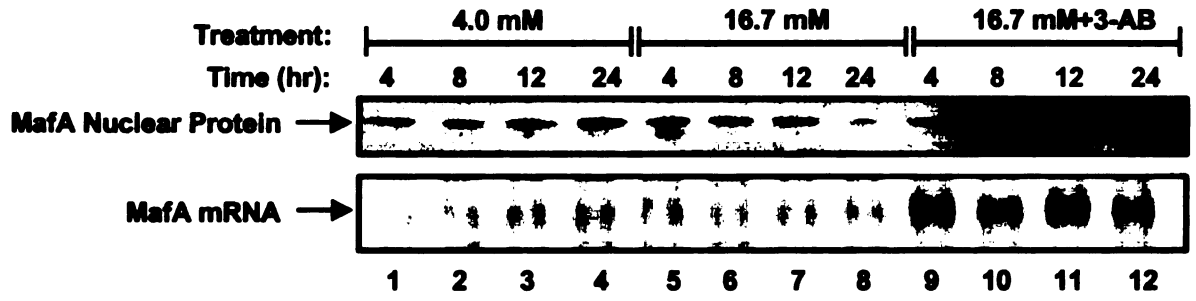
Figure 27. Lp-PARPi rapidly upregulate MafA gene expression in INS-1 cells treated with high glucose. A) INS-1 cells were treated with 4.0 mM glucose, 16.7 mM glucose or 16.7 mM glucose plus 10 mM 3-AB. The cells were harvested at 4, 8, 12, or 24 hr for MafA nuclear protein and mRNA levels, which were detected by Western blot analysis (n=3) and Northern blot analysis (n=4), respectively. Shown here are two representative blots. B) Summary of quantified data for MafA mRNA levels (n=4). Data were plot as the fold change in MafA mRNA relative to β-actin mRNA and normalized to 4.0 mM glucose. Values are mean ± SEM. * 4.0 mM Glu at 4 hr versus other time points, # 16.7 mM Glu plus 3-AB versus 16.7 mM Glu, $p<0.05$.

A

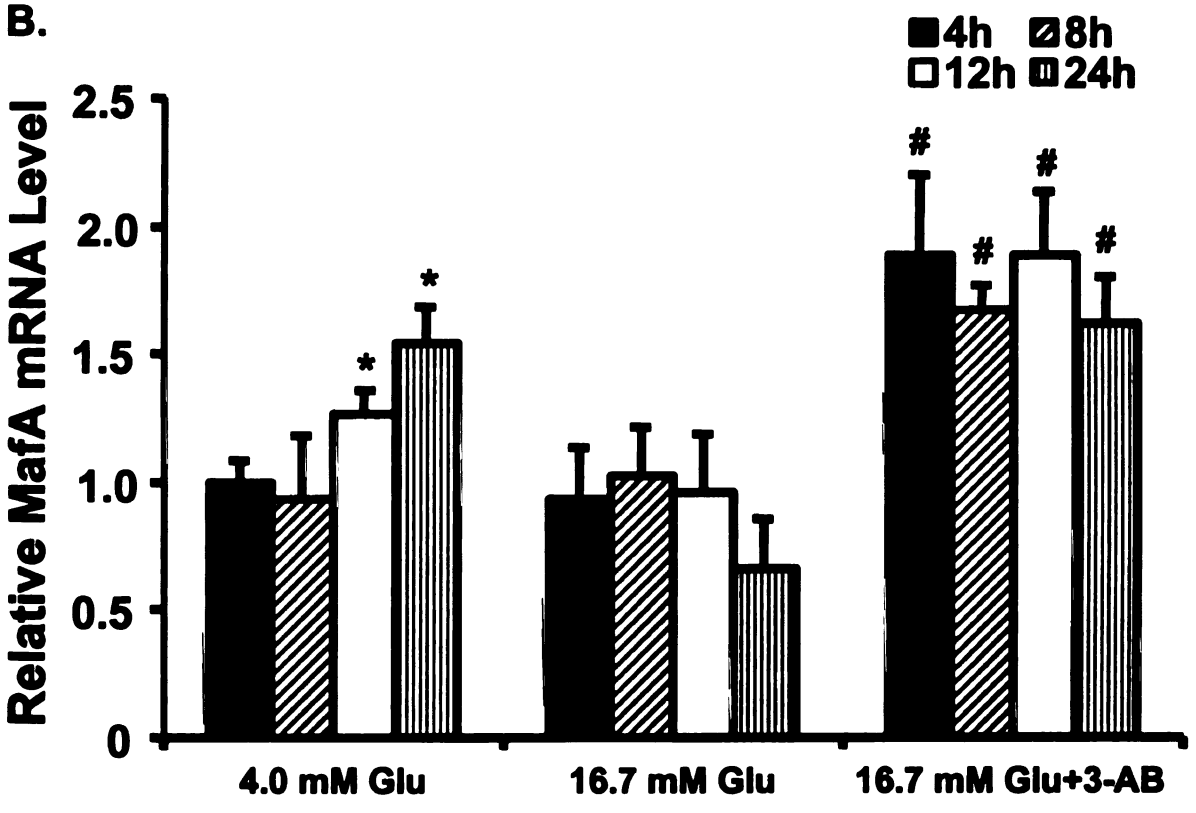
B

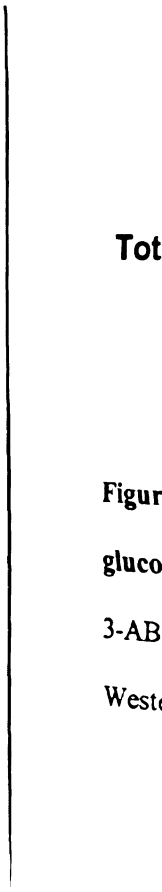
Relative MafA mRNA Level

A.


Treatment:
4.0 mM
16.7 mM
16.7 mM+3-AB
Time (hr):
4 8 12 24 4 8 12 24 4 8 12 24
MafA Nuclear Protein
MafA mRNA
1 2 3 4 5 6 7 8 9 10 11 12

B.


4h 8h
12h 24h
Relative MafA mRNA Level
2.5
2.0
1.5
1.0
0.5
0
*
*
#
#
#
#
4.0 mM Glu
16.7 mM Glu
16.7 mM Glu+3-AB



Figur

gluco

3-AB

West

Figure 28. Lp-PARPi increase MafA protein levels in INS-1 cells cultured in low glucose. INS-1 cells were treated with 4.0 mM glucose, or 4.0 mM glucose plus 10 mM 3-AB. The cells were harvested at 12, 20, 24, or 36 hr for total MafA protein levels by Western analyses. Shown here is a representative blot of two independent experiments.

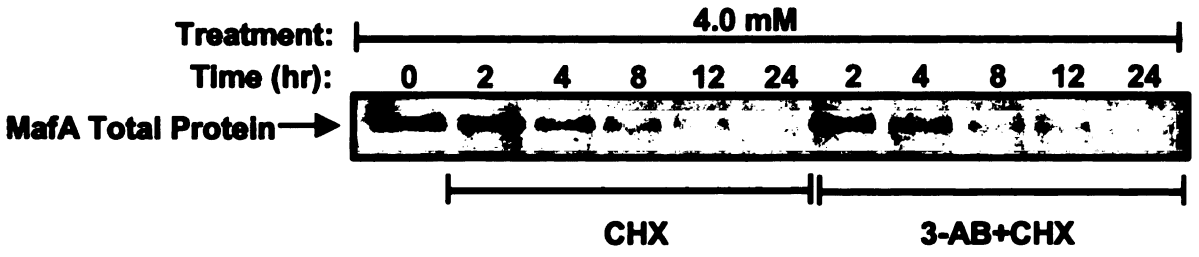


Figure 29. Lp-PARPi do not affect MafA protein stability. INS-1 cells were incubated in 4.0 mM glucose for 12 hrs. 40 μM cycloheximide (CHX) and/or 10 mM 3-AB were then added to the cells. Cells were harvested at the time point shown on the figure after 12-hr incubation with 4.0 mM glucose. Whole cell lysates were prepared. Western blots were performed to detect MafA protein levels. Shown here is a representative blot of two independent experiments.

Discussion

The present study showed that INS-1 cells cultured in elevated glucose for two days led to a significant reduction of insulin mRNA levels (Fig. 18). *In vitro* and *in vivo* studies from other laboratories have also shown that chronic hyperglycemia reduced insulin mRNA levels (17, 20, 21, 103). Lp-PARPi attenuated glucose-suppression of insulin mRNA levels in INS-1 cells, and this was due to increased insulin promoter activity by lp-PARPi. Deletion study indicated that the sequences between –201 bp to –114 bp mediated the majority of lp-PARPi induction of insulin promoter activity (Fig. 22). Within the sequences between –201 bp to –114 bp, the A1 and C1 elements are known to mediate glucose-suppression of insulin promoter activity (17, 18, 20). Mutation study identified that the C1 element and CRE mediated the lp-PARPi induction of –230 insulin promoter (Fig. 23). Both elements are known MafA binding sites, indicating that lp-PARPi may increase MafA binding to these elements. Indeed, lp-PARPi restored C1 binding complex formation in cells cultured in high glucose (Fig. 24A), and this is through the increased MafA protein levels (Fig. 26A). Mutation of CRE did not cause as large of a reduction of lp-PARPi induced insulin promoter activity as mutation of the C1 element (Fig. 23). This is because that MafA only binds weakly to the CRE (28). Moreover, other nuclear proteins have been identified to bind to the CRE, including CREM, CREB and c-Jun (75-77). These nuclear proteins may compete with MafA for binding to the CRE, thus reducing lp-PARPi stimulatory effect at the CRE. Another reason for the poor activity of MafA binding to CRE could be due to another nuclear protein NF-Y binding to the overlapping binding site CCAAT located directly downstream of the CRE (77). These reports suggest that MafA is not the main binding

factor to the CRE. Therefore, the CRE may not play an important role in mediating lp-PARPi stimulatory effects on insulin promoter activity, and the C1 element is the main element involved in mediating lp-PARPi-induction of –230 insulin promoter activity.

Although the C1 element mediated the lp-PARPi induction of insulin promoter, removal of the C1 element by deletion to –114 bp of insulin promoter did not completely abolish lp-PARPi effect (Fig. 22). This observation suggests that other regulatory elements such as the A1 and E1 elements might mediate some of the lp-PARPi effect. The E1 is a strong regulatory element of insulin promoter (61). Removal of E1 element caused a completely diminution of insulin promoter activity (61), thus it was hard to estimate the effects of lp-PARPi on E1 element through deletion studies (data not shown). The E1 activation activity, however, was not enhanced by lp-PARPi in INS-1 cells transfected with CAT reporter gene containing multimerized (three copies) E1 elements (data not shown). These data indicate that lp-PARPi stimulatory effects were not mediated through the E1 element. Mutation of the A1 and A3 elements did not reduce lp-PARPi induction of insulin promoter (Fig. 23A), indicating that the A1 and A3 elements were not important in lp-PARPi induction of insulin promoter. Consistently, PDX-1 binding to the A3 element was not affected by lp-PARPi under either low or high glucose (Fig. 24B). These data support the conclusion that the C1 element is the main element involved in mediating lp-PARPi-induction of the –230 insulin promoter activity.

Lp-PARPi induced MafA binding to the C1 element through the increase of MafA protein levels. Lp-PARPi completely restored nuclear MafA protein levels in cells cultured in high glucose (Fig. 26A). Consistently, immunochemical staining of INS-1 cells with MafA antibodies showed a complete loss of MafA staining in the nuclei of

cells cultured with high glucose and a recovery of nuclear staining with lp-PARPi (unpublished data by Mei-Hui Tai). Measuring total MafA protein levels showed that chronic hyperglycemia significantly reduced total MafA protein levels, and lp-PARPi markedly induced total MafA protein levels (Fig. 26A). Therefore, lp-PARPi induce nuclear MafA protein levels through an overall increase in total MafA protein levels. Moreover, these data also showed that lp-PARP did not increase nuclear MafA protein levels through the enhanced MafA translocation from cytosol to nucleus. Consistently, lp-PARPi also increased MafA binding to an additional MafA binding site, the A5/core, in INS-1 cells cultured in high glucose (Fig. 25A). Furthermore, the A5/core transactivation activity was also enhanced by lp-PARPi (Fig. 25A). Taken together, these data show that lp-PARPi increase MafA binding to the C1 and A5/core elements through the enhanced MafA protein levels in INS-1 cells cultured in high glucose.

The present study showed that chronic hyperglycemia reduced nuclear MafA protein levels in INS-1 cells, in part, through decreased MafA mRNA levels (Fig. 26). Harmon et al recently demonstrated that nuclear MafA protein levels were also diminished in HIT-T15 cells chronically exposed to high glucose (16). In HIT-T15 cells, however, chronic hyperglycemia reduction of nuclear MafA protein levels were not due to decreased MafA mRNA levels (16). In the HIT-T15 cell study, cells were cultured in high glucose for about a year. Chronic culturing of HIT-T15 cells in high glucose may lead to phenotypic changes of cells and cause colony selection. High glucose usually causes glucotoxicity in normal isolated islets within 4 to 6 days (21, 167). Culturing INS-1 cells for two days in high glucose are sufficient for the development of glucotoxic characteristics that are observed with normal isolated islets. Therefore, two-day high

glucose mediated changes in INS-1 cells are probably better representation of chronic hyperglycemic effects on normal β-cells than HIT cells. Further studies with isolated islets should be performed to determine whether chronic hyperglycemia also decreased MafA protein and mRNA levels.

The mechanisms whereby lp-PARPi increase of MafA protein levels in INS-1 cells cultured in elevated glucose involved increased MafA mRNA levels. This was demonstrated by the ability of 3-AB to induce MafA mRNA levels by 4-hr prior to the increase in MafA protein levels by 8-hr in INS-1 cells cultured in high glucose (Fig. 27). This rapid induction of MafA mRNA levels could be either due to increased MafA promoter activity and/or increased MafA mRNA stability. Lp-PARPi, however, did not delay MafA mRNA degradation in INS-1 cells treated with the RNA synthesis inhibitor, actinomycin D (unpublished data by Mei-Hui Tai). These data suggest that lp-PARPi induce MafA mRNA levels through stimulation of MafA promoter activity. MafA promoter has not been characterized, and whether MafA promoter is responsive to glucose and lp-PARPi remains unknown.

Since lp-PARPi-induction of MafA mRNA levels was small compared to MafA protein levels, it was possible that lp-PARPi also increased MafA protein stability. In cells incubated with protein synthesis inhibitor-cycloheximide, the rate of MafA protein degradation is similar in cells treated with or without 3-AB (Fig. 29). These data rule out the possibility that lp-PARPi increase MafA protein stability. Consistent with this conclusion, proteasome inhibitors lactacystein and MG132 did not increase MafA protein levels in cells cultured in high glucose (unpublished data by Mei-Hui Tai), indicating that lp-PARPi do not increase MafA protein levels through the inhibition of proteasome

activity. Taken together, both inhibition of protein synthesis and protein degradation studies show that lp-PARPi do not increase MafA protein stability.

Although lp-PARPi induced insulin promoter activity and insulin mRNA levels in INS-1 cells cultured in either low or high glucose (Fig. 18 and 19), MafA protein levels were the same in cells cultured in 4.0 mM glucose plus lp-PARPi or 16.7 mM glucose plus lp-PARPi by 48-hr (Fig. 26, and unpublished data). Moreover, lp-PARPi led to a greater induction of insulin gene expression in INS-1 cells cultured in high glucose compared to the cells cultured in low glucose. Time course studies showed that 4.0 mM glucose slightly increased MafA protein level by 24-hr (Fig. 27A). INS-1 cells were normally maintained in 11.1 mM glucose, which is also high glucose, for promoting proliferation, thus MafA gene expression was chronically reduced. Switching INS-1 cells back to low glucose restored MafA protein levels, indicating that adverse effects caused by chronic hyperglycemia were reversible in INS-1 cells. Consistent with our results, the changes in gene expression profile have been shown to be partially reversed in 90% partial pancreatized rats and ZDF diabetic rats by normalization of blood glucose (95, 289). In cells cultured in low glucose, addition of 3-AB also increased MafA protein levels by 12-hr (data not shown), and then the MafA protein levels decreased to the level of cells cultured with 4.0 mM glucose alone by 36-hr (Fig. 28). Since by 48-hr, MafA protein levels were the same in cells treated with 4.0 mM, 4.0 mM glucose plus 3-AB, and 16.7 mM glucose plus 3-AB (Fig. 26A), one can predict that the expression of MafA has reached equilibrium by 48-hr in all three conditions. Since 3-AB increased MafA protein levels in INS-1 cells cultured in low glucose (by 12-hr) later than the cells culture in high glucose (by 8-hr), one can predict that MafA would be on the insulin promoter for

a longer period of time in cells cultured in high glucose compared to cell cultured in low glucose. Therefore, lp-PARPi have more profound effects on insulin gene expression in cells cultured in high glucose compared to the cells cultured in low glucose.

Since the deletion to –114 bp of insulin promoter did not completely remove lp-PARPi-induction of insulin promoter activity, it was also possible that lp-PARPi just had a general effects on the gene transcription in a cell. This was not the case since in the control study with thymidine kinase promoter CAT reporter gene, lp-PARPi did not modulate thymidine kinase promoter activity (Fig. 19B). Moreover, lp-PARPi reduced glucose-induction of fatty acid synthase and liver-pyruvate kinase promoter activities (data not shown). These data support the conclusion that lp-PARPi selectively affect gene transcription, possibly genes altered by chronic hyperglycemia.

Lp-PARPi have been shown to inhibit the six known PARP isoforms through NAD binding site of PARPs (227, 232, 234, 242-244, 250, 253, 290), however, PARP-1 is the PARP that is known to regulate gene expression (186). The possible role of PARP-1 in regulating insulin promoter activity was tested by using potent PARP-1 inhibitors PJ34 and INO-1001. These inhibitors did not stimulate insulin promoter activities, and higher concentration of PJ34 or INO-1001 led to cytotoxicity (data not shown). Consistently, PJ34 did not enhance C1, A3 or A5/core binding complexes formation in INS-1 cells cultured in high glucose (Fig. 24 and 25). These data indicate that lp-PARPi induction of insulin promoter is likely to be independent of PARP-1 enzymatic activity. A number of studies showed that PARP-1 regulation of gene transcription were independent of PARP-1 enzymatic activity (218, 220). Hassa et al showed that lipopolysaccharides (LPS) failed to stimulate NF-κB activation in PARP-$1^{-/-}$ fibroblasts

(220), indicating that PARP-1 was a coactivator of NF-κB. 3-AB, however, did not attenuate NF-κB mediating gene transcription in PARP-$1^{+/+}$ fibroblasts (220). Further analyses showed that neither the enzymatic activity nor the DNA binding activity of PARP-1 was required for NF-κB mediated gene transcription (220). This study indicated that PARP-1 direct interaction with NF-κB was required for PARP-1 coactivation function. If the PARP-1 coactivation effect is dependent solely on the protein-protein interaction, lp-PARPi and potent PARP-1 inhibitors should have the same effects on insulin gene expression in INS-1 cells.

The different effects of lp-PARPi and potent PARP-1 inhibitors on insulin gene transcription could be due to their different chemical structures. Current PARP inhibitors can be divided into three categories including monocyclic carboxamides (NAM and 3-AB), bicyclic lactams (PD) or polycyclic lactams (PJ34) (291). The amide group of NAM or 3-AB is free to rotate relative to the plane of the aromatic ring, making them less potent (291). Constraining mono-aryl carboxamide into heteropolycyclic lactams usually increases potency, thus PD is more potent than NAM and 3-AB (291). Moreover, three or more ring structures increase the potency of PARP-1 inhibitor, thus PJ34 is more potent than lp-PARPi (291). The oxygen atom from the carbonyl group of PARP inhibitors serves as hydrogen acceptor and the hydrogen atom from the amide or imide group serves as a proton donor in the hydrogen bond interaction with the enzyme (291). PJ34 contains more carbonyl and amide groups, thus PJ34 binds to the enzyme tighter than lp-PARPi (292). The structure of INO-1001 is not available to the public, thus we can not compare its structure with lp-PARPi or PJ34. Because of the differences in chemical structure, lp-PARPi binding to PARP-1 may lead to a conformational change of

PARP-1 that favors PARP-1 coactivation of insulin promoter activity. In contrast, PJ34 may lead to a conformational change that does not favor of PARP-1 binding to insulin promoter. Consistent with this hypothesis, several studies showed that unlike 3-AB, PJ34 prevented PARP-1 coactivation with NF-κB. Pre-treating the animals with PJ34 before the LPS challenge attenuated NF-κB activation in liver and lung (293). In endothelial cells cultured in high glucose, PJ34 also reduced NF-κB activation (294). These reports suggest that inhibition of PARP-1 by PJ34 prevents NF-κB activation, and lp-PARPi and PJ34 can act differently in regulating the same set of genes. To more thoroughly explore the role of PARP-1 in regulating insulin promoter activity, dominant negative PARP-1 or small interfering RNA (SiRNA) for PARP-1 can be used to determine whether or not PARP-1 plays a role in regulating insulin promoter activity. Dominant negative PARP-1 can dimerize with endogenous PARP-1 and prevent it binding to DNA. SiRNA of PARP-1 should knock down endogenous PARP-1 expression. Both techniques should be feasible since PARP-1 knockout animals are usually healthy except that they are more sensitive to DNA damage (196).

The different effects of lp-PARPi and potent PARP-1 inhibitors could be also explained by their different potency to the NAD binding site of PARP-1. As described earlier, lp-PARPi are less potent, thus higher concentrations of lp-PARPi must be used to inhibit PARP-1. This may raise the possibility of their inhibition of other NAD-binding enzymes such as Sir2, and CtBP (295-298). The effects of lp-PARPi on Sir2 and CtBP were explored in the Chapter 6.

It has been suggested that chronic hyperglycemia may cause increased oxidative stress, leading to reduced insulin gene expression (109). NAM and 3-AB at the

concentrations used in this study can act as antioxidants (286, 287), thus it is possible that lp-PARPi restore insulin promoter activity simply by acting through an antioxidant mechanism. Lp-PARPi markedly induced insulin promoter activity whereas NAC, quercetin and lipoic acid only marginally stimulated insulin promoter activity (Fig. 20A). In agreement with these findings, NAC has been shown to marginally increase insulin promoter activity in HIT –T15 cells chronically exposed to high glucose (112). In ZDF diabetic animals, 6 weeks NAC administration led to a small increase in insulin mRNA levels compared to the rats treated with vehicle (112). In both cases, NAC did not lead to complete recovery of insulin gene expression. In contrast, lp-PARPi were able to completely restore insulin gene expression in INS-1 cells cultured in high glucose (Fig. 18, 19 and 21). Moreover, NAC induction of insulin mRNA levels in ZDF diabetic rats could be simply due to lowering the whole body oxidative stress instead of a direct effect on insulin gene transcription. All these data suggest that lp-PARPi stimulate insulin gene transcription more effectively than antioxidants. Control experiments demonstrated that NAC and quercetin at the concentration used functioned as antioxidants (Fig. 20B). Lipoic acid, NAM and 3-AB were only weak antioxidants in INS-1 cells. NAM and 3-AB are known to be weak antioxidants, but lipoic acid is supposed to be a strong antioxidant. One explanation is that reduced form of lipoic acid, dihydrolipoic acid, has pro-oxidant properties (299). Furthermore, this study also showed that PD was not an antioxidant but it still stimulated insulin gene expression (Fig. 21). These control experiments further support the conclusion that lp-PARPi are not good antioxidants in INS-1 cells cultured in high glucose, and they are unlikely to stimulate insulin gene expression through an antioxidant mechanism.

Besides increasing insulin promoter activity, lp-PARPi may also restore insulin mRNA levels by increasing insulin mRNA stability in INS-1 cells cultured in high glucose. NAM has been shown to increase insulin biosynthesis through increased polyamine content in fetal porcine islets (15). Spermine, one of the polyamines, stimulates insulin biosynthesis by increasing insulin mRNA stability (274). NAM enhance polyamine synthesis by stimulating the activities of several enzymes involved in polyamine biosynthesis (15). It is possible that 3-AB and PD also affect the same enzymes activities since NAM, 3-AB and PD have very similar chemical structures. Therefore, in INS-1 cells cultured in high glucose, lp-PARPi could also increase insulin mRNA through enhanced insulin mRNA stability. Further study should be conducted to determine whether this is an additional mechanism of lp-PARPi-induction of insulin gene expression.

Consistent with increased insulin gene expression, lp-PARPi increased cellular insulin content and this may account for the partial improvement of GSIS in INS-1 cells cultured under high glucose. Incubating INS-1 cells under high glucose led to a significant reduction of insulin accumulation over a 24-hr or 48-hr period (Fig. 15). Both 4.0 mM glucose and 16.7 mM glucose are stimulatory glucose concentrations of INS-1 cells (163). Therefore, the reduced insulin accumulation in medium in INS-1 cells cultured with 16.7 mM reflects the impairment in GSIS, which has also been demonstrated to be impaired with acute GSIS (Fig. 16). In agreement with these findings, chronically culturing human islets or rat islets with high glucose decreases GSIS (21, 300). The chronic hyperglycemia mediated reduction of GSIS is caused, in part, by decreased insulin gene expression and cellular insulin content (17, 20, 21, 103) (Fig. 17).

Lp-PARPi increase insulin accumulation in medium, basal and acute GSIS, and total cellular insulin content in INS-1 cells chronically cultured in high glucose (Fig. 15-17). Although lp-PARPi increase cellular insulin content and GSIS, the effects of lp-PARPi are minimum in INS-1 cells cultured under high glucose (Fig. 16, 17). This is because 16.7 mM glucose was a stimulatory concentration to INS-1 cells, and insulin was continuously secreted into the medium. Consistent with our findings, 3-AB increases GSIS in human fetal islets (11), and NAM improves GSIS in ZDF diabetic rats (4) and partial pancreatized dogs (3). In human fetal islets (11) and the present study, the improved GSIS is partially due to increase of insulin gene expression. In ZDF diabetic rats, NAM effects on β-cell function were suggested to be associated with lowered blood glucose and FFAs levels (4). Whether lp-PARPi directly target insulin gene expression in ZDF diabetic rats needs to be further explored. In partial panreatized dogs, the improved GSIS was associated with increased cellular insulin content and β-cell regeneration (3). Although this study demonstrated that lp-PARPi increased insulin gene expression and cellular insulin content and improved GSIS, Sandler et al showed that chronic culturing adult mouse islets with NAM failed to increase insulin content and inhibited GSIS (301). The discrepancy between the present study and Sandler's study could be because the adult mouse islets were not chronically cultured in high glucose and they were not dedifferentiated (loss of function and phenotype). The expression of β-cell specific genes such as insulin, MafA and PDX-1 might not be downregulated in adult mouse islets. Therefore, the fold increase of insulin gene expression might not be as large as if they were chronically cultured in high glucose. Chronic culturing adult mouse islets with NAM inhibited GSIS, and this was associated with a decrease of ATP level

(301), indicating that chronic NAM treatment may have adverse effects on normal adult islets. Furthermore, it was shown that in normal human subjects, NAM did not affect insulin secretion (302). Taken together, lp-PARPi may only provide beneficial effects in the β-cells with differentiation potential including fetal islets and embryonic stem cells (10-15) or dedifferentiated β-cells.

In conclusion, lp-PARPi induce insulin promoter activity through the enhanced MafA gene expression in INS-1 cells cultured in high glucose. This study also demonstrated that lp-PARPi induction of insulin promoter activity was independent of PARP-1 activity or an antioxidant mechanism. Lp-PARPi improve INS-1 cell function through the increased insulin gene expression.

Chapter 6. Effects of Low-potency Poly(ADP-ribose) Polymerase Inhibitors (lp-PARPi) on Transcription Factors, Coactivators and Repressors Action at Insulin Promoter

Abstract

Insulin is selectively expressed in β-cells, and this restricted expression is due to a unique combination of nuclear factors at insulin promoter. Transcription factors, coactivators and repressors interplay to control insulin gene expression. Before the identification of MafA as the target of lp-PARPi, we hypothesized that lp-PARPi affected the activities of several transcription factors, coactivators and repressors. This chapter summarizes the findings of these studies.

The present study showed that overexpression of p300, MafA, and BETA2 significantly induced insulin promoter activity, and lp-PARPi potentiated the stimulatory effects of these nuclear proteins. Effects of lp-PARPi on NAD-binding proteins including C-terminal binding protein (CtBP) and silent information regulator 2 (Sir2) were also examined. Overexpression of CtBP reduced lp-PARPi potentiation of p300 activity. Overexpression of Sir2 minimally reduced lp-PARPi induction of insulin promoter. Overexpression of CtBP or Sir2, however, did not completely remove lp-PARPi induction of insulin promoter, indicating lp-PARPi-induction of insulin promoter activity was not through inhibition of CtBP or Sir2. In conclusion, this study suggests that lp-PARPi may induce insulin promoter activity through potentiation of MafA, p300, and BETA2 activation at insulin promoter.

Introduction

Insulin is selectively expressed in the β-cells, and this restricted expression is due to a unique combination of nuclear factors at insulin promoter. Transcription factors, coactivators and repressors interplay to control insulin gene expression. Upon acute glucose stimulation, PDX-1 and BETA2 are phosphorylated and bind to insulin promoter (45, 74). The interaction of PDX-1 with BETA2/E47 recruits p300, which acetylates histone H4 or H3 (43, 80). Acetylation of histone H4 and H3 makes the chromatin more accessible for transcription binding complexes. Binding of MafA enhances PDX-1/BETA2/E47/p300 stimulatory effects on insulin promoter (59, 60). When cells are switched from high glucose to low glucose, histone deacetylases (HDAC), HDAC-1 and HDAC-2, are recruited to insulin promoter by dephosphorylated PDX-1 (48). HDAC-1 and HDAC-2 deacetylate histone H4 and decrease insulin gene transcription (48). The effects of chronic hyperglycemia on nuclear protein binding complexes formation at insulin promoter are not well understood. Impaired PDX-1 and p300 interaction in β-cells, however, limits insulin production and leads to the development of diabetes (280). Chronic hyperglycemia has also been suggested to enhance binding of insulin promoter repressor C/EBPβ at C1 element, leading to reduced insulin promoter activity (283). These studies indicate that chronic hyperglycemia reduces insulin promoter activity, in part, through interruption of activation binding complexes formation. Therefore, effects of lp-PARPi on p300, PDX-1, BETA2 and MafA activation of insulin promoter were examined in the present study.

Effects of lp-PARPi on the activities of two NAD-binding repressors C-terminal binding protein (CtBP) and yeast silent information regulator 2 (Sir2) were also studied.

CtBP is a redox sensing transcriptional regulator, and its activity may be altered by the energy status of a cell during development and in diseases (303). The mechanisms of CtBP repression of gene transcription are not fully understood. Recent studies indicate that CtBP may repress gene transcription in a histone deacetylase (HDAC)-dependent or –independent manner (295, 297, 298). Binding of NAD^+ can lead to conformational change of CtBP and transcriptional repression of gene promoter (304). Moreover, CtBP can reduce p300 activation at a number of gene promoters (305, 306). Sir2 is an NAD-dependent histone deacetylase (296). Binding of NAD^+ to Sir2 leads to deacetylation of various proteins, leading to gene silencing (296). Mammalian homologue of Sir2, SIRT1, has been shown to deacetylate and repress p53, NFκB, and p300 transcriptional activation (307-310). Since CtBP and Sir2 use NAD as a cofactor for the transcriptional repression on gene promoter, we hypothesized that lp-PARPi might inhibit NAD binding site of CtBP and Sir2, thus preventing CtBP or Sir2 repression of insulin promoter activity.

The results show that lp-PARPi may induce insulin promoter activity through potentiation of MafA, p300, and BETA2 activation at the insulin promoter. Overexpression of CtBP or Sir2 suppresses lp-PARPi-induction of insulin promoter activity, however, they can not completely remove lp-PARPi induction. These data indicate that lp-PARPi-inhibition of CtBP and Sir2 may not play important roles in lp-PARPi-induction of insulin promoter under high glucose.

Results

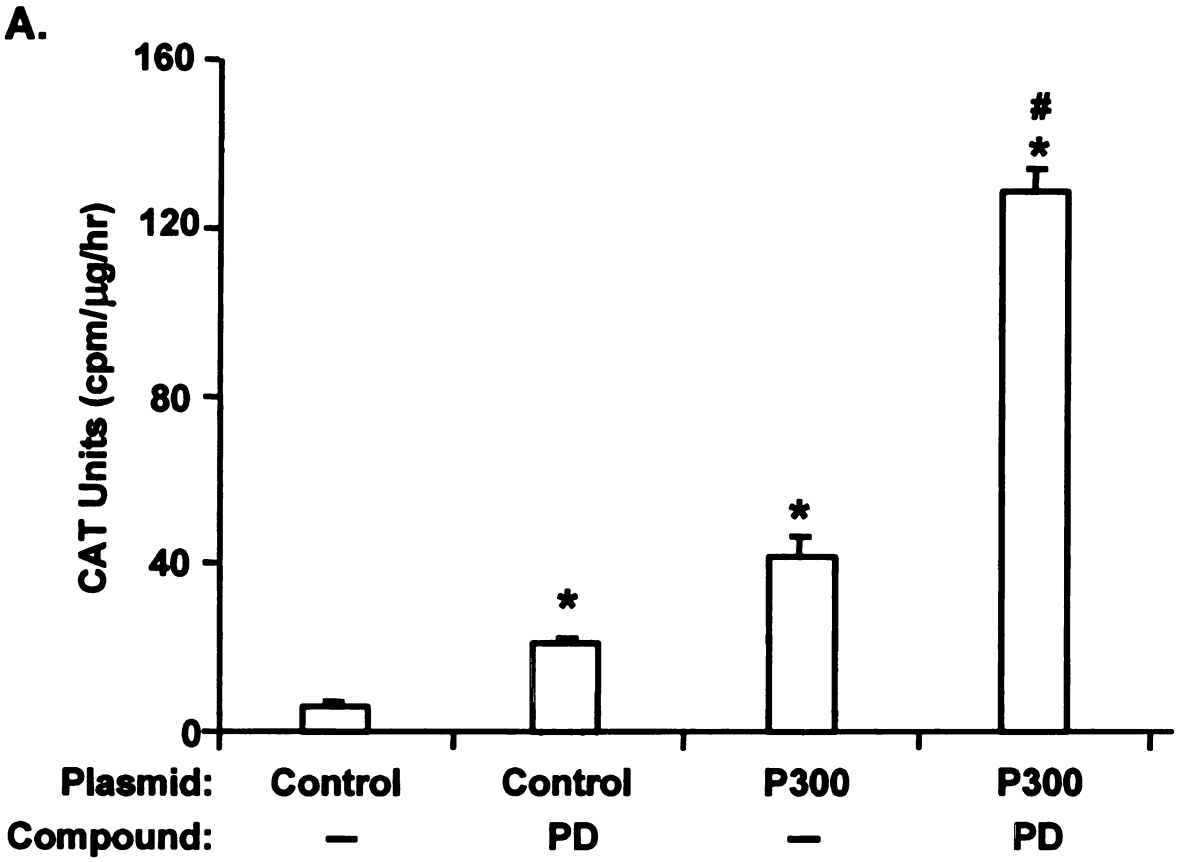
1. Lp-PARPi potentiate p300 induction of insulin promoter activity.

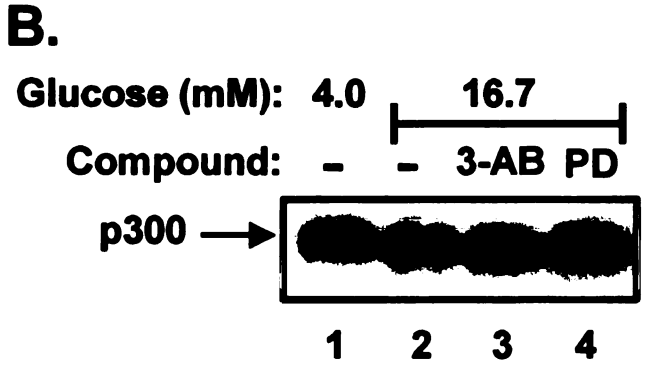
Coactivator p300 interacts with PDX-1 and BETA2, and potentiates PDX-1 and BETA2 activities at insulin promoter (44, 70, 280). To determine whether lp-PARPi affected p300 activity at insulin promoter, p300 was overexpressed in INS-1 cells transfected with INS(-230)CAT. The cells were then treated with high glucose with or without PD. PD and p300 induced insulin promoter activity significantly compared to controls (Fig. 30A). PD potentiated p300 stimulation of INS(-230)CAT expression. To determine whether lp-PARPi potentiated endogenous p300 levels, p300 protein levels were measured. In INS-1 cells cultured in low glucose, high glucose, or high glucose with 3-AB or PD, lp-PARPi did not affect p300 protein levels (Fig. 30B). These data show that lp-PARPi can potentiate p300 activation of insulin promoter without increase of endogenous p300 protein levels.

2. Lp-PARPi potentiate MafA and BETA2 activation of insulin promoter.

To determine whether lp-PARPi can affect MafA, BETA2, and PDX-1 activation of insulin promoter, BETA2, PDX-1 or MafA was overexpressed in INS-1 cells transfected with INS(-230)CAT. The cells were then treated with high glucose or without lp-PARPi. Overexpression of p300, BETA2 or MafA led to a 2.2-, 1.6- or 6.6-fold increase of insulin promoter activity, respectively (Fig. 31A). In contrast, PDX-1 overexpression caused a 40% reduction of promoter activity (Fig. 31B). Addition of PD to INS-1 cells transfected with p300 or BETA2 expression vectors caused a 1.7- or 2.9-fold further induction of insulin promoter activity (Fig. 31A). 3-AB also potentiated

Figure 30. Lp-PARPi potentiate p300 induction of insulin promoter activity. A) INS-1 cells were transfected with 1 μg INS(-230)CAT, and 0.5 μg of pCR3.1-CMV (control), or pCI-FLAG-p300. Cells were then treated with or without 500 μM PD under 16.7 mM glucose for 48 hrs. Cells were harvested and CAT assays performed (n=4). Values are mean ± SEM. * PD, p300, and p300 plus PD versus Control, and # p300 plus PD versus Control plus PD, $p<0.05$. B) INS-1 cells were treated with 4.0 mM glucose, or 16.7 mM glucose in the presence or absence of 10 mM 3-AB or 500 μM PD for 48 hrs. Western blot analyses were performed to determine p300 protein levels. Shown here is a representative blot of three independent experiments.


A.
160
120
80
40
0
CAT Units (cpm/µg/hr)
*
*
#
*
Plasmid: Control Control P300 P300
Compound: — PD — PD

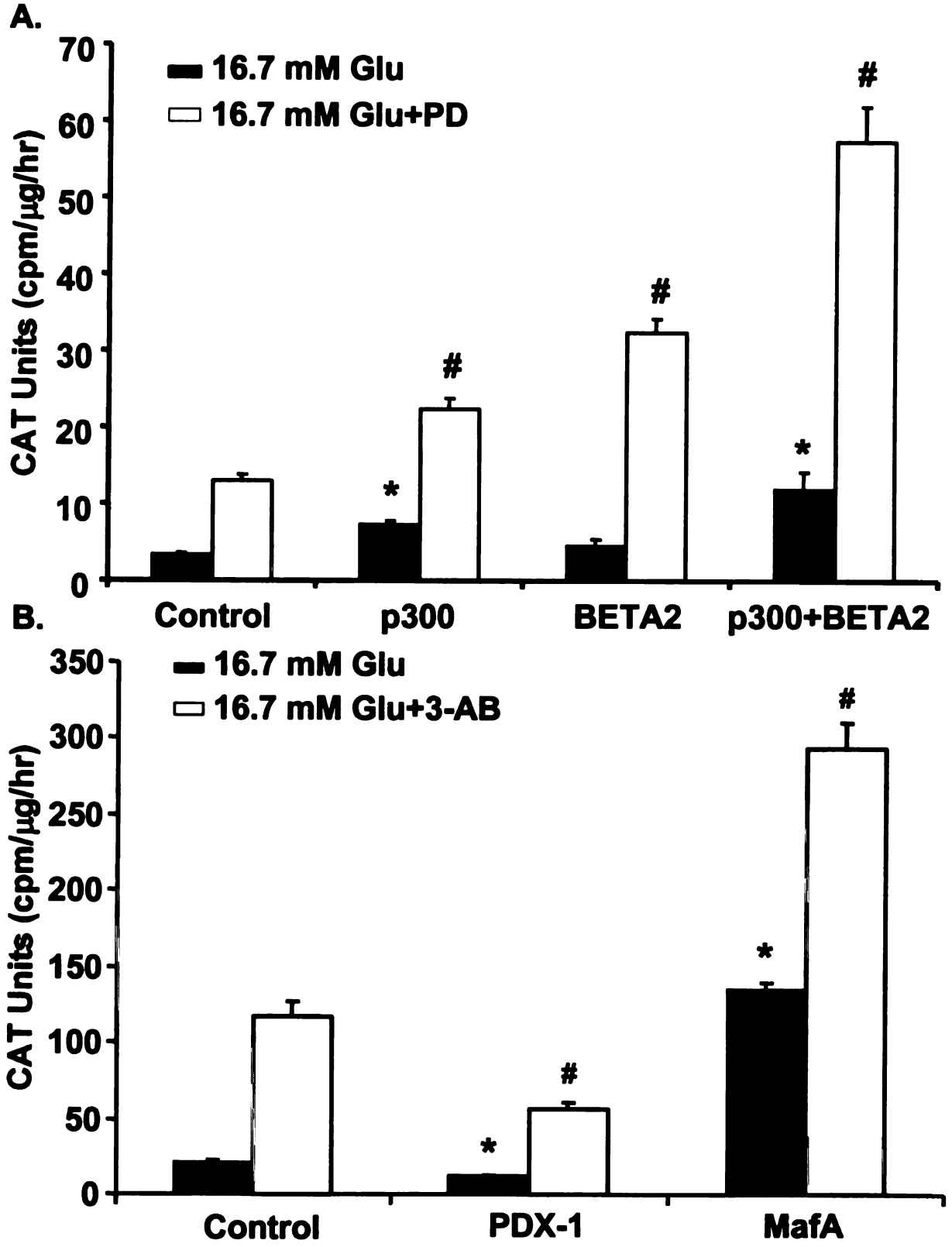

B.
Glucose (mM): 4.0 16.7
Compound: - - 3-AB PD
p300
1 2 3 4

MafA activation of insulin promoter (2.5-fold) (Fig. 31B). PDX-1 suppression of insulin promoter activity was reversed by 3-AB (Fig. 31B). In cells that p300 and BETA2 were both overexpressed, there was a 3.7-fold stimulation of the promoter activity, and a 5.4-fold induction with cells treated with PD (Fig. 31A). These data suggest that lp-PARPi can augment promoter activity stimulated by p300, MafA or BETA2.

3. Overexpression of CtBP significantly reduces lp-PARPi potentiation of p300 activity at insulin promoter.

CtBP is a NAD-binding enzyme and binding of NAD^+ can lead to conformational change of CtBP and transcriptional repression of gene prmoter (304). CtBP has been shown to reduce p300 activation at a number of gene promoters (305, 306). Lp-PARPi inhibit PARPs at their NAD-binding site. CtBP also uses NAD^+ as a substrate for its enzymatic activity, thus by analogy, we hypothesized that lp-PARPi could also inhibit CtBP and interfer its repression of p300 activity at insulin promoter. Measurement of CtBP protein levels showed that CtBP was an abundant nuclear protein in INS-1 cells, and glucose and lp-PARPi did not alter CtBP protein levels (Fig. 32A). In INS-1 cells transfected with INS(-230)CAT, overexpression of CtBP did not decrease insulin promoter activity under high glucose (Fig. 32B). PD, p300 or combination of both significantly activated insulin promoter. CtBP overexpression did not significantly reduce PD-stimulation of insulin promoter activity, however, it reduced activation of insulin promoter by p300, or p300 plus PD (Fig. 32B). These data suggest that CtBP can block p300-potentiated insulin promoter activity, but does not efficiently block lp-PARPi activation of promoter activity.

Figure 31. Lp-PARPi potentiate MafA and BETA2 activation of insulin promoter. INS-1 cells were transfected with 1 μg INS(-230)CAT, and 0.5 μg of pCR3.1 CMV (control), pCI-FLAG-p300, and/or pCR3.1-BETA2 (A), pCR3.1-CMV-PDX-1, or pCR3.1-CMV-MafA (B). Equivalent amounts of expression plasmid were transfected per well by the addition of pCR3.1-CMV containing no insert. Cells were then incubated in medium containing 16.7 mM glucose with or without 500 μM PD or 10 mM 3-AB. After 48 hrs treatments, cells were harvested and CAT assays performed (n=4). Values are mean ± SEM. * 16.7 mM Glu: Control versus other overexpression, and # 16.7 mM Glu+PD: Control versus other overexpression, $p<0.05$.


A.
16.7 mM Glu
16.7 mM Glu+PD
CAT Units (cpm/µg/hr)
70
60
50
40
30
20
10
0
*
#
Control
p300
BETA2
p300+BETA2
B.
16.7 mM Glu
16.7 mM Glu+3-AB
CAT Units (cpm/µg/hr)
350
300
250
200
150
100
50
0
Control
PDX-1
MafA

To test this further, increasing amounts of CtBP were expressed in INS-1 cells with a constant amount of p300. Increasing expression of CtBP showed a gradual reduction of p300 induction of insulin promoter activity (slope=9.35), suggesting that CtBP can compete with p300 (Fig. 32C). PD potentiation of p300 activity was significantly suppressed by increasing level of CtBP (slope=19.9) but not completely (Fig. 32C). These data support the conclusion that CtBP is not a direct target of lp-PARPi, but that CtBP can reduce p300 activity in β-cells.

4. Overexpression of Sir2 reduces lp-PARPi induction of insulin promoter activity.

Sir2 is an NAD-dependent histone deacetylase, and binding of NAD^+ to Sir2 leads to deacetylation of various proteins and gene silencing (296). NAM can inhibit Sir2 through its binding to NAD-binding site, thus blocking the deacetylation activity of Sir2 (311). Therefore, we hypothesized that lp-PARPi could inhibit Sir2 function in INS-1 cells, leading to increased insulin gene transcription. In INS-1 cells overexpressed with increasing amounts of Sir 2, insulin promoter activity was significantly reduced at the highest dose of Sir2 (slope=2.66) (Fig. 33). NAM or PD stimulation of insulin promoter activity was also significantly reduced with the highest dose of Sir2 overexpression but not completely ($slope_{NAM}$ =11.3, $slope_{PD}$ =8.48) (Fig. 33).

Figure 32. Overexpression of CtBP significantly reduces lp-PARPi potentiation of p300 activity at the insulin promoter. A) INS-1 cells were treated with 4 mM glucose or 16.7 mM glucose with or without 10 mM NAM, 10 mM 3-AB or 500 μM PD. Nuclear proteins were isolated and Western blot analyses were performed by using CtBP antibodies. Shown here is a representative blot of three independent experiments. B) INS-1 cells were transfected with INS(-230)CAT and overexpressed with 0.5 μg pCl-FLAG-p300 and/or 0.5 μg pCDNA3-CtBP in the presence or absence of 500 μM PD under high glucose (n=3). Cells were then harvested and CAT assays performed. Values are mean ± SEM. * Control versus other conditions, and # p300 plus PD versus p300 plus CtBP and PD, $p<0.05$. C) INS-1 cells were transfected with INS(-230)CAT and overexpressed with 0.5 μg pCl-FLAG-p300 and/or 0.5 μg, 0.75 μg, 1 μg, 1.25 μg, or 1.5 μg pCDNA3-CtBP in the presence or absence of 500 μM PD under high glucose (n=3). Cells were then harvested and CAT assays performed. Values are mean ± SEM. * Control versus p300, ∞ p300 versus p300 plus CtBP, and # p300 plus PD versus p300, PD plus CtBP, $p<0.05$.

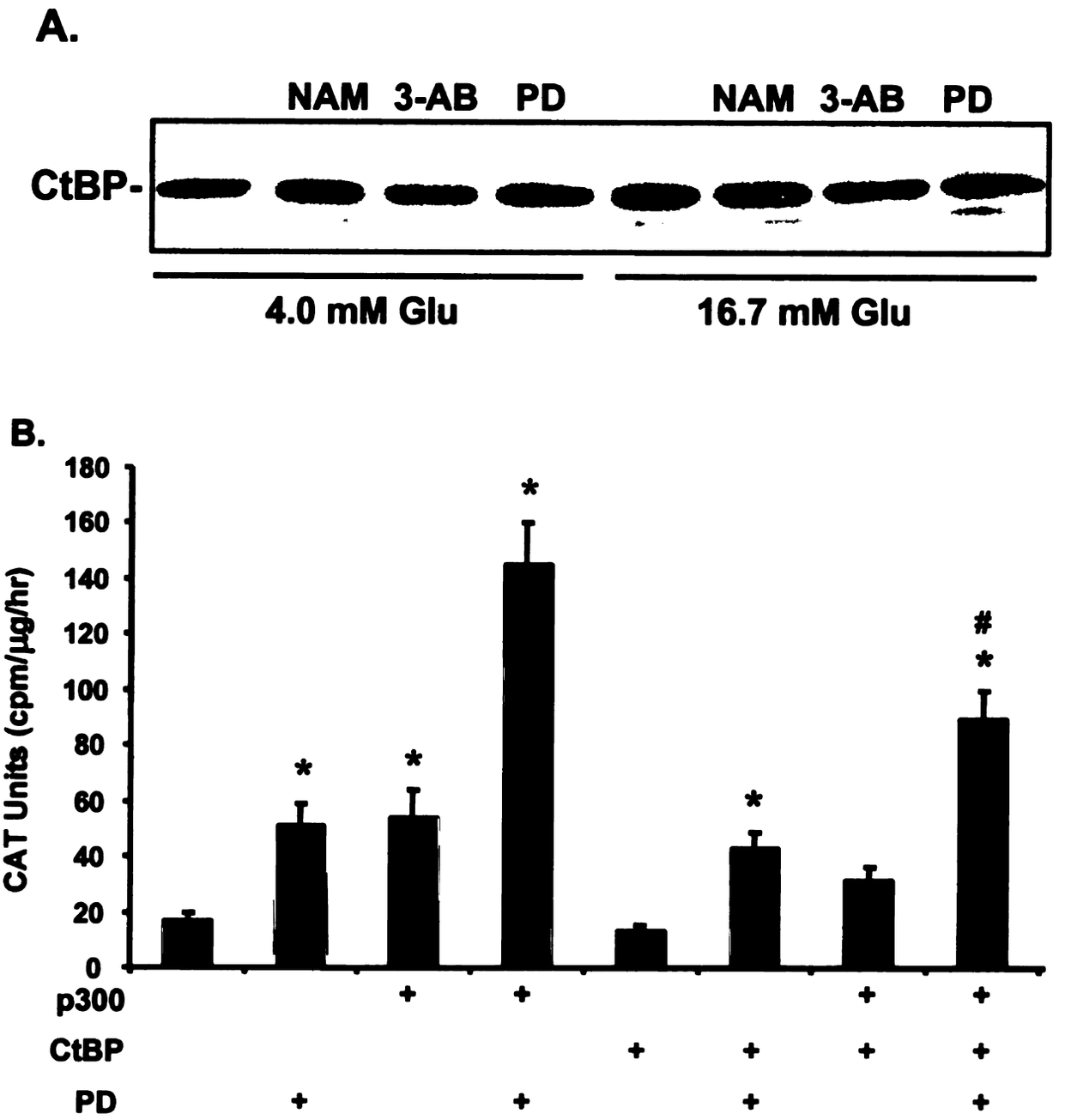
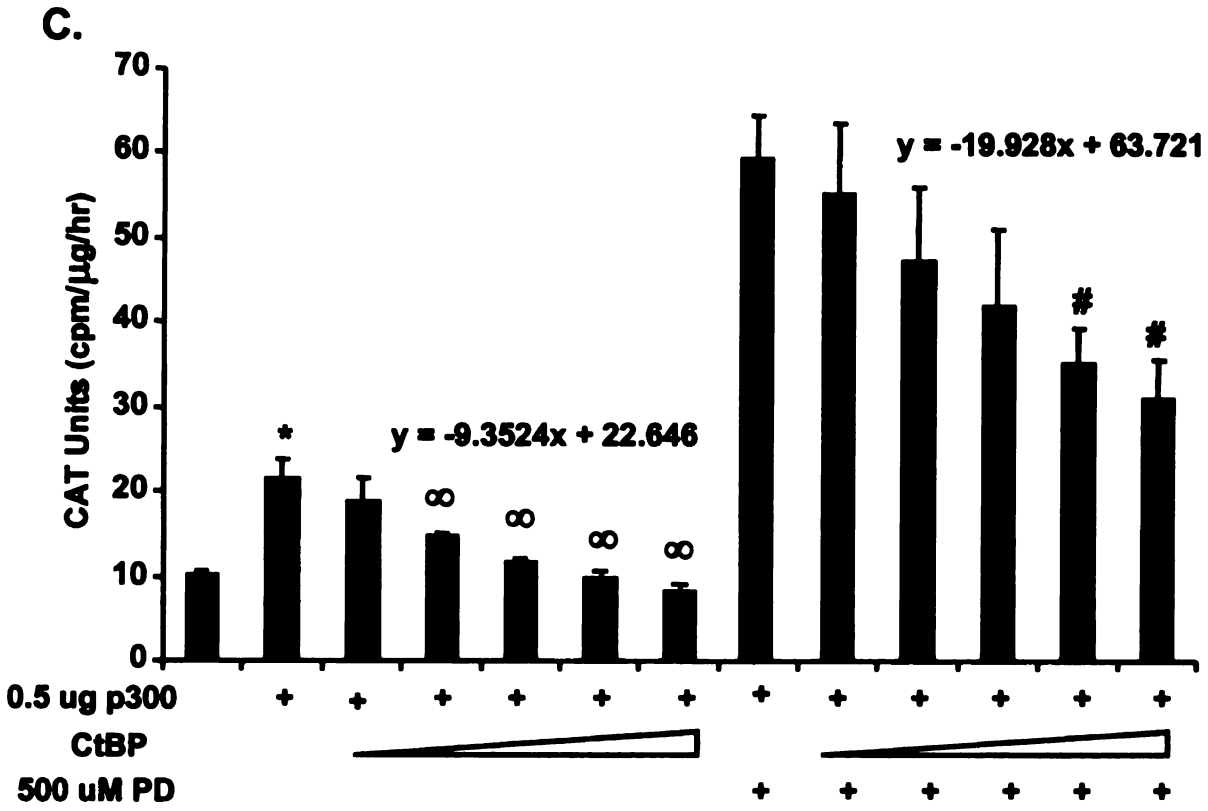

A.
NAM 3-AB PD
NAM 3-AB PD
CtBP-
4.0 mM Glu
16.7 mM Glu
B.
CAT Units (cpm/µg/hr)
180
160
140
120
100
80
60
40
20
0
p300
CtBP
PD

Figure 32 (cont'd)



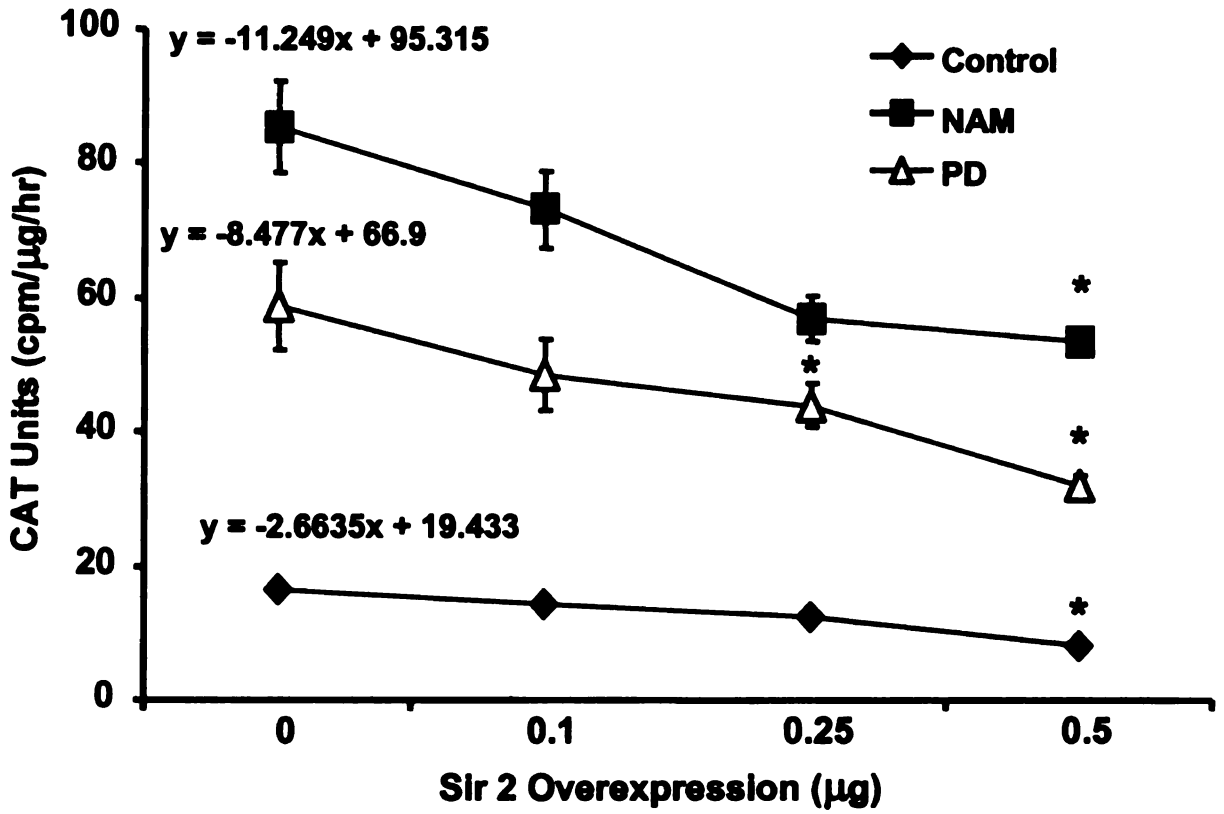
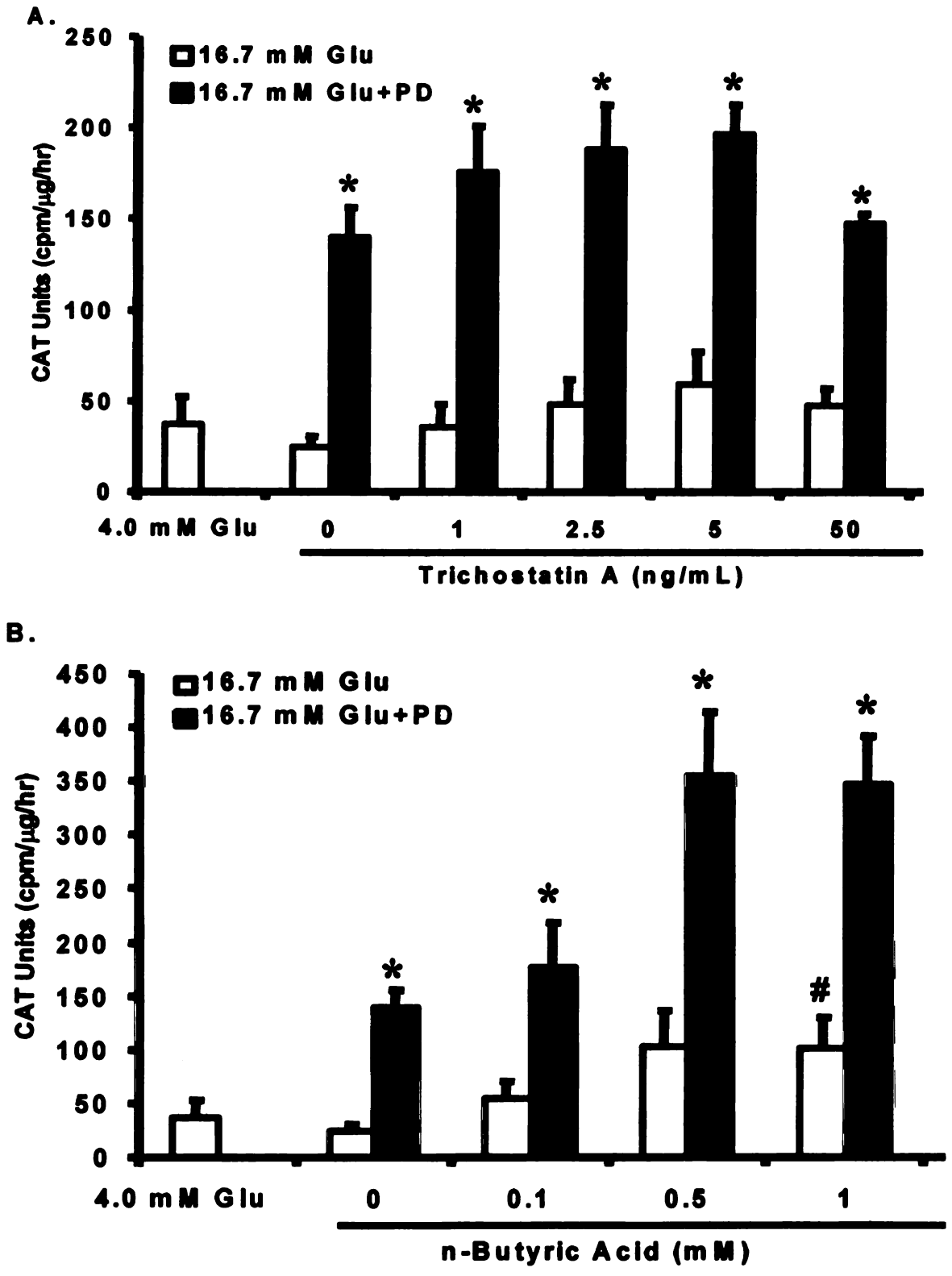


Figure 33. Overexpression of Sir2 reduces lp-PARPi induction of insulin promoter activity. INS-1 cells were transfected with INS(-230)CAT and Sir2 in the presence or absence of 10 mM NAM or 500 µM PD under high glucose concentration (n=4). Cells were then harvested after 48-hr treatment and CAT assays performed. Values are mean ± SEM. *Control Sir 2 (0 µg) versus cells overexpressed with different amount of Sir 2, $p<0.05$.

5. Lp-PARPi do not elevate insulin promoter through a mechanism involving inhibition of general deacetylases.

Chromatin conformation can be changed by acetylation or deacetylation of histone, making chromatin more or less accessible for transcriptional machinery. Histone acetylation and deacetylation are carried out by histone acetyltransferases and deacetylases, respectively. The activity of insulin promoter can be regulated by histone acetylation and deacetylation in β-cells (43, 48). Moreover, acetylation of BETA2 has been shown to be important for BETA2 activation of insulin promoter (73). Lp-PARPi induction of insulin promoter could occur through inhibition of general deacetylases, leading to inhibition of the deacetylation of histones and BETA2. To test this hypothesis, INS-1 cells were transfected with INS(-230)CAT and then treated with general deacetylase inhibitor trichostatin A (TSA) or n-butyric acid in the presence or absence of PD. Trichostatin A and n-butyric acid were shown to modestly activate insulin promoter activity under high glucose (Fig. 34). Lp-PARPi were still able to further induce insulin promoter activity. These data suggest that lp-PARPi are unlikely to stimulate insulin promoter activity through a mechanism involving inhibition of general deacetylases (Fig. 34).

Figure 34. Lp-PARPi do not elevate insulin promoter through a mechanism involving inhibition of general deacetylases. INS-1 cells were transfected with INS(-230)CAT, and then treated with 4 mM glucose, 16.7 mM glucose, 16.7 mM glucose plus 500 μM PD or 16.7 mM glucose plus 500 μM PD and increasing concentration of trichostatin A (A) or n-butyric acid (B) (n=3). Cells were harvested after 48-hr treatment and CAT assays performed. Values are mean ± SEM. * 16.7 mM Glu versus 16.7 mM Glu plus PD and trichostatin A, and # 16.7 mM Glu versus 16.7 mM Glu plus PD and n-butyric acid, $p<0.05$.


A.
16.7 mM Glu
16.7 mM Glu+PD
CAT Units (cpm/µg/hr)
250
200
150
100
50
0
4.0 mM Glu
0
1
2.5
5
50
Trichostatin A (ng/mL)
B.
16.7 mM Glu
16.7 mM Glu+PD
CAT Units (cpm/µg/hr)
450
400
350
300
250
200
150
100
50
0
4.0 mM Glu
0
0.1
0.5
1
n-Butyric Acid (mM)

Discussion

The present study was designed to determine whether lp-PARPi-induction of insulin promoter activity was through modulating activities of transcription factors, coactivators or repressors. The experiments in this study were performed in INS-1 cells cultured under high glucose because the fold-induction by lp-PARPi was the greatest. We showed here that lp-PARPi enhanced p300, BETA2 and MafA activation of insulin promoter. The roles of CtBP, Sir2 and deacetylases in mediating lp-PARPi-induction of insulin promoter activity were also eliminated.

This study showed that overexpression of p300 or MafA significantly induced insulin promoter activity in INS-1 cells cultured in high glucose (Fig. 31). In contrast, overexpression of BETA2 or PDX-1 did not stimulate insulin promoter activity (Fig. 31). Lp-PARPi enhanced insulin promoter activity in INS-1 cells overexpressed with p300, BETA2, MafA and PDX-1 (Fig. 30 and 31). All these lp-PARPi stimulatory effects could be simply due to increased MafA protein levels by lp-PARPi in INS-1 cells (Fig. 26). In INS-1 cells overexpressed with MafA, 3-AB only led to a small increase of insulin promoter activity (Fig. 31B). This could be because the promoter activity is at its maximum due to increased MafA protein levels by both overexpression and 3-AB. In INS-1 cells, lp-PARPi further stimulated insulin promoter activity in cells overexpressed with both p300 and BETA2 under high glucose (Fig. 31A). From all these data, I propose that lp-PARPi stabilize p300/PDX-1/MafA/BETA2 binding complex formation through increased MafA nuclear protein levels in INS-1 cells incubated with high glucose (Fig. 35). Chronic hyperglycemia has been shown to reduce NFAT (nuclear factor of activated T cells) and MafA binding to A2 and C1 elements, resulting in increased

Chronic hyperglycemia:

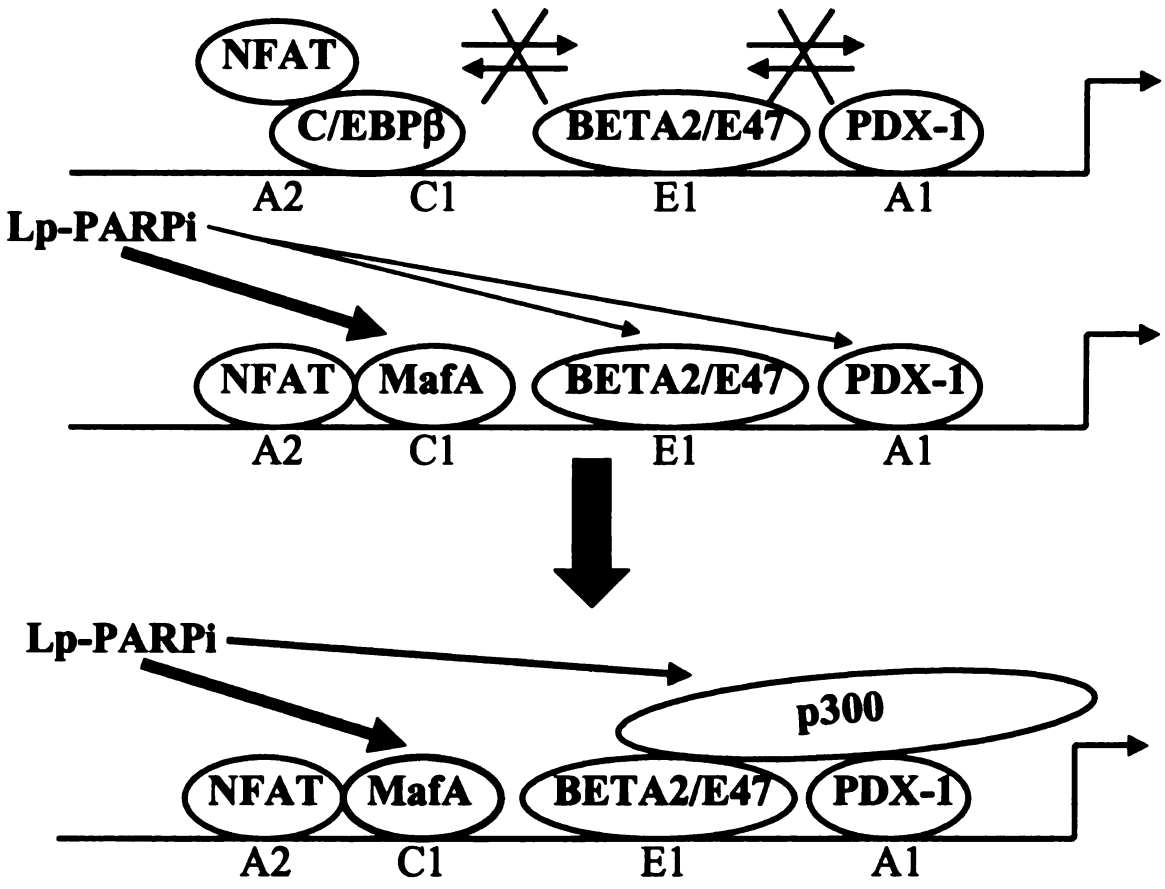


Figure 35. Model for the lp-PARPi regulation of activity of A2/C1/E1/A1 region in INS-1 cells prolonged cultured with elevated glucose. During chronic hyperglycemia, MafA protein levels are low and C/EBPβ protein levels are high, resulting in increased binding of C/EBPβ to insulin promoter and reduced insulin promoter activity. Lp-PARPi increase MafA protein levels and enhance MafA binding to C1. The enhanced MafA binding leads to increased BETA2/E47 and PDX-1 binding to E1 and A1, respectively. BETA2 and PDX-1 can further recruit p300, which enhances BETA2, MafA and PDX-1 stimulatory effects at insulin promoter. Lp-PARPi may also directly increase p300, BETA2 and PDX-1 activity at insulin promoter.

binding of repressor C/EBPβ (283). The increased C/EBPβ binding to insulin promoter was proposed to attenuate E47/BETA2 and PDX-1 binding to E1 and A1, respectively, thus leading to reduced insulin promoter activity (283) (Fig. 35). Lp-PARPi increased MafA protein may lead to increased MafA binding to insulin promoter and compete against C/EBPβ, thus enhanced insulin promoter activity (Fig. 35). Certainly, the present study does not prove the whole theory, and further study should be done with CHIP assays to determine how lp-PARPi affect p300/PDX-1/MafA/BETA2 binding complex formation at the insulin promoter. Preliminary data from ChIP assays showed that lp-PARPi enhanced MafA and p300 binding to insulin promoter under high glucose (data not shown), suggesting that lp-PARPi possibly stabilize p300/PDX-1/BETA2/MafA binding complex formation at the insulin promoter.

The effects of lp-PARPi on other NAD-binding enzymes, mainly CtBP and Sir2, were explored. CtBP is a redox sensing transcriptional regulator, and its activity may be altered by the energy status of a cell during development and in diseases (303). Binding of NAD^+ can lead to conformational change of CtBP and transcriptional repression of gene promoter (304). The present study showed that overexpression of CtBP did not suppress insulin promoter activity (Fig. 32B) and this could be due to already high level of CtBP proteins or limited amounts of p300 in INS-1 cells. Overexpression of CtBP, however, dose-dependently reduced p300 activity at the insulin promoter (Fig. 32C), suggesting CtBP and p300 compete for the regulation of insulin promoter activity. Consistent with this observation, CtBP has been shown to repress p300-mediated transcriptional activation by directly binding to p300 (305). In doing so, CtBP blocks p300 interaction with histones and thus prevents acetylation of histones and transcription

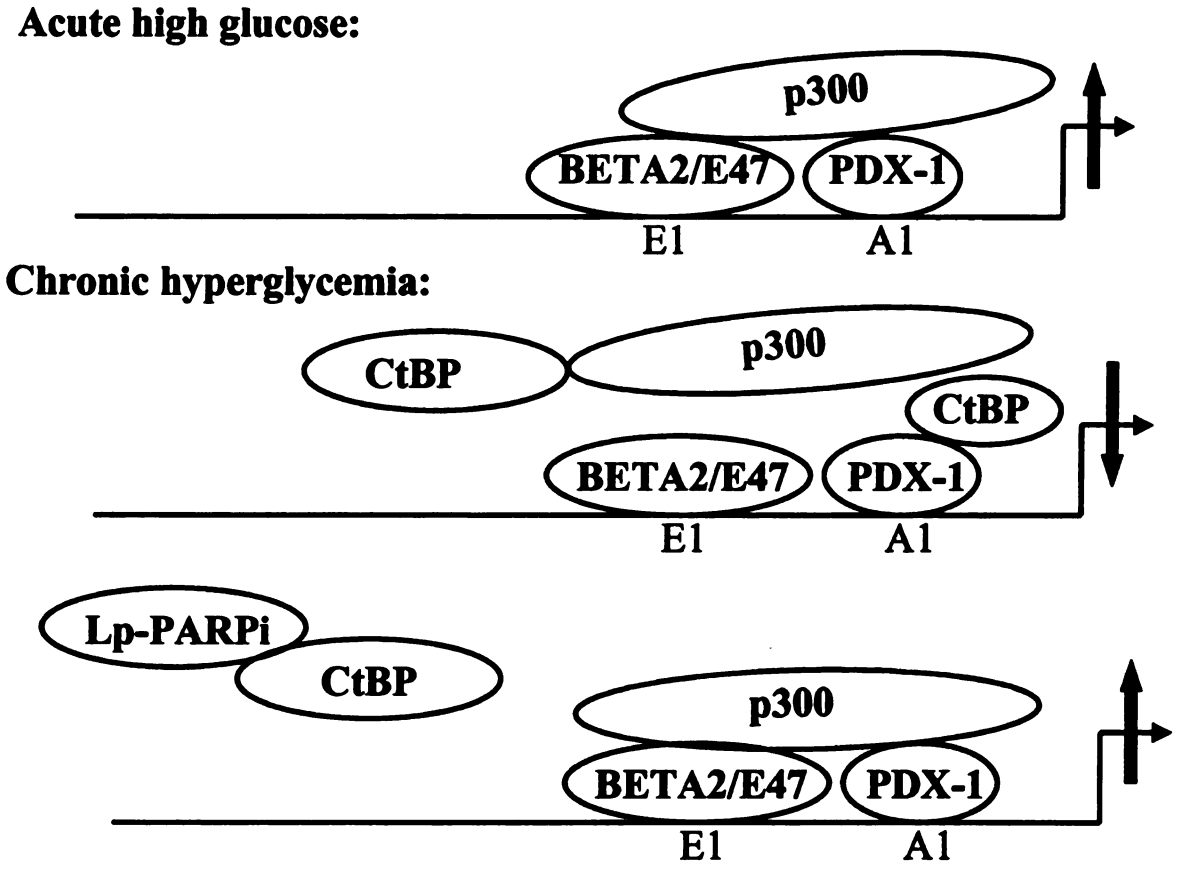


Figure 36. Hypothetic role of lp-PARPi in relief of CtBP suppression of p300 activation of insulin promoter. Acute high glucose stimulates p300/BETA2/PDX-1 binding complexes formation and induction of insulin promoter activity. Chronic hyperglycemia may lead to increased CtBP/p300 or CtBP/PDX-1 binding, blocking insulin promoter activity. Lp-PARPi inhibition of CtBP may lead to conformational change of CtBP, and p300 and PDX-1 are free to form binding complexes with other transcription factors. Therefore, lp-PARPi inhibition of CtBP should lead to increased activation of insulin promoter. Increased CtBP protein levels should be able to overcome PD inhibition of CtBP, resulting in reduced insulin promoter activity.

activation (305). In β-cells, BETA2 and histone H4 are known to be acetylated by p300-associated factor (PCAF) and p300, respectively (73). CtBP may also directly interact with p300 in INS-1 cells, preventing acetylation of BETA2 and histone H4, causing reduced activation of insulin promoter (Fig. 36). CtBP is known to interact with proteins through a well conserved PXDLS motif and interfere protein binding to gene promoter (305, 312, 313). Therefore, CtBP may also interact with PDX-1 or BETA2 and inhibit p300 recruitment to the insulin promoter by PDX-1 and BETA2. A search for a protein containing this motif was performed to determine whether PDX-1 or BETA2 could contain this motif. PDX-1 seems to contain a motif with similar sequences, PPDIS, which is well conserved in human, rat and mouse PDX-1 proteins. Thus, CtBP may also compete with p300 for the binding to PDX-1, leading to repressing of insulin promoter (Fig. 36).

In INS-1 cells, increased overexpression of CtBP led to a gradual but limited decrease of PD and p300 induction of insulin promoter (Fig. 32C). If CtBP competes with p300, one would predict that overexpression of CtBP can completely remove PD potentiation of p300 activity. The slope of CtBP reduction of combined PD and p300 activities was slightly larger than the slope of p300 activity alone (Fig. 32C), suggesting that overexpression of CtBP marginally attenuated PD potentiation of p300 activation of insulin promoter. Moreover, overexpress of CtBP did not completely block PD potentiation of p300 activity, indicating PD may enhance p300 activity by a different mechanism. One of the possibility is that lp-PARPi may enhance MafA binding to insulin promoter, and thus increase p300 recruitment to the insulin promoter and activation of insulin promoter. Furthermore, CtBP had no effect on PD-induction of

insulin promoter in the absence of p300 overexpression (Fig. 32B), indicating that CtBP only affected p300 activity. Taken together, these data show that lp-PARPi do not potentiate p300 activity through inhibition of CtBP.

Sir2 has been shown to interact and deacetylate transcription factors and coactivators, leading to decreased gene transcription (307-310). Mammalian homologue of Sir2, SIRT1, has been shown to deacetylate and repress p53, NFκB, and p300 transcriptional activation (307-310). NAM is an inhibitor of Sir2 and prevents Sir2 deacetylation activity (311, 314). Therefore, one could predict that if lp-PARPi inhibit Sir2 activity, there would be an increase of insulin promoter activity. In INS-1 cells, the insulin promoter activity was gradually decreased with increasing expression of Sir2 under high glucose condition (Fig. 33). The promoter activity was not significantly reduced except at the highest Sir2 overexpression (Fig. 33). One possible reason is that insulin promoter activity is already low in cells cultured in high glucose. If the cells were cultured in low glucose, we might have seen more Sir2-mediated repression of insulin promoter activity. Overexpression of Sir2 reduced lp-PARPi induction of insulin promoter activity but not completely (Fig. 33). The slopes of Sir2 repression of insulin promoter in cells treated with lp-PARPi are slightly higher than the control (Fig. 33), indicating that Sir2 can marginally attenuate lp-PARPi-induction of insulin promoter activity. If lp-PARPi induce insulin promoter activity by inhibition of Sir2, one can predict that overexpression of Sir2 can completely remove lp-PARPi stimulatory effects. Therefore, these data suggest that Sir2 is not a target of lp-PARPi. This conclusion is supported by several current studies, which show that Sir2 plays positive roles in regulating insulin gene expression and β-cell function (315, 316) instead of negative roles

in β-cells as I have hypothesized originally. Kitamura et al have shown that the forkhead protein FoxO1 protects β-cells against glucose-induced oxidative stress by forming a complex with the promyelocytic leukemia protein Pml and Sirt1, the mammalian homolog of Sir2, to activate expression of BETA2 and MafA (315). If Sir2 increases expression of BETA2 and MafA in β-cells, one can predict that overexpression of Sir2 should increase insulin gene expression in INS-1. Alternatively, insulin promoter activity was slightly decreased in INS-1 cells overexpressed with Sir2, indicating that too higher amount of Sir2 may lead to suppression of insulin promoter. Nevertheless, these data indicate that lp-PARPi is unlikely to induce insulin promoter activity through the inhibition of Sir2.

Chromatin conformation can be altered by acetylation or deacetylation of histone, making it more or less accessible for transcriptional machinery. Histone acetylation and deacetylation are carried out by histone acetyltransferases and deacetylases, respectively. Transcription of insulin is controlled by histone acetylation and deacetylation depending on the glucose concentration (48, 79). When MIN6 pancreatic β-cells were acute stimulated with high glucose, p300 was recruited to insulin promoter (43). p300 is a histone acetyltransferase and increased p300 present at insulin promoter was associated with increased acetylation of histone H4 (43). When MIN6 cells were switched from high glucose to low glucose, HDACs were recruited to insulin promoter, leading to reduced acetylation of histone H4 (48). Transcription factor such as BETA2 can be acetylated by p300-associated factor (PCAF) (73). To determine whether preventing deacetylation of histones or transcription factors will lead to increased insulin promoter activity, INS-1 cells were incubated with high glucose containing general deacetylase

inhibitors TSA or n-butyric acid. TSA or n-butyric acid dose-dependently increased insulin promoter activity in INS-1 cells cultured in high glucose (Fig. 34). In agreement with this observation, treating MIN6 cells with TSA or n-butyric acid led to increased acetylated-histone H4 association of insulin promoter and increased insulin mRNA levels (79, 80). Plasmids, however, are not packaged like genomic DNA with histones, thus lp-PARPi stimulation of insulin promoter activity does not likely involve the enhanced acetylation of histones in INS-1 cells. The small increased expression of INS(-230)CAT reporter gene by TSA or n-butyric acid could be due to enhanced BETA2 acetylation. The profound induction of insulin promoter activity, however, indicates that lp-PARPi increase insulin promoter activity by additional mechanisms.

Overall, we examined other possible mechanisms of lp-PARPi induction of insulin promoter activity. The results show that lp-PARPi enhance p300, BETA2 and MafA activation of insulin promoter. Previous chapter showed that lp-PARPi increased MafA expression in INS-1 cells, therefore, the lp-PARPi stimulatory effects on p300, BETA2 or MafA activation of insulin promoter could be solely due to the increased MafA levels. We propose that loss of MafA is the main mechanism of glucose-suppression of insulin promoter activity, and lp-PARPi-induction of MafA protein levels may facilitate p300/PDX-1/BETA2/MafA binding complex formation. This study also suggests that lp-PARPi do not enhance insulin promoter activity through inhibition of CtBP, Sir2 or general deacetylases.

Chapter 7. General Conclusion and Future Studies

Chronic hyperglycemia alters the expression of genes in pancreatic β-cells (16, 95-97, 103, 154, 155, 166). Expression of β-cell specific genes, including insulin and PDX-1, GLUT2, glucokinase, voltage-gated Ca^{2+} channel, and K_{ATP} channel is decreased (16, 17, 38, 95, 96, 165). In this thesis, the expression of another β-cell specific gene, MafA, has been shown to be downregulated by chronic hyperglycemia. The decreased MafA protein levels by high glucose were due to reduced MafA mRNA levels. Interestingly, a group of compounds called lp-PARPi have been shown to increase insulin gene expression through the upregulation of MafA gene expression in INS-1 cells cultured in high glucose in this study.

The mechanisms whereby chronic hyperglycemia attenuates insulin promoter activity are not well understood. Recently, Lawrence et al has proposed that acute high glucose activates ERK1/2 and calcineurin through the increased intracellular Ca^{2+} levels, leading to NFAT and MafA binding complex formation at the A2/C1 elements and activation of insulin promoter activity (283) (Fig. 37). Chronic hyperglycemia also activates ERK1/2, but it stimulates NFAT and C/EBPβ binding complex formation at the A2/C1 elements and inhibition of insulin promoter activity (283) (Fig. 37). C/EBPβ is a known repressor of insulin promoter, and islet C/EBPβ protein levels have been shown to be increased during the development of type 2 diabetes in ZDF rats (97, 103). The present study demonstrated that MafA protein levels were reduced by chronic hyperglycemia. Therefore, I propose that chronic hyperglycemia decrease MafA protein levels but increase C/EBPβ protein levels, leading to increased C/EBPβ binding to insulin promoter and repression of insulin promoter activity. Indeed, increasing MafA

protein levels by lp-PARPi enhanced MafA binding to the C1 and A5/core elements, stimulating insulin promoter activity. Moreover, these data suggest that increased MafA may compete against C/EBPβ, leading to increased insulin promoter activity in INS-1 cells exposed with elevated glucose.

Lp-PARPi have also been shown to enhance MafA, BETA2 and p300 activation of insulin promoter under high glucose in this study. I propose that lp-PARPi stabilize binding complex formation at A2/C1/E1/A1 region mainly through the increased MafA protein levels (Fig. 37). Increased MafA may help to recruit BETA2 and PDX-1 to insulin promoter. BETA2 and PDX-1 are readily phosphorylated in INS-1 cells cultured with high glucose since high glucose can stimulate SAPK2, ERK1/2 and PI3K activities (43, 45, 46, 74). Phosphorylated BETA2 and PDX-1 can then recruit coactivators such p300/CBP to insulin promoter (43, 44), augmenting BETA2, PDX-1, and MafA activation of insulin promoter. Therefore, MafA plays a central role in stabilizing p300/PDX-1/BETA2/MafA complex formation. This hypothesis is supported by the observation that overexpression of MafA significantly potentiates p300/PDX-1/BETA2 activation of insulin promoter (59, 60). This hypothesis is also supported by the observation that increases of MafA protein levels by lp-PARPi or overexpression stimulates insulin promoter activity regardless of glucose concentrations in INS-1 cells. Further study should be performed to determine whether lp-PARPi enhance p300/PDX-1/BETA2/MafA binding complexes formation at insulin promoter with ChIP assay.

The present study also shows that the expression of MafA is glucose responsive. Consistent with this conclusion, acute increases in glucose concentrations enhance MafA nuclear protein and mRNA levels (25, 55). Moreover, the present study indicated that

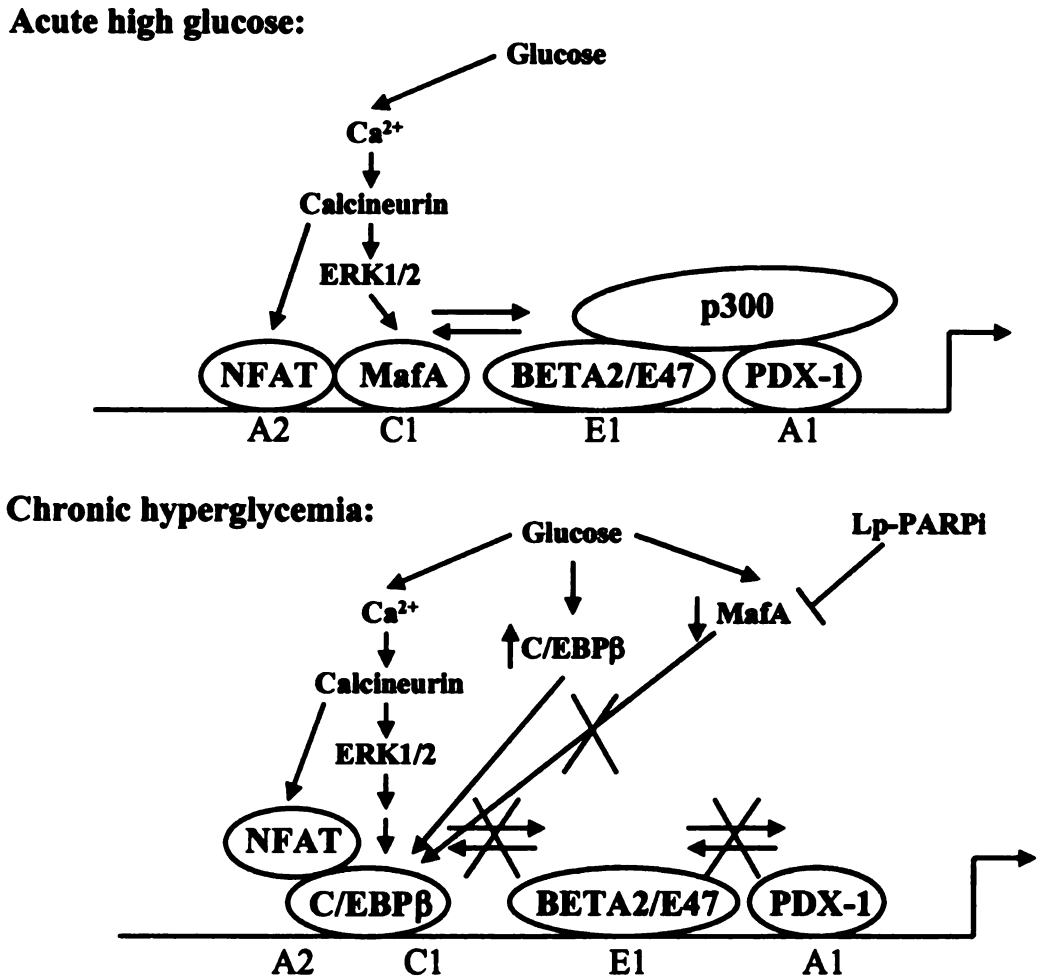


Figure 37. Model for glucose and lp-PARPi regulation of the A2/C1/E1/A1 region of insulin promoter activity. Acute high glucose activates ERK1/2 and calcineurin, leading to MafA/NFAT binding complex formation at A2/C1 elements. MafA binding enhances p300/BETA2/PDX-1 binding complex formation and activation of insulin promoter activity. During chronic hyperglycemia, MafA protein levels are low and C/EBPβ protein levels are high, resulting in increased binding of C/EBPβ to A2/C1 and reduced insulin promoter activity. Lp-PARPi increase MafA protein levels by stimulating MafA promoter. MafA competes against C/EBPβ for the binding at the C1 element. Increased MafA binding to the C1 element enhances p300/BETA2/PDX-1 binding complex formation at insulin promoter, increasing insulin promoter activity.

chronic hyperglycemia attenuatesd MafA gene expression by reducing MafA promoter activity, and lp-PARPi was proposed to stimulate MafA promoter activity. MafA promoter has not been characterized so far, thus one of future studies should be identifying cis-elements regulated by glucose and lp-PARPi in β-cells.

Lp-PARPi-induction of MafA may also play an important role in restoring β-cell phenotype during chronic hyperglycemic insult (Fig. 38). MafA deficient mice have reduced insulin, PDX-1, BETA2 and GLUT2 expression, and impaired GSIS (317), indicating that MafA is important in regulating the expression of these β-cell specific genes. I propose that lp-PARPi upregulate β-cell specific genes such as insulin, PDX-1, BETA2, and GLUT2 through the increased MafA gene expression. Since the β-cell specific genes mentioned above are all involved in insulin secretion, lp-PARPi may improve β-cell function. Consistent with this hypothesis, lp-PARPi improved basal insulin secretion as well as GSIS in INS-1 cultured in high glucose. Lp-PARPi have also been shown to increase GSIS in human fetal islets (11), and ZDF diabetic rats (4) and partial pancreatized dogs (3). The increased insulin gene expression and cellular insulin content account for the improvement of GSIS by lp-PARPi. Furthermore, lp-PARPi may increase PDX-1 gene expression since MafA has been shown to bind to PDX-1 promoter (318), suggesting that MafA may regulate PDX-1 promoter activity. Increased PDX-1 expression may lead to increased expression of GLUT2 and glucokinase since PDX-$1^{+/-}$ islets have reduced GLUT2 and glucokinase protein levels (23). In INS-1 cells cultured in high glucose, lp-PARPi induced MafA protein levels within 8 hrs. One will expect that increased MafA will lead to increases of nuclear PDX-1 protein levels. Alternatively, nuclear PDX-1 protein levels were not induced by lp-PARPi in INS-1 cells cultured in

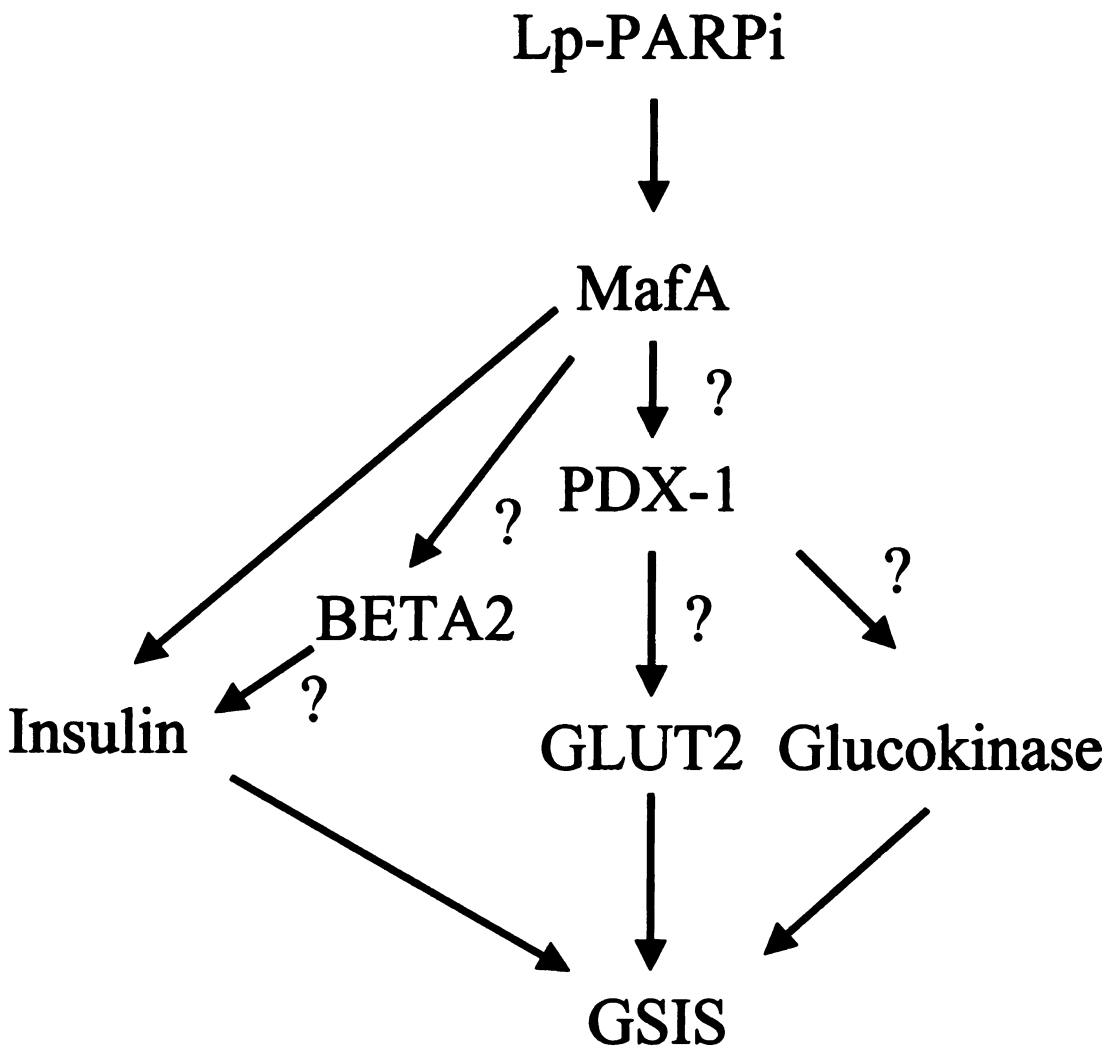


Figure 38. A schematic diagram describing the potential mechanisms of lp-PARPi in restoring β-cell phenotype. Lp-PARPi induce the expression of MafA, which can induce expression of insulin, BETA2 and PDX-1. Increased BETA2 may enhance insulin gene expression. Increased PDX-1 may induce GLUT2 and glucokinase protein levels in β-cells. The enhanced expression of insulin, GLUT2 and glucokinase may improve GSIS.

high glucose. This paradoxical observation could be explained by a slower induction of PDX-1 promoter activity by the gradual increase of MafA protein levels. Time course of lp-PARPi effects on PDX-1 gene expression should be performed to address this issue. Nevertheless, it should be worthy to investigate lp-PARPi effects on gene expression profile in INS-1 cells cultured in high glucose.

In summary, lp-PARPi increase insulin gene expression through the enhanced MafA gene expression. I propose that the lp-PARPi induction of MafA is one of the mechanisms that lp-PARPi restore β-cell phenotype during chronic hyperglycemic insult. The implication of this study is broad since NAM has been long used for the differentiating fetal islets, pancreatic precursor cells, and embryonic stem cells into insulin producing cells. Moreover, lp-PARPi have been shown to provide beneficial effects in several diabetic animal models. Further investigation of the mechanisms whereby lp-PARPi induce MafA gene expression will assist us to design more specific compounds to target the cellular processes affected by lp-PARPi.

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