

The anti-diabetic mechanisms by isoflavone genistein

By

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ABSTRACT

Diabetes is growing public health problem in the United States. Both in Type 1 and Type 2 diabetes, the deterioration of glycemic control over time is largely due to insulin secretory dysfunction and significant loss of functional β -cells. As such, the search for novel agents that promote β -cell survival and preserve functional β -cell mass are one of the essential strategies to prevent and treat the onset of diabetes. Genistein, a flavonoid in legumes and some herbal medicines, has various biological actions. It was recently shown that dietary intake of foods containing genistein improves diabetes in both experimental animals and humans. However, the potential anti-diabetic mechanisms of genistein are unclear.

In the present study, we first investigated the effect of genistein on β -cell insulin secretion and proliferation and cellular signaling related to these effects *in vitro* and *in vivo*. We then determined its anti-diabetic potential in insulin-deficient and obese diabetic mouse models. The results in our study showed that exposure of clonal insulin secreting (INS1E) cells or isolated pancreatic islets to genistein at physiologically relevant concentrations (1-10 μ M) enhanced glucose-stimulated insulin secretion (GSIS), whereas insulin content was not altered, suggesting that genistein-enhanced GSIS is not due to a modulation of insulin synthesis. This genistein's effect is protein tyrosine kinase- and K_{ATP} channel-independent. In addition, genistein had no effect on glucose transporter-2 expression or cellular ATP production, but similarly augmented pyruvate-stimulated insulin secretion in INS1E cells, indicating that genistein improvement of insulin secretion in β -cells is not related to an alternation in glucose uptake or the glycolytic pathway. Further, genistein (1-10 μ M) induced both INS1 and human islet β -cell

proliferation following 24 h of incubation, with 5 μ M genistein inducing a maximal 27% increase. The effect of genistein on β -cell proliferation was neither dependent on estrogen receptors, nor shared by 17 β -estradiol or a host of structurally related flavonoid compounds. Pharmacological or molecular intervention of PKA or ERK1/2 completely abolished genistein-stimulated β -cell proliferation, suggesting that both molecules are essential for genistein action. Consistent with its effect on cell proliferation, genistein induced cAMP/PKA signaling and subsequent phosphorylation of ERK1/2 in both INS1 cells and human islets. Furthermore, genistein induced protein expression of cyclin D1, a major cell-cycle regulator essential for β -cell growth. Dietary intake of genistein significantly improved hyperglycemia, glucose tolerance, and blood insulin levels in both insulin deficient type 1 and obese type 2 diabetic mice, concomitant with improved islet β -cell proliferation, survival, and mass. These changes were not due to alternations in animal body weight gain, food intake, fat deposit, plasma lipid profile, or peripheral insulin sensitivity. Collectively, these findings provide better understanding of the mechanism underlying the anti-diabetic effects of genistein.

Loss of functional β -cell mass through apoptosis is central to the development of both T1D and T2D and islet β -cell preservation and regeneration are very important components of β -cell adaptation to increased apoptosis and insulin resistance and therefore holds promise as a treatment for this disease. In this context, these findings may potentially lead to the development of novel low-cost natural agents for prevention and treatment of diabetes.

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ATTRIBUTIONS

Each author listed in this thesis has participated sufficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, and conduction of the experiments and for data interpretation.

In chapter 3, Zhuo Fu carried out the studies, performed sample and data analyses, and drafted the manuscript. Dongmin Liu participated in the design and data analysis of the study, coordinated the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

In chapter 4, Zhuo Fu and Wen Zhang carried out the studies, performed sample and data analyses, and drafted the manuscript. Wei Zhen and Zhengquan Jia helped to carry out the studies. Hazel Lum, Jerry Nadler, Josep Bassaganya-Riera, Yanwen Wang, and Hara Misra helped to draft the manuscripts. Dongmin Liu participated in the design and data analysis of the study, coordinated the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

In chapter 5, Zhuo Fu and Elizabeth R. Gilbert carried out the studies, performed sample and data analyses, and drafted the manuscript. Liliane Pfeiffer, Yangling Zhang, and Yu Fu helped to carry out the studies. Dongmin Liu participated in the design and data analysis of the study, coordinated the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

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LIST OF ABBREVIATIONS

A

AGEs advanced glycation endproducts
AMPK: AMP-activated kinase
ATF: activating transcription factor

B

BrdU: bromodeoxyuridine;

C

CaMK: calmodulin-dependent protein kinase
CAMPS: cAMP sensor
CHX: cycloheximide
ClC-3: chloride channel -3
CMRL: CMRL-1066 medium;
CPT-1: carnitine palmitoyl transferase-1
CRE: c-AMP response element
CREB: cAMP response element binding protein

D

DAG: diacylglycerol
DHAP: dihydroxyacetone phosphate
DHP: dihydropyridines
DMSO: dimethyl sulfoxide;

E

ER: estrogen receptor
ER: endoplasmic reticulum

F

FBS: fetal bovine serum
FFA: free fatty acid
FFAR-1: free fatty acid receptor -1

G

G6P: glucose-6-phosphate
GABA: Aminobutyric acid
GH: Growth hormone
GHR: growth hormone receptor
GIP: insulinotropic polypeptide
GISS: glucose-induced insulin release
GLP-1: glucagon-like peptide-1
GLUT2: glucose transporter 2
Gly3P: glycerol-3-phosphate
GPx: glutathione peroxidase
GS-3: glycogen synthase kinase

GSH: glutathione
GSIS: glucose-stimulated insulin secretion
GSSG: Oxidized GSH
gSUR: granule SUR
GTT: glucose tolerance test

H

HCSP: highly Ca²⁺-sensitive pool
HLH: a helix-loop-helix domain
HSL: hormone-sensitive lipase
HVA I: high voltage-activated

I

IAPP : islet amyloid polypeptide
IBMX: 3-isobutyl-1-methylxanthine
ICA512: islet cell autoantigen 512
IFN- γ : Interferon- γ
IGF-1: insulin-like growth factor-I
IL-1 β : interleukin-1 β .
iNOS: inducible nitric oxide synthesis
INS1E: insulin secreting cell line
IP3: inositol 1,4,5-trisphosphate
IRE: interferon response element
IRS-2: receptor substrate
ITT: insulin tolerance test

J

JNK: c-Jun NH₂-terminal kinase

K

K_{ATP} channel: ATP-sensitive potassium channel
KRBB: Krebs-Ringer bicarbonate buffer

L

LVA: low voltage-activated

M

MAPKs: mitogen-activated protein kinases
MCP-1: monocyte chemoattractant protein-1

N

NF- κ B: factor nuclear factor - κ B
NO: Nitric oxide
NOD: none-obese diabetic

O

OAA: oxaloacetate
ODNs: oligodeoxynucleotides

P

PDE: phosphodiesterase
PI3K: phosphatidylinositol 3-kinase
PIP2: phosphatidylinositol 4,5-bisphosphate
PKA: protein kinase A
PKB or Akt: protein kinase-B
PKC: protein kinase C
PKG: cGMP/cGMP-dependent protein kinase
PP1: protein phosphatase 1
PTBPs: Polypyrimidine tract binding proteins
PTK: protein tyrosine kinase

R

REM: Ras exchange motif
rER: rough endoplasmic reticulum
Rim2: Rab3-interacting molecule 2
RIPE3b: Rat insulin promoter element 3b
ROS : reactive oxygen species
RRP: readily releasable pool

S

SERCA-2b: sarcoendoplasmic reticulum Ca²⁺ ATPase type 2b
SHP: small heterodimer partner
SNAP-25: synaptosomal-associated protein of 25 kDa
SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOD: superoxide dismutase
SREBP1c: sterol-regulatory-element-binding-protein-1c
SRP: signal recognition particles
STZ : streptozotocin

T

T1D: type 1 diabetic
T2D: type 2 diabetic
TGs: Triglycerides
Th-1: T helper-1
TNF- α : tumor necrosis factor- α

U

UCP-2: uncoupling protein-2

X

XBP: X-box binding protein

CHAPTER ONE

Introduction

Background

Diabetes mellitus is a growing public health problem in the US and worldwide. It is estimated that at least 25.8 million or 8.3% of Americans presently suffer from diabetes, and 79 million people have pre-diabetes as of 2011 [1]. As reported in 2007, there are 5.4 million of the diabetic populations are type 1 diabetes as reported in 2010 [2], the dominant form of diabetes in children (>95%) [4]. While the availability of novel drugs, techniques, and surgical intervention has improved the survival rate of individuals with diabetes, the prevalence of diabetes is still rising in Americans, with the number of people with diabetes projected to double by 2025 [5].

Diabetic patients are categorized into type 1 (T1D), type 2 (T2D), and gestational diabetes. The gestational diabetic patients can be recovered completely after delivery. T1D is a T-cell-mediated autoimmune disease resulting from selective destruction of pancreatic β -cells. As of 2010, there is no known cure for this disease. Successful islet transplantation is a promising approach to T1D treatment. However, the lack of sufficient islets, loss of islet cell mass after islet isolation and potential autoimmune destruction of the transplanted islets prevent the widespread use of this procedure. In addition, islet transplantation is accompanied by significant side effects from the immunosuppressive drugs [6]. Therefore, the search for novel and cost-effective agents that can prevent or treat T1D is extremely important to decrease the burden of morbidity and mortality from this disease. T2D is a result of chronic *insulin resistance and loss of β -cell mass and function* [7]. Both in experimental animals and people, obesity is a leading pathogenic factor for developing insulin resistance, Constant insulin resistance will progress to T2D only when β -cells are unable to secrete adequate amount of insulin to compensate for decreased insulin sensitivity [7].

Therefore, pancreatic β -cells are playing central roles in glycemic regulation for both type 1 and type 2 diabetic patients. In obesity-linked type 2 diabetic patients it is proposed early obesity can stimulate β -cell growth and boost insulin secretory function to compensate for peripheral insulin resistance [8-11]. This explains why 70–75% of obese individuals do not develop type 2 diabetes [12]. An associated compensatory increase in β -cell function and mass was reported to cope with the increased demand of insulin [13]. With the increase of time and/or severity of insulin resistance, a decline in β -cell adaptability appears, which will lead to decreased β -cell mass and function. Together with β -cell destruction caused by hyperlipidemia as well as inflammations, this will result in an inadequate supply of insulin which results in failure of compensation for insulin resistance, and diabetes ensues [9, 14, 15]. In contrast, type 1 diabetes is characterized by autoimmune destruction of β -cells by infiltrated CD4+ and CD8+ T cells and macrophages [16], which is free of peripheral insulin resistance. The mechanisms involved in the destruction of β -cell include direct lysis of β -cells by CD8+ T cells or other lytic cells such as macrophages as well as the damaging effect of cytokines produced by T-lymphocyte cells. β -cell destruction can also be induced by non-T cells that release innate inflammatory mediators such as interleukin (IL)-1 β . IL-1 β , also secreted by T helper (Th)-1 cell, can damage β -cell directly and break immune tolerance by activating adaptive response [17-23]. Thus both in type 1 and type 2 diabetes loss of β -cell mass and/or function is the origin of diseases. Therefore, to increase β -cell mass by limiting apoptosis or by promoting β -cell growth and/or to maintain residual β -cells insulin secretory function provides promising strategies in prevention and treatment of both type 1 and type 2 diabetes.

Genistein, a major isoflavone in soy, received considerable attention over the past 10 years, because of its protective function against multiple chronic diseases, such as cardiovascular

disease [24], osteoporosis [25, 26], and certain hormone related cancers [27, 28]. It was well-established that genistein is a weak estrogen receptor agonist [29], and a tyrosine kinase inhibitor [30]. Studies on whether genistein has an effect on diabetes are very limited, and carefully controlled studies to determine this effect are lacking. Recent studies performed in animals and humans have shown that ingestion of isoflavones containing soy protein moderates hyperglycemia [31, 32]. However, it is not clear whether isoflavones primarily contributes to this beneficial effect. Emerging studies reported that administration of isoflavones lowered plasma glucose in diabetic animals [33, 34] and postmenopausal women [35] independent of its effect on food intake or weight gain, suggesting that genistein may be a novel plant-derived anti-diabetic agent, although the mechanism whereby genistein exerts such a beneficial effect on diabetes is unknown. Although studies are limited, the available data shows that genistein may have a direct effect on pancreatic β -cells. Several earlier studies demonstrated that genistein stimulates insulin secretion from a clonal pancreatic β -cell line [36] and cultured islets [37, 38] while other studies have found an inhibitory effect on insulin secretion [39, 40]. These discrepant data may be the result of variations in the experimental conditions and model used. We recently in our lab discovered for the first time that genistein at physiologically achievable concentrations (0.1-5 μ M) activated cAMP/PKA signaling by stimulating adenylate cyclase activity in β -cells and islets [41]. cAMP as second messenger is critically involved in many signaling pathways of insulin synthesis and secretion [42-44]. It is also actively involved in β -cell survival [45, 46]. Therefore it is promising to investigating that if genistein could have an anti-diabetic effect via promoting β -cell insulin secretory function and β -cell survival. Therefore, in this dissertation research, studies are designed to elucidate the fundamental role for genistein in the regulation of β -cell function and survival.

Hypotheses

Recently, emerging studies reported that administration of isoflavones lowered plasma glucose in diabetic animals [33, 34] and postmenopausal women [35] independent of its effect on food intake or weight gain, suggesting that genistein may be a novel plant-derived anti-diabetic agent. However most published trials using isoflavones or genistein have focused largely on elucidating the effect of isoflavones on lipid profiles, and therefore data from recent studies suggest an anti-diabetic effect of genistein presumably by a hypolipidemic effect [34], thereby increasing insulin sensitivity. But studies investigating genistein's effect on plasma lipid profiles have shown either only a moderate positive effect [47-50] or a neutral effect [51-54]. Additionally, the result of a recent meta-analysis by the American Heart Association showed that isoflavones have no significant effect on human plasma lipids [55]. Indeed, studies demonstrated that isoflavone administration lowered plasma glucose, but lipid profile or insulin sensitivity was unaffected in obese and diabetic animals [33] and humans [35]. These data therefore do not support the concept that isoflavones exerts an anti-diabetic effect through lowering plasma lipids or increasing insulin sensitivity. Previous study in our lab had shown that genistein at physiologically achievable concentrations (0.01-5.0 μ M) acutely activates cAMP/protein kinase A (PKA) signaling by stimulation adenylyl cyclase activity, leading to rapid insulin secretion both in clonal β -cells and mouse islets [56]. These genistein effects are not related to estrogen receptors or protein tyrosine kinase inhibition. However it is not clear if genistein's anti-diabetic effect is mediated by potentiating β -cell secretory function directly in physiological conditions, given the extended exposure time of β -cells after genistein ingestion. Thereby as the first project of my study, **I tested the hypothesis if genistein could enhance β -cell insulin secretory**

function after long term exposure in vitro and ex vivo, and if the potentiating effect is associated glucose uptake or glucose metabolism.

Recent studies provide evidence that β -cells have the potential to regenerate by proliferation of pre-existing β -cells in both physiological and pathological conditions [57, 58]. As such, a strategy that induces β -cell proliferation, thus preserving functional β -cell mass, could be one of the essential strategies to prevent the onset of diabetes [7, 57, 59-62]. Several earlier studies reported that genistein directly acts on β -cells, leading to insulin secretion [36, 37], whereas other studies have found an inhibitory effect [39, 40]. As mentioned above we had discovered that genistein is a cAMP signaling agonist by activation of adenylate cyclase in pancreatic β -cells [56]. It has been recently shown that several growth factors induce β -cell proliferation and exerts their anti-diabetic effects via activation of cAMP signaling [45, 46]. Therefore, secondly, **I tested the hypothesis if genistein could promote β -cell cell proliferation in vitro as well as in streptozotocin (STZ) induced diabetic mouse model and thereby it could prevent diabetes in this diabetic mouse model.**

Although massive and functionally β -cell loss are the common feature shared between type 1 and type 2 diabetes, insulin resistance, dyslipidemia, and their adverse effect on β -cells are pathogenic factors that are unique to type 2 diabetes [20]. It is not clear if genistein's beneficial effect on β -cell's function and survival could be preserved with insulin resistance and dyslipidemia stepping in with type 2 diabetes. **Therefore, as the third project of my study, I tested the hypothesis that genistein can improve β -cell mass and prevent or ameliorate hyperglycemia in obese diabetic mice.**

Abbreviation

IL-1 β : interleukin-1 β .
PKA: protein kinase A
STZ: streptozotocin
T1D: type 1 diabetes
T2D: type 2 diabetes
Th-1: T helper-1

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CHAPTER TWO

Literature Review

Abstract

Pancreatic β -cell dysfunction plays an important role in the pathogenesis of both type 1 (T1D) and type 2 diabetes (T2D). Insulin is a critical regulator of metabolism and is produced exclusively in β -cells. Insulin is synthesized as preproinsulin and processed to proinsulin. Proinsulin is then converted to insulin and C-peptide and stored in secretory granules awaiting release on demand. Insulin synthesis is regulated at both the transcriptional and translational level. The cis-acting sequences within the 5' flanking region and trans-activators including paired box gene 6 (PAX6), pancreatic and duodenal homeobox-1(PDX-1), MafA, and B-2/Neurogenic differentiation 1 (NeuroD1) regulate insulin transcription, while the stability of preproinsulin mRNA and its untranslated regions (UTRs) control protein translation. Insulin secretion involves a sequence of events in β -cells that lead to fusion of secretory granules with the plasma membrane. Insulin is secreted primarily in response to glucose, while other nutrients such as free fatty acids and amino acids can also augment glucose-induced insulin secretion. In addition, various hormones, such as melatonin, estrogen, leptin, growth hormone, and glucagon like peptide-1 also regulate insulin secretion. Thus, the β -cell is a metabolic hub in the body, connecting nutrient metabolism and the endocrine system. Although an increase in intracellular $[Ca^{2+}]$ is the primary insulin secretory signal, recent studies show that the cAMP signaling-dependent mechanisms are also critical in the regulation of insulin secretion. This chapter reviews the current knowledge on how β -cells synthesize and secrete insulin. In addition, this

review presents evidence that genetic and environmental factors can lead to hyperglycemia, dyslipidemia, inflammation, and autoimmunity, resulting in islet β -cell dysfunction, thereby triggering the pathogenesis of diabetes.

1 Pancreatic β -cell physiology

1.1 Insulin biosynthesis

The secreted insulin consists of 51 amino acids with a molecular weight of 5.8 kDa. However, the insulin gene encodes a 110-amino acid precursor known as preproinsulin. As with other secreted proteins, preproinsulin contains a hydrophobic N-terminal signal peptide, which interacts with cytosolic ribonucleoprotein signal recognition particles (SRP) [1]. SRP facilitates preproinsulin translocation across the rough endoplasmic reticulum (rER) membrane into the lumen. This process occurs via the peptide-conducting channel [2, 3], where the signal peptide from preproinsulin is cleaved by a signal peptidase to yield proinsulin [4]. Proinsulin then undergoes folding and formation of three disulfide bonds [5], a process requiring a diverse range of endoplasmic reticulum (ER) chaperone proteins such as the protein-thiol reductase. [6]. Subsequent to maturation of the three dimensional conformation, the folded proinsulin is transported from the ER to the Golgi apparatus. In the Golgi apparatus, proinsulin enters immature secretory vesicles, where it is cleaved to yield insulin and C-peptide. Insulin and C-peptide are then stored in these secretory granules together with islet amyloid polypeptide (IAPP or amylin) and other less abundant β -cell secretory products [7, 8].

Although insulin biosynthesis is controlled by multiple factors, glucose metabolism is the most important physiological event that stimulates insulin gene transcription and mRNA translation [9]. In 3-day fasted rats, glucose injection could increase relative proinsulin mRNA

levels by three- to four-fold within 24 h, and this effect can be blocked by pharmacological inhibition of transcription with actinomycin D [10]. This study suggests that glucose plays a central role in regulation of insulin biosynthesis which is controlled at least partially via alterations in proinsulin mRNA expression. In addition, glucose is an important factor for maintaining insulin mRNA stability. In vitro studies have shown that insulin mRNA stability reduced under lower glucose concentrations and increased under higher glucose concentrations [11, 12]. Interestingly, elevation of intracellular cAMP levels can prevent this reduction [13].

Most animals have only a single copy of the insulin gene, but rodents have two non-allelic insulin genes (insulin I and II). They differ in their number of introns and chromosomal locations [14]. In all insulin genes the 5'-flanking region determines its tissue- and cell-type-specific expression [15]. The transcriptional factor binding sites that determine insulin's exclusive expression in β -cells are located between -520 and +1 base pairs (bp) relative to the transcription initiation site both in rat and human insulin genes [9, 15, 16]. Among mammalian insulin genes, there is a conserved sequence located from -350 bp to the initiation site, which controls cell-type-specific expression of insulin. Most transcriptional regulation occurs through interactions within these conserved sequences. Studies have shown that the sequence between -340 and +91 is the major insulin gene transcription enhancer region, which determines cell-specific and glucose-regulated insulin gene expression [17-21].

1.2 regulation of insulin transcription

Insulin biosynthesis is regulated both at transcriptional and translational levels. In a mouse β -cell, there are 13,000 insulin granules on average. They occupy more than 10% of the total cell volume [22]. Each granule contains approximately 200,000 insulin molecules [23].

However, insulin content in β -cells is highly dynamic. Insulin accumulates in the presence of nutrients and decreases in response to nutrient deprivation. The ability of β -cells to quickly respond to cellular signals is generally due to transcriptional regulation. A number of discrete sequence elements within the promoter region of insulin gene, named A, C, E, Z, and CRE elements determine localization of insulin in β -cells and also serve as binding sites for several β -cell transcription factors to regulate insulin gene expression [24]. The transcription factor binding sites that are located within a region spanning \sim -400 base pairs (bp) relative to the transcription initiation site are determinants of β -cell-specific expression of insulin [24].

A number of cis- and trans- transcriptional factors are associated with the activation of the insulin enhancer region. In all characterized insulin enhancer sequences the A, C, and E elements are contained in core binding motifs [25].

A elements: The A elements are multiple A/T rich elements located in the conserved control region of insulin gene [26]. There is a TAAT core in each of these A elements that serves as the central DNA binding recognition motif for homeodomain proteins [27, 28], including duodenal homeobox-1(PDX-1) [29-31], Cdx2/3 [32], and Isl-1 [33]. PDX-1 is the predominant binding factor detected with insulin A element probes in pancreatic β -cell extracts [29, 34, 35]. . This factor was first characterized as an insulin [29, 34-36] and somatostatin [43,44] transcriptional factor. The expression of PDX-1 in adult pancreas is essentially restricted to islet β -cells (\sim 91%). Only a small subset of δ -cells (\sim 15%) express PDX-1 and its levels in exocrine acinar cells are extremely low [31, 36-38]. The Cdx2/3, while expressed in β -cells and α -cells, appears to play a less important role in islet function, since Cdx2/3 mutant mice only have defects in intestinal function [39]. The Isl-1 is present in all types of islet cells [40] and can

activate somatostatin [41], glucagon [42], and IAPP [43] gene expression. It also plays an essential role in islet formation during embryo development [44].

C element: There are two C elements in insulin gene promoter. The C1 element is located between approximately -118 and -107 bp upstream of the insulin transcription start site [45]. Rat insulin promoter element 3b (RIPE3b)1 and RIPE3b2 [45, 46] are two factors that form protein-DNA complexes within the C1 element. RIPE3b2 consists of the p58, p62, and p110 subunits [47]. RIPE3b2 do not contribute to β -cell-specific expression of the insulin gene [45, 47], and RIPE3b2-binding activity is present in a variety of other tissues [45]. The DNA-binding component of the RIPE3b1 was recently identified as MafA [48-50], which is expressed exclusively in β -cells [51]. MafA also mediates glucose-regulated and fatty acid-inhibited insulin expression. Prolonged exposure of islets to fatty acid and high glucose inhibits insulin gene transcription by impairing nuclear cellular expression of MafA. [52-54]. MafA deficient animals had no defects in β -cell development, but the impaired insulin expression in adult islets was observed [55].

The C2 element, which is located at -317/-311 bp in the rat I insulin gene, was termed the pancreatic islet cell enhancer sequence (PISCES). It was found to contribute to insulin, glucagon, and somatostatin transcription in α -, β -, and ϵ -cells, respectively [56]. Later studies further showed that PISCES is a binding site for PAX6 both in insulin and glucagon genes [57]. Like other PAX transcriptional factors, PAX6 contains a paired box bipartite DNA binding domain. PAX6 is required for normal transcription of insulin genes and islet development [57]. Besides PAX6, PAX4 is also a paired/homeodomain protein expressed in the pancreas. Although both PAX4 and PAX6 can bind to PISCES [58], PAX4 is only detected transiently in β -cells during early development and absent in adult β -cells [59]. PAX4 is reported to suppress PAX6-

induced trans-activation; however it is not clear if PAX4 regulates insulin expression in vivo because of its rare existence.

E element: The E elements (5'-GCCATCTG-3') are two separated mini-enhancer units within the insulin enhancer [17, 45, 60]. Rodents have two E elements (-241 to -233 bp and -112 to -104 bp) in the insulin I gene; while other mammals have only one (approximately -100 to -91 bp) [25]. The core insulin E element (5'-CANNTG-3') is also found in the heavy-chain immunoglobulin and muscle creatine kinase control elements [61-63]. The factors that activate E element are composed of a helix-loop-helix domain (HLH) that is important in facilitating protein-protein interactions, and a contiguous amino terminal basic region (b) that is necessary for DNA-protein binding. This motif is shared by a number of transcriptional factors required in cell type determination including the muscle determination proteins MyoD [64], Myf-5 [65], myogenin [66], and the proteins of the drosophila achaete-scute complex, which are important in neural development [67]. The E element activators include BETA2/NeuroD1, E2/5, E12, and E47. BETA2/NeuroD1 is enriched in islets [68, 69], while E2/5, E47 [45, 70, 71], and E12 [72] are widely distributed. BETA2/NeuroD1 is important in regulating insulin gene expression and β -cell survival, and the endocrine pancreas-specific deficiency of BETA2/NeuroD in mice causes massive β -cells apoptosis and subsequent diabetes and early death [73, 74].

Z element: The Z element is located upstream of the A element (-292 to -243 bp) and is unique to human insulin. A glucose-sensitive DNA-binding complex termed ZaI binds to the region of -287 to -271 bp within the Z element in primary islet cells [75]. Z element also functions as a transcriptional repressor in transformed β -cell lines and primary fibroblast cells [75, 76]. Recent studies show that A element activation depends on the presence of the Z element

[77]. PDX-1 and MafA regulate insulin gene transcription through activation of the Z element [78].

Cyclic AMP response element (CRE): Human insulin gene promoter contains four CRE sites: CRE1 at -210 bp, CRE2 at -183 bp, CRE3 at +18 bp, and CRE4 at +61 bp [79]; within the core of each CRE there is a sequence similar to the CRE consensus sequence [80]. A variety of transcription factors can regulate insulin gene transcription by binding to the consensus CRE sequence of 5'-TGACGTCA-3' [80]. These transcriptional factors are members of CRE binding protein (CREB)/ATF family [81]. The CREB/ATF family of transcription factors are basic region leucine zipper (bZIP) proteins that share a common cluster of basic amino acids at the N-terminus of the bZIP domain, which binds to the CRE site to initiate insulin transcription [82].

Modes of gene regulation could be species-specific, and thus interpretation of data from animal models and extrapolation to humans must be exercised with caution. For example, hepatocyte nuclear factor (HNF)-1 [83] and Isl-1 [84] can bind to the A elements to stimulate rat insulin-I gene transcription. In addition, Cdx-3 [32] and HMGI(Y) [85] bind specifically to the A3/A4 element, which is unique to rat insulin-I. Besides regulation at the gene promoter regions as described above, control of ER load, granule counting, and cell cooperation provide essential feedback loops for controlling insulin transcription [86], which will be further elaborated in detail in this chapter.

1.3 Regulation of insulin translation

In response to nutrients, β -cells enhance their overall speed of protein translation, which might be controlled by dephosphorylation of eukaryotic initiation factor 2a (eIF2a) via protein phosphatase 1 (PP1) [87]. For example, exposure of β -cells to high glucose for 2 hours

significantly decreases the ratio of phosphorylated eIF2a to eIF2a [88]. However, there should be additional mechanisms to regulate glucose-induced insulin translation, because a study found that overall protein translation induced by glucose in β -cells was only increased by about 3-fold, while proinsulin translation was elevated by up to 8-fold compared to the fasting state [89].

The pancreatic ER kinase (PERK), plays an important role in regulating translational events of insulin. It phosphorylates eIF2a [90], thereby regulating insulin translation [91]. PERK phosphorylation of eIF2a can be partially compensated for by other kinases [92, 93]. PERK mutation results in Wolcott-Rallison syndrome associated with permanent neonatal diabetes in humans [94]. PERK-deficient mice not only develop severe defects in insulin synthesis, but also in β -cell proliferation and differentiation, leading to permanent neonatal diabetes as seen in humans. Interestingly, PERK expression in β -cells at the adult stage seems not important for maintaining β -cell functions [95]. The Wolfram Syndrome gene WFS1, taking its name from the Wolfram syndrome, is unregulated by ER stress via Inositol-Requiring Protein 1(IRE1)-a and PERK [96]. [97]. At the beginning of glucose exposure, IRE1 stimulates insulin synthesis via WFS1, while after prolonged exposure, it might reduce insulin production via X-box-binding protein 1 (XBP1) [98]. The β -cells have evolved a mechanism to detect the amount of insulin stored and secreted and adjust insulin synthesis accordingly. A granule transmembrane protein called islet cell autoantigen 512 (ICA512), is a crucial part of this feedback control. Insulin granules travel a long distance on tubulin tracks before arriving to the peripheral actin network [99]. Before becoming linked to the cytoskeleton, insulin granules are anchored to actin cortex via ICA512 and β 2-synthrophin [100]. Upon activation, the granule membrane fuses transiently to the cell membrane to release insulin. Elevated Ca^{2+} level in the mean time activates the protease μ -calpain to cleave away a cytosolic fragment from ICA512. The free ICA512 cytosolic

fragment then moves to the nucleus and binds to the tyrosine-phosphorylated transcriptional factor STAT5 to prevent STAT 5 from dephosphorylation, which in turn upregulates insulin transcription [101]. Nuclear free ICA512 cytosolic fragments also bind to sumoylating enzyme PIAS γ . The sumoylation of ICA512 by PIAS γ reverses the binding of ICA512 to STAT5 [101]. Hence, the release of insulin from secretory granules is communicated to the nucleus, which serves as a positive feedback mechanism to initiate insulin translation for maintaining an adequate amount of stored insulin.

In addition to transcriptional regulation, β -cells are also able to adjust insulin production in response to immediate environmental triggers through regulating the speed of insulin translation. For example, a study showed that exposure of rat islets to 25 mM glucose for 1 hour can increase intracellular proinsulin levels by up to ten-fold from baseline (2.8 mM glucose), whereas proinsulin mRNA quantities remained the same [102]. Consistently, an earlier study had shown that this acute glucose-stimulated insulin synthesis is independent of mRNA synthesis within the first 45 min because blockage of transcription only slowed insulin accumulation after that time frame [103]. In addition, insulin mRNA stability, which is subject to nutrient state, is an important factor that influences insulin protein synthesis [10]. In vitro studies have shown that insulin mRNA stability decreases under lower glucose concentrations, while increases under high glucose conditions [11, 12]. In the absence of glucose, insulin mRNA levels in β -cells decrease sharply, which could be reversed by elevation of intracellular cAMP levels [13]. The same phenomena were observed in animal studies. Rats fasted for 3 days have only 15-20% of the levels of pancreatic insulin mRNA measured in the fed control animals [10]. Therefore post-transcriptional regulation controls the modulation of immediate insulin synthesis, while the regulations at transcription level contribute to the modulation of delayed insulin synthesis.

Polypyrimidine tract binding proteins (PTBPs) are the proteins that regulate mRNA translation. They are involved in exon repression while mRNA is undergoing splicing in nuclei and stabilization and ribosome recruitment in the cytosol [104-106], They upregulate translation both by extending mRNA viability and by stimulating the initiation of translation. Cytosolic PTBP1 binds to a CU-rich sequence in the 3' UTR of proinsulin, which stabilizes proinsulin mRNA [104-106]. PTBP1 also upregulates translation of several insulin granule proteins. PTBP1 binding to ICA512 mRNA decreases 3' UTR decay. Deletion of the PTB binding site substantially reduced prohormone convertase 2 (PC2) translation. Insulin and insulin granule mRNA share a similar affinity to the RNA binding protein PTBP1, which enables their gene-specific activation by glucose. It was recently found that there is a conserved region (40-48nt) from the 5' UTR of proinsulin mRNA which plays an essential role in glucose regulation of proinsulin translation, because removal of this region blocked glucose-stimulated proinsulin translation [89].

1.3 Regulation of insulin secretion

Insulin is an important hormone required for normal metabolism. In healthy subjects, insulin is released in exquisitely exact amounts to meet the metabolic demand. Specifically, β -cells sense changes in plasma glucose concentration and respond by releasing corresponding amount of insulin [107]. To sense the nutritional state, β -cells are clustered in islets that strategically connect to the vasculature. Islets form a dense network with small blood vessels and receive 10 times the amount of blood than cells in the surrounding exocrine regions. Capillaries surrounding islets show a remarkable number of small pores called fenestrae that allow for a greater nutrient exchange between the circulation and surrounding tissues. This structure

enhances permeability, allowing unrestricted nutrient access so that β -cells can sense the nutritional state quickly. Fenestrations also permit rapid insulin diffusion into the blood [86].

Glucose and insulin secretion: The β -cells respond to many nutrients in the blood circulation, including glucose, other monosaccharides, amino acids, and fatty acids. Glucose is evolutionarily the primary stimuli for insulin release, because it is a principal food component, and can accumulate immediately after food ingestion, and is the obligate fuel source for a number of cells [86]. Indeed, the amplitude of insulin secretion induced by glucose is much larger compared with that stimulated by protein and fat. Approximately oral ingestion of 75 g of glucose will cause plasma insulin to rise from a basal level (20-30 pmol/L) to 250-300 pmol/L in 30 min, while intake of a similar amount of fat or a fat plus protein diet will only increase plasma insulin levels to 50 and 60 pmol/L, respectively in human subjects [108].

β -cells do not appear to contain membrane-bound glucose receptors but are equipped with several sensing devices that measure circulating glucose. Glucose transporter 2 (GLUT2), constitutively expressed in β -cells, is the first encountered glucose sensor in β -cells. Glucose equilibrates in β -cells via GLUT2-mediated facilitated diffusion. GLUT2 is the only form of glucose transporter expressed in β -cells. It is also expressed in the liver, and to a lesser extent, in renal and intestinal absorptive cells. Unlike GLUT4, which is primarily expressed in muscle and fat cells, mobilization of GLUT2 to the plasma membrane is insulin-independent and it has a low substrate affinity, ensuring high glucose influx. After entering β -cells, glucose is phosphorylated by the rate-limiting enzyme glucokinase, a subtype of hexokinase. Glucokinase is expressed in only four types of mammalian cells: hepatic cells, β -cells, enterocytes, and glucose-sensitive neurons [86]. Two important properties enable glucokinase to function as a glucose sensor in β -cells, distinguishing it from other hexokinases. The first property is its relatively lower affinity

for glucose than other hexokinases. Its K_m is only 6 mmol/L, falling in the middle of the normal blood glucose range (4-10 mmol/l), while other hexokinases function at maximal velocity at this glucose concentration. The second property is that it is not inhibited by its product, often a regulatory feature in metabolism. This feature enables its continued activity despite of high glycolysis load. Glucokinase is thus the rate-limiting step in β -cell glucose metabolism and it is considered to be an important glucose sensor [86].

Following glycolysis, glucose is further hydrolyzed to generate pyruvate, which is oxidized through the tricarboxylic acid *cycle* by mitochondria in β -cells to produce ATP. In other type of cells, pyruvate can be converted to lactate by pyruvate dehydrogenase. However, because β -cells are lack of this enzyme, pyruvate is mainly metabolized to produce metabolic coupling factors through 2 routes: 1) After metabolized to acetyl-coA it enters glucose oxidation and 2) anaplerosis. Pyruvate oxidation through the tricarboxylic acid *cycle* (TCA) by mitochondria is the major signaling pathway coupled to “ATP-sensitive potassium (K_{ATP}) channel-dependent insulin release”, which increase intracellular ATP/ADP ratio, sequentially leading to closure of K_{ATP} channels, depolarization of the plasma membrane, opening of voltage-dependent Ca^{2+} channels, influx of Ca^{2+} , and eventual activation of exocytosis of insulin-containing granules. Anaplerosis serves to replenish the carbon pool in the TCA cycle. After the cycle is filled with intermediates, these carbons can exit via cataplerosis. Some products derived in these processes can act as insulin secretion signals, which include NADPH, malonyl-CoA, and glutamate. These molecules reportedly amplify K_{ATP} channel-dependent insulin secretion [109, 110]. A third glucose signal results from the formation of glycerol-3-phosphate (Gly3P). After glucose is phosphorylated into glucose-6-phosphate (G6P) by glucokinase, G6P can enter glycolysis to generate pyruvate . It can also be metabolized into dihydroxyacetone phosphate (DHAP) part of

the way through the pathway to provide Gly3P. Gly3P is important for generating lipid metabolic coupling factors such as long-chain acyl-CoA and diacylglycerol (DAG), which augments insulin secretion. Gly3p/DAG is an alternative pathway that is independent of mitochondria metabolism of glucose to produce metabolic coupling factors to stimulate insulin release. Gly3P can also replenish NAD⁺ for promote β -cell glycolysis via the mitochondrial Gly3P NADH shuttle process to activate mitochondrial energy metabolism to trigger insulin secretion[111, 112].

Amino acids and insulin secretion: Individual amino acids at physiological concentrations are poor insulin secretagogues. However, certain combinations of amino acids at physiological concentrations or higher can augment GSIS [113]. For example, glutamine alone does not stimulate insulin secretion or enhance GSIS, but a combination of glutamine with leucine can enhance GSIS from β -cells [114]. Leucine can activate glutamate dehydrogenase, which converts glutamate to α -ketoglutarate. Glutamine, after converted into glutamate by glutaminase in the cytosol, can enter the TCA cycle via α -ketoglutarate, which results in ATP production, thereby insulin secretion [113]. Without leucine, glutamine is only metabolized to γ -aminobutyric acid (GABA) and aspartate. Moreover, some amino acids can indirectly influence β -cell insulin secretion. During the fasting period, proteins in skeletal muscle are catabolized and amino acids are subsequently metabolized for generating energy. Free amino acids, including alanine and glutamine, are released into the blood and serve as potent glucagon secretagogues. This results in elevation of blood glucose levels, which then trigger insulin secretion. Dietary amino acids can also induce insulin secretion via incretin-dependent mechanisms. Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are the two major incretin hormones secreted from the gastrointestinal tract. Ingestion of nutrients in the gut, including

glucose and amino acids, stimulates secretion of these hormones from intestine K-cells and L-cells. These hormones then directly act on β -cells by binding to their specific receptors located on cell surface, augmenting GSIS [115-117].

Fatty acids and insulin secretion: Free fatty acids (FFAs) also influence β -cell secretion of insulin. They potentiate insulin secretion to compensate for increased insulin need because of insulin resistance in type 2 diabetes [118-120]. FFA can also enhance GSIS. Islets deprived of fatty acids lose GSIS, which can be reversed by replacement with exogenous fatty acids [121-123]. Recently, it was discovered that β -cells have a free fatty acid receptor, free fatty acid receptor (FFAR)-1, through which FFA can influence β -cell function [124, 125]. The intracellular metabolism of FFA is the source for synthesis of lipid signal molecules such as long-chain acyl-CoA [120] and DAG [120, 126]. Long-chain acyl-CoA could acylate essential proteins in insulin granule fusion, such as synaptosomal-associated protein-25 (SNAP-25) and synaptogamin [127, 128]. DAG is an important molecule in activating protein kinase C, which is implicated in insulin secretion [129]. It also binds to synaptic vesicle priming protein Munc-13 to promote insulin secretion [130]. A schematic illustration of nutrient regulated insulin secretion was shown in Fig 1.

1.4 Cellular signaling transduction pathways in regulation of insulin secretion

Insulin secretion is a process that involves the fusion of insulin granules with the plasma membrane and exocytosis of granule content. Insulin secretion shows a characteristic biphasic pattern that consists of a transient first phase followed by a sustained second phase. In humans, when plasma glucose is ~ 7 mM, first phase insulin secretion peaks at 1.4 nmol/min. The first phase lasts for ~ 10 min and it is then followed by the second phase with the secreting rate at ~ 0.4

nmol/min [131]. However, the biphasic pattern is less prominent in mice than in rats and humans. This might be explained by the relatively higher basal plasma blood insulin levels in mice (8-9 mmol/L in mice vs. 4-5 mmol/L in rats and humans) [132, 133]. Thus, even insulin secretion is induced by 10 mM glucose in mouse islets, the clear peak of a first phase of insulin release was missing in mouse. It is likely that biphasic insulin secretion and insulin exocytosis have the same cellular background. Although no clear boundary exists, insulin granules can be categorized into distinct functional pools [134, 135]. A small fraction of the granules (1%) are immediately available for release, named the readily releasable pool (RRP), which contribute to the rapid insulin release triggered by glucose [136]. The remaining granules (99%) belong to the reserve pool. When the RRP depletes, it is refilled from the reserve pool. Granules in reserve pool have to undergo the preparatory reactions before becoming a RRP granule. The priming process, involving both granule modification and translocation toward the plasma membrane, is the rate limiting step for insulin exocytosis. The following observations suggest the relationship between biphasic insulin secretion and pools of granules: 1). Both the first phase of insulin secretion and the exocytosis from RRP can occur even in the absence of nutrients, while both the second phase of insulin secretion and RRP replacement are strictly metabolic-product-dependent; 2), The total number of granules in RRP is positively related to the amount of insulin released in the first phase of secretion [137]; and 3). Ablation of Munc13-1 selectively suppresses second phase insulin secretion and insulin granule exocytosis, but does not affect the first phase and insulin exocytosis from the RRP [138, 139]. However, there is discrepancy in kinetics. The replacement of RRP is in less than 1sec, while the first phase of insulin secretion can last for about 10 min.

Several proteins participate in insulin exocytosis. The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) plays an essential role in insulin granule membrane

fusion. Four SNARE motifs form the extremely stable helical β -cell exocytotic core complex. The central part of this complex contains four highly conserved amino acids contributed by the four SNARE motifs: three glutamine (Q), and one arginine (R) residue [140]. In β -cells, the fusion of the insulin granules with the plasma membrane involves the assembly of a complex consisting of VAMP-2 (R-SNARE) on the granule membrane, syntaxin-1a (Qc-SNARE) on the plasma membrane, and the membrane-associated protein SNAP-25 (Qa-Qb SNARE). The assembly of this complex can be regulated by other accessory factors to achieve elegant regulation of insulin granule fusion. Tomosyn-1 is such a regulatory factor that can replace VAMP2 in the process of assembly [141]. It is required for granule fusion and/or priming of the granules, but its absence does not influence insulin granule transport and docking [142, 143].

The priming and fusion of insulin granules, which results in insulin exocytosis, is triggered by elevation of intracellular $[Ca^{2+}]$. Insulin exocytosis can proceed at the rate of 500 granules per second when intracellular $[Ca^{2+}]$ is increased to 17 mmol/L, but it only proceed at the rate of 3-4 granules per second when $[Ca^{2+}]$ is at 0.17 mmol/L. Exocytosis occurring at low $[Ca^{2+}]$ is due to a small portion of granules capable of releasing insulin, which are referred to as the high Ca^{2+} -sensitive pool (HCSP). The exocytosis occurring at different rates is controlled by two Ca^{2+} sensing mechanisms: the low affinity Ca^{2+} sensor and the high affinity Ca^{2+} sensor. Exocytosis occurring at high $[Ca^{2+}]$ are controlled by the low affinity Ca^{2+} sensor. Synaptotagmin IX has been reported to be a high-affinity Ca^{2+} sensor in β -cells [144] and it remains to be determined if synaptotagmin IX also functions as a low-affinity Ca^{2+} sensor. Another putative Ca^{2+} sensor is piccolo, which facilitates rapid Ca^{2+} - induced exocytosis by interacting with essential proteins, including cAMP-regulated guanine nucleotide exchange factor /exchange proteins activated directly by cyclic AMP (cAMPGEFII/ Epac2), sulfonylurea receptor1 (SUR1),

and the L-type Ca^{2+} channels [133, 145, 146]. Thus, piccolo might act as a low-affinity Ca^{2+} sensor.

Intracellular $[\text{Ca}^{2+}]$ is determined by the open and closure of plasma membrane Ca^{2+} channels. Three subfamilies of voltage-gated Ca^{2+} channels exist: 1). L-type high voltage-activated (HVA) Ca^{2+} channels. They are sensitive to dihydropyridines (DHP) and include (1) $\text{CaV}1.1$, 1.2, 1.3, and 1.4 channels [147-149]; (2) the non-L-type HVA channels $\text{CaV}2.1$ (P/Q-type), 2.2 (N-type) and 2.3 (R-type) [147, 148, 150]; and (3) the low voltage-activated (LVA) T-type Ca^{2+} channel ($\text{CaV}3.1$, 3.2, and 3.3). LVA differs from HVA Ca^{2+} channels electrophysiologically. The LVA open transiently upon modest depolarization [151, 152]. They are pacemakers in most cell types [153]. A mixture of voltage-gated Ca^{2+} channels were reported to present in β -cells [154-156]. The existence of L-type Ca^{2+} channels was first confirmed about 25 years ago by radioisotopic and electrophysiological measurements [157]. Later the expression of $\text{CaV}1.2$ of the L-type Ca^{2+} channels, and P/Q, N and R-type Ca^{2+} channels were confirmed by single-cell PCR [156]. The first phase of insulin secretion couples to the activation of L-type $\text{CaV}1.2$ channel, whereas the second phase secretion depends on R-type ($\text{CaV}2.3$ channels), which mediates a moderate global increase in intracellular $[\text{Ca}^{2+}]$. The R-type Ca^{2+} channel-mediated Ca^{2+} influx is insufficient to cause insulin exocytosis, but accelerates granule mobilization, and increases the size of RRP [158].

Glucose induces insulin secretion by both triggering (i.e. involving closure of the K_{ATP} channels) and amplifying (i.e. post K_{ATP} channel closure) effects. Although the increase in intracellular $[\text{Ca}^{2+}]$ is the primary signal that triggers insulin exocytosis by glucose, there are other cell signals activated by glucose that also play roles in this process, such as cAMP, cGMP, inositol 1,4,5-trisphosphate (IP3), and DAG [158]. Among those signaling molecules, cAMP

may be the most important one for potentiating insulin secretion [129, 159-162]. Around 50 years ago, it was found that an oral glucose load elicits greater insulin secretion than an intravenous glucose load even though similar circulating glucose levels were achieved by these two methods [162]. The potentiated insulin secretion by orally ingested glucose was due to the action of GIP and GLP-1, incretin hormones secreted by enteroendocrine K cells and L cells, respectively upon glucose ingestion [163, 164]. Incretin hormones augment GSIS by stimulating the cAMP signaling pathway. Cyclic AMP's action was generally thought to be mediated exclusively by the activation of protein kinase A (PKA), which phosphorylates proteins involved in insulin exocytosis [165]. However, the insulinotropic effect of cAMP can only be blocked partially by inhibition of PKA activity, suggesting that an alternative mechanism exists that mediates, in part, the cAMP effect on insulin exocytosis. Recently it was discovered that cAMP stimulates exocytosis of insulin granules from a RRP, an effect that was unaffected by PKA inhibition, suggesting a PKA-independent mechanism [166]. A cAMP-binding protein called CAMPS (cAMP sensor) was identified by yeast two-hybrid screening of the insulinoma cell line MIN6 in the search for intracellular molecules that interact with the sulfonylurea receptor SUR1 [167]. CAMPS was later identified using a BLAST-search approach as a mouse homolog of rat cAMP-GEFII/Epac2 which is an isoform of cAMP-GEFI/Epac1 [168, 169]. Studies using a yeast two-hybrid system later further confirmed the interaction between cAMP-GEFII/Epac2 and SUR1 [167, 170], revealing a novel cAMP-GEFII/Epac-dependent pathway activated by cAMP.

The cAMP-binding protein, cAMP-GEF/Epac participates in potentiating insulin secretion in a PKA-independent manner. cAMPGEFII/Epac2 is abundant in the brain and neuroendocrine and endocrine tissues including pituitary, adrenal, and pancreatic islets, while

cAMP-GEFI/Epac1 is expressed at high levels in adult tissues including thyroid, kidney, ovary, skeletal muscle, and heart, and at low levels in the brain [167-169]. In addition to SUR1 which is the regulatory subunit of the K_{ATP} channel, cAMPGEFII/ Epac2 also binds to Rab3-interacting molecule 2 (Rim2) [167] and Piccolo [146]. Rim2 is the target of the small G-protein Rab3 which is involved in exocytosis [171]. Piccolo defines and organizes the site of neurotransmitter release in neurons [172]. Piccolo also forms both homodimers and heterodimers with Rim2 in a Ca^{2+} -dependent manner [146]. cAMP-GEF/Epac, which has a higher dissociation constant for cAMP (1.2-4 μ mol/L, PKA: 5-25 μ mol/L), could be a cAMP sensor when PKA activity is fully saturated [173-175]. The cAMP-GEF/Epac might be localized in cAMP compartments distinct from PKA, since a much higher concentration of cAMP is required for activating the cAMP-GEF/Epac-mediated signaling than that for stimulating PKA activity. In the absence of cAMP, Ras exchange motif (REM) binds to GEF/Epac to stabilize GEF/Epac and inhibit its activity. Cyclic AMP activates GEF/Epac by binding to its regulatory region. Activated GEF/Epac then activates Ras-like small GTP-binding proteins, Rap1 and Rap2 [168, 169, 173, 176]. Cyclic AMP-GEFII/Epac2 may be involved in both the first and second phase of insulin release, since treatment with antisense oligodeoxynucleotides (ODNs) against GEFII/Epac2 in pancreatic islets reduced both first and second phases of cAMP- potentiated insulin secretion [177]. Interaction between SUR1 and cAMP-GEFII/Epac2 is an essential step in this PKA-independent cAMP potentiated insulin secretion. This PKA-independent effect on cAMP-regulated insulin secretion is impaired in SUR1 knockout islets [178, 179] and early PKA-independent exocytosis is absent in SUR1 knockout β -cells [145]. Being recruited to the plasma membrane by SUR1, cAMP-GEFII/Epac2 mediates the cAMP-dependent activation of Rap GTPase activity. Rap then stimulates phospholipase C (PLC)- ϵ , which catalyzes hydrolysis of

phosphatidylinositol 4,5-bisphosphate (PIP₂). The hydrolyzation of PIP₂ cause inhibition of K_{ATP} channels [180]. Therefore, the interaction between cAMP-GEFII/Epac2 and SUR1 is independent of intracellular ATP concentration [181]. After activated by cAMP, cAMP-GEFII/Epac2 dissociates from the SUR1-cAMP-GEFII/Epac2 complex, which then release its inhibition on K_{ATP} channels. There is another hypothesis that after activated by cAMP, cAMP-GEFII/Epac2 will disassociate from granule SUR (gSUR, a putative SUR on insulin granules) to open a chloride channel (ClC-3), which is coupled with gSUR. Opening of ClC-3 channel allows Cl⁻ influx to promote granular acidification, which allows insulin granule priming and refilling of the RRP [182]. Accumulating evidence also suggests that activation of cAMP-GEFII/Epac2 mobilizes Ca²⁺ from intracellular Ca²⁺ storage, thereby increasing insulin secretion [183-186]. Three possible mechanisms might mediate this Ca²⁺ mobilization: 1). cAMP-GEFII/Epac2 interacts with IP₃ receptors and ryanodine receptors to increase intracellular Ca²⁺ channel sensitivity to Ca²⁺ or Ca²⁺ mobilizing signals; 2). cAMP-GEFII/Epac2 might act through Rap and extracellular signal regulated kinase to sensitize intracellular Ca²⁺ release; and 3). cAMPGEFII/Epac2 acts through Rap to stimulate PLC-ε, thereby hydrolyzing PIP₂ to release IP₃, which signals release of Ca²⁺ from the ER [187].

1.5 Hormone regulation of insulin secretion

Estrogen: β-cells are not considered classic estrogen targets; however, estrogen receptors are present in islets [188] and the effects of 17β-estradiol on β-cells has been known for a long time [189]. The main physiological consequence of 17β-estradiol action on β-cells is the enhancement of insulin secretion [190]. In humans, 17β-estradiol can increase insulin secretion in postmenopausal women [191, 192]. This insulinotropic effect is mediated by potentiating

glucose-stimulated insulin secretion (GSIS) [193]. The effects of estradiol are initiated by its binding to estrogen receptors. Two types of estrogen receptor (ER) are present in β -cells: 1). the nuclear ERs ($ER\alpha$ and $ER\beta$) and 2). the membrane ER ($ER\gamma$) [194]. It is reported that at physiological concentrations, 17β -estradiol can significantly decrease K_{ATP} channel activity in a reversible manner [190], which causes membrane depolarization and subsequent opening of voltage-gated Ca^{2+} channels, thereby potentiating glucose-induced intracellular $[Ca^{2+}]$ oscillations. The modulation of K_{ATP} channel activity by estradiol may be mediated by activation of the cGMP-dependent protein kinase (PKG) pathways [195]. The activated PKG can directly phosphorylate transcriptional factor CREB. After phosphorylation, CREB can bind to CRE, which in turn modulates transcription of genes containing cAMP/ Ca^{2+} response elements to potentiate glucose-induced intracellular $[Ca^{2+}]$ oscillations to influence insulin secretion [196-199].

Melatonin: Melatonin is a hormone secreted by pineal gland, which helps adjust the timing or reinforces oscillations of the biological clock [200]. The direct effect of melatonin on β -cells was confirmed by the discovery of melatonin receptors on both clonal β -cells [201, 202] and human islets [203]. But its effect on insulin secretion is controversial in the reported literature. There are studies showing that melatonin either exert an inhibitory [204, 205], a neutral [206], or stimulatory effect on insulin secretion [207]. However the inhibitory effect is consistent in replicated experiments with clonal β -cells [201, 203, 207, 208]. It has been reported to attenuate glucose- and KCl-stimulated insulin secretion in rat islets [209]. The inhibitory effect of melatonin on insulin release was later confirmed in rat islets [210]. Consistently, chronic melatonin administration can ameliorate hyperinsulinemia in vivo [211].

It was reported that melatonin receptor is coupled to G_i , which inhibits G protein [212]. G protein activation will further activate adenylate cyclase to catalyze cAMP production. Indeed melatonin is found to block the enhanced insulin secretion by cAMP agonist forskolin or GLP-1 [201, 202]. In contrast, cAMP levels in human islets are not influenced by melatonin, whereas the formation of cAMP in MIN-6 cells is impaired in the presence of melatonin [203]. It is also found that melatonin decreases cGMP level to inhibit insulin secretion. This effect is mediated by activation of melatonin receptor (MTNR) 1B [213]. However, when G_i coupling is blocked by pertussis toxin, MTNR1A also mediated a stimulatory effect on insulin secretion by coupling to Gq/11. The activation of Gq/11 provokes the release of IP3 by activating PLC- ϵ to potentiate insulin secretion [207, 214, 215].

GLP-1: GLP-1 secreted from small intestine L-cells together with GIP is an incretin hormone in response to nutrient load [216, 217]. Incretin is responsible for an augmentation of insulin secretion to meet the increased demand for insulin after a meal. Experiments have shown nutrient load from the oral route stimulates more insulin secretion than intravenous nutrient load [218]. The analogs of both GLP-1 and GIP have been explored as a potential therapy for T2D for many years, and the long-lasting GLP-1 analog exenatide was introduced to clinics in 2005 [219], which is now a prescription drug for T2D treatment. Upon binding to GLP-1 GLP-1 receptor (GLP-1R) will be activated, which will further activate adenylyl cyclase and cAMP is generated [220]. The elevated cAMP then potentiates GSIS. This insulinotropic effect is dependent on glucose. When the extracellular glucose concentration is in the normal fasting range (lower than 4 mmol/L), GLP-1 is inactive in stimulating insulin secretion [219]. Such glucose-dependent action of GLP-1 is very important because it will not cause hypoglycemia.

Leptin: Leptin is secreted by adipocytes and is known to influence insulin action in fat and liver cells [221, 222]. It is generally accepted that leptin exerts an inhibitory effect on insulin secretion. Leptin deficiencies are associated with hyperinsulinemia in both mice and humans [221, 223]. A large body of literature shows that leptin plays an inhibitory role in insulin secretion in clonal β -cells [224-226], cultured rodent islets [225-233], human islets [225, 234, 235], perfused rodent pancreas [224, 236], as well as in mice [225]. It is hypothesized that leptin's inhibitory effect is through antagonizing the action of elevated intracellular cAMP [237], since it was reported that leptin inhibits insulin secretion induced by 3-isobutyl-1-methylxanthine (IBMX), which elevates cAMP content by inhibiting phosphodiesterases (PDEs) [232], the enzymes catalyzing the hydrolysis of cAMP. Leptin also potently inhibits glucocorticoid- or GLP-1-induced insulin secretion, which augments GSIS by activation of the cAMP signaling pathways [226, 236]. Leptin was shown to inhibit insulin secretion by activating PDE 3B, a subtype of PDE [226].

Growth hormone: Growth hormone (GH) has targets in variety of cells but one of its best-known actions is to generate insulin-like growth factor-I (IGF-I) and its binding proteins [238]. Recombinant human IGF-I was shown to decrease serum levels of insulin and C-peptide in normal human subjects [239]. Ex-vivo studies using isolated rat islets confirmed that IGF-1 directly suppresses insulin secretion [240]. This inhibitory effect is possibly mediated through activation of PDE3B [241], which is responsible for breaking down cAMP in β -cells, as stated above.

1.6 Pancreatic β -cell apoptosis and regeneration

Both T1D and T2D are characterized by progressive β -cell destruction, of which apoptosis is the main form. Although β -cell loss is caused by excessive nutrient in type 2 diabetes, while autoimmune reaction caused β -cell damage in type 1 diabetes, similar cellular signal final pathways are shared between the two types of diabetes [242].

T1D is a T-cell-mediated autoimmune disease resulting from selective destruction of pancreatic β -cells. The incidence of T1D is estimated to increase from 4.4 million in 2000 to about 5.4 million in 2010 [243]. However the pathogenic mechanisms and T-cell mediated autoimmune process that destroy pancreatic β -cells in T1D are complex and are not fully defined yet, which are subjects of many excellent reviews [243-246]. It is clear from past studies that infiltration of inflammatory cells, such as T helper type 1 (Th1) cells and macrophages, into the islets in response to islet associated antigens and subsequent insulinitis are hallmarks of the pathogenesis of T1D. Activated T cells and macrophages release several proinflammatory cytokines, such as interleukin-1 β (IL-1 β), interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), which are believed to be important mediators leading to β -cell destruction in T1D [247-252]. These cytokines act on β -cells through their specific receptors to induce several signal transduction pathways that lead to alternations in gene and protein expressions [243]. Research evidence suggests that activation of NF- κ B may be a common and crucial step for various cytokine-stimulated β -cell dysfunction. Activation of NF- κ B will lead to induction of its downstream gene inducible nitric oxide (NO) synthase (iNOS) and subsequent NO production [253]. Consistently, cumulative evidence shows that IL-1 β , IFN- γ and TNF- α induce the overexpression of iNOS in β -cells, leading to the overproduction of NO that causes the cytotoxicity to β -cells [252], suggesting an important role for NO in the pathogenesis of diabetes.

Indeed, transgenic mice overexpressing iNOS in β -cells developed insulin-dependent diabetes [254]. Conversely, inhibition or knockout of iNOS in the islets protects β -cells in vitro and in vivo from the cytotoxic effects of cytokines [255-257]. While additional mechanisms may also be involved in the pathogenesis of T1D, these data clearly indicate that iNOS derived NO is at least partially responsible for cytokines-mediated destruction of β -cells, which is central to the development of T1D [258-261]. Recent studies found that β -cell proliferation is increased during the pathogenesis of diabetes in humans and non-obese diabetic (NOD) mice, an animal model for human T1D [262, 263], which however, cannot adequately restore autoimmune-mediated destruction of β -cells [264, 265]. As such, identifying agents that can simultaneously induce β -cell proliferation and survival in T1D milieu can provide novel treatment for T1D.

β -cell apoptosis in the course of insulinitis in type 1 diabetes is caused by direct contact with islet infiltrated T cells and macrophages and exposure to soluble mediators secreted by those infiltrated immune cells such as oxygen free radicals, NO, and cytokines including IL-1 β , IFN- γ and TNF- α [266]. IL-1 β and IFN- γ are considered to be two major soluble factors which mediate β -cell damage. In vitro cell culture had shown exposure of β -cell to IL-1 β or IL-1 β +IFN- γ can cause β -cell destruction similar to those observed in pre-diabetic patients [267]. In response to the stimulation of IL-1 β and INF- γ around 700 genes could be up- or down-regulated [268].

It is well studied that in β -cells IL-1 β can activate transcription factor nuclear factor (NF)- κ B [266]. Although basal NF- κ B activity is required in maintaining β -cell normal insulin secretory function [269], excessive activated NF- κ B will result in up-regulated expression of inducible nitric oxide synthesis (iNOS)[270]. iNOS can produce massive amount of NO which will result decreased expression level of other transcription factors responsible for β -cell

differentiation and function (e.g., PDX-1 and Isl-1) [271, 272]. This cytokine mediated NF- κ B also up-regulates chemokines such as monocyte chemoattractant protein-1 (MCP-1) [273, 274] and down-regulates the Ca²⁺ pump sarcoendoplasmic reticulum Ca²⁺ ATPase type 2b (SERCA-2b) [272, 275] Decreased SERCA-2b expression leads to ER calcium depletion and severe ER stress which results in β -cell apoptosis [272, 276-278].

Exposure to IL-1 β also activates the c-Jun NH2-terminal kinase (JNK), a member of the mitogen-activated protein kinases (MAPKs)[279, 280]. JNK has been shown to JNK play an important role in the intracellular events during β -cell loss [281]. Cell-permeable peptide inhibitors of JNK prevent cytokine-induced β -cell apoptosis [282]. IFN- γ is reported to have synergize with IL-1 β to trigger β -cell apoptosis [266]. IFN- γ binds to cell surface receptors and activates JAK1 and JAK2, which phosphorylate the transcription factor STAT-1. Upon activation STAT-1 forms dimer and translocates into nucleus to activate γ -activated site of diverse genes including up-regulating iNOS expression [266, 270].

Cytokine and hyperglycemia share some common mechanism to alter β -cell gene expressions. C-Myc, A20, and heme-oxygenase are induced in both of the conditions. And hyperglycemia was reported to increase the production of IL-1 β from β -cell [283]. However, the pattern of hyperglycemia-induced genes in β -cells are not exactly the same as that induced by cytokines [268, 272, 284] suggesting the diverge mechanisms of β -cell apoptosis of these two conditions. NF- κ B dependent genes which are strictly activated by IL-1 β remained unchanged after high glucose exposure, while lactate dehydrogenase A, the mitochondrial uncoupling protein (UCP)-2, and the transcription factor cAMP-responsive element modulator (CREM) can be induced in hyperglycemia [284, 285]. It has been shown that β -cell glucotoxicity at least in

part results from increase of β -cell oxidative stress and subsequent JNK activation is NF- κ B independent [286, 287]. The main source of oxidative stress probably comes from the mitochondrial electron transport chain [288, 289]. In addition, ER stress and sustained elevation of cytosolic calcium concentration could also be possible explanations of β -cell viability loss [290].

Besides glucotoxicity, high plasma concentration of free fatty acid (FFA) is another risk factor for β -cell destruction. The effect of dyslipidemia depends on the lipid profile. Saturated fatty acids such as palmitate are highly toxic at long-term exposure, whereas monounsaturated fatty acids such as oleate protect β -cells from palmitate and high glucose-induced β -cell apoptosis [291, 292]. FFA's toxicity on β -cells is suggested to be iNOS/NO independent, because the absence of iNOS expression or NO production in FFA-mediated β -cell apoptosis [293, 294]. It is also reported that FFA's toxic effect is oxidative stress independent [294]. Moreover, oleate or palmitate did not activate NF- κ B in β -cells [293]. FFA-induced β -cell toxicity might occur at ER level, in which FFA are esterified. Both oleate and palmitate can induce ER stress markers including alternative splicing of XBP-1, activation of ATF-6, and induction of the ER chaperone BiP [293]. Besides, FFA might also impair ER calcium handling [295]. Therefore, in the condition of increased insulin demand such as high glucose, ER stress induced by FFA might be amplified.

In mammal, β -cells are slowly renewed tissue. In healthy adult individuals, extremely low levels of β -cells are replicating to apoptotic ones [296]. However β -cells proliferation can be increased in obese or/and insulin resistant individuals [297-299] and during the progression of autoimmunity in type 1 diabetes [300]. The population of β -cells can be increased by at least two mechanism: replication of the existing β -cells, and neogenesis from pancreatic precursor

'stem cells' [301]. Besides the increase in cell number, an increase in cell size also contributes to increased β -cell mass to meet higher insulin requirement. The mechanism by which β -cells expands is hypothesized to involve up-regulation of protein synthesis, but the exact molecular mechanism is largely unclear [302].

β -cell proliferation and differentiation can be influenced by certain nutrients and various growth factors. Nutrients which simulate insulin secretion and synthesis can also increase β -cell proliferation. Among the nutrients glucose is the most physiological relevant β -cell growth nutrient [303, 304]. Some of the growth factors which can stimulate β -cell growth such as IGF-1 and GH are glucose-dependent [304, 305]. However glucose-mediated β -cell growth is relatively an acute effect. Chronic exposure to high glucose will evoke β -cell apoptosis [306].

GH stimulates β -cell by binding to growth hormone receptor (GHR) present on β -cell which leads to JAK-mediated tyrosine phosphorylation and activation of STAT5a and 5b [307, 308]. Activated STAT5a and 5b then up-regulate cyclin D2 expression, which is an essential regulator of β -cell proliferation [309, 310]. In some types of cell, GH's effect is mediated by increasing local IGF-1 production. But β -cell IGF-1 and GH signal transduction pathways are independent [305, 311]. IGF-1 mediated β -cell mitogenesis requires the induction of phosphatidylinositol 3-kinase (PI3K) activity, which is located downstream of insulin receptor substrate (IRS)-2 [304]. IGF-1 will activate protein kinase-B (PKB; also known as Akt) which is important for β -cell survival [312, 313]. PKB in turn can phosphorylate glycogen synthase kinase (GSK)-3 [314, 315] leading to inhibition of GSK-3 [316]. Although the consequences of GSK3 inactivation in are currently unclear, GSK-3 is considered to be able to control general protein synthesis and cell differentiation which contribute to β -cell hypertrophy and neogenesis [316].

2 The pathogenesis of T1D

A decrease in both mass and insulin secretory function of β -cells is the common characteristic shared in both type 1 and type 2 diabetic patients. Autoimmunity plays a critical role in the development of type 1 diabetes. The classical type 1 diabetes is characterized by the presence of antibody (humoral) and T-cell (cellular) responses to self-islet proteins (antigens) [317-321]. Histological analysis of the pancreas from patients with type 1 diabetes shows the presence of immunological activity [322]. Drugs that suppress the immune response such as cyclosporine and azathioprine can slow the progression of β -cell destruction pointing to the critical role of immune activity in development of type 1 diabetes [323, 324]. Although the development of type 1 diabetes is influenced by dietary factors including early infant feeding status [325], Vitamin D and omega 3 polyunsaturated fatty acid intake [326], and duration of exposure to gluten [327]. People with genetic predispositions have a higher risk to develop overt type 1 diabetes. Human leukocyte antigen (HLA) which encodes cell surface proteins that interact with immune cells is an important gene family that contributes up to 40% of T1D risk. The HLA Class II region is considered to be the most influential. In Caucasians, HLA types DR3-DQA 0501-DQB1 0201 and DR4-DQA1 0301-DQB1 0302 are strongly associated with risk, while DQB1 0602 is associated with protection [328].

Innate immune response signaling is involved in the initiation of the autoimmune process. However the molecular pathways of innate immunity linked to type 1 diabetes development is yet to be uncovered [329]. Adaptive immunity is known to play a critical role in β -cell destruction in type 1 diabetes development. Humoral and cellular immunity are the two major facets of adaptive immunity. In type 1 diabetes, the appearance of multiple autoantibodies is believed to reflect progressive β -cell autoimmunity [330, 331]. Although the autoantibodies can

be markers for type 1 diabetes, whether they contribute to pathogenesis is not confirmed [331]. β -cell destruction is partly mediated by the cellular immune response.

T lymphocytes are reported to be the primary mediator in type 1 diabetes progression [332], though macrophages and dendritic cells infiltrate islets before T lymphocytes [333]. The indispensable role of T lymphocytes is supported by their presence in insulinitis, and detection of circulating autoreactive T lymphocytes in clinical overt type 1 diabetes patients, and the observation that immunosuppressive drugs specifically against T lymphocytes delay disease progression [334]. Although type 1 diabetes is T lymphocytes dependent, paradoxically both NOD mice and human type 1 diabetes patients are lymphopenic [335-338]. The decreased number of T lymphocytes drives T lymphocyte homeostatic expansion [338]. This homeostatic expansion results in increased effector/memory T lymphocytes instead of naive T lymphocytes [339]. These effector/memory T lymphocytes can generate new effector cells more efficiently, which precipitate autoimmune disease [340]. Two subsets of T lymphocytes, CD4+ and CD8+ are both involved in type 1 diabetes development. The precise role of CD4+ and CD8+ T lymphocytes in β -cell destruction are controversial. It is generally accepted that CD4+ T lymphocytes contribute to provide proper homing for CD8+ effector cells as well being effector cells themselves [341].

Naive CD4+ T lymphocytes reside as T helper (Th) 0 cells in secondary lymphoid organs before they encounter antigens. After antigen encountering they differentiate into functional subsets namely Th1 secreting IL-2, INF- γ , and TNF- α [342, 343] and Th2 secreting IL-4, IL-5, IL-10 and IL-13 [344-347]. Studies about the correlation of diabetes and T helper cell phenotypes lead to the idea that Th1 and their cytokines promote diabetes [347, 348]. Th1 cells can either cause β -cell destruction directly [349, 350] or by secreting Th1 cytokine (INF- γ) to

recruit and activate macrophages and CD8⁺ T lymphocytes that exert toxic functions [351]. In contrast, Th2, through secretion of the cytokine IL-4, is generally considered protective [348, 352]. Although there is evidence that Th2 plays a role in causing β -cell damage, it is through IL-10 instead of IL-4 [353-355].

Th1/Th2 differentiation is influenced by the antigen concentration, ligation of co-stimulatory molecules, and cytokine circumstance; but eventually transcription factors T-bet and GATA-3 control T helper cell differentiation [356-358]. Cells dominated by T-bet will differentiate into Th1 cells, while Th2 differentiation is directed by GATA-3 expression [358]. The activation of Th1 and Th2 cells can be suppressed by a specialized subpopulation of CD4⁺ T cells named regulatory T (Treg) cells. Treg cells, which are protective, contribute to immune suppression by suppressing activity of both CD4⁺ T lymphocytes and CD8⁺ cytotoxic T lymphocytes [359-361]. Treg cells comprise 5-10% of the peripheral CD4⁺ T lymphocyte population in mice and humans [362]. Naturally occurring Treg cells (nTreg) are generated in thymus and express surface markers CD4 and CD25 and an intracellular marker, transcription factor forkhead box P3 (Foxp3) [363]. The other Treg subpopulation named induced Treg (iTreg) is generated in response to antigen. They are not CD25⁺ by default. But they share features with nTreg in terms of Foxp3 expression and bystander (non-antigen-specific) immune suppression [363].

Similar to CD4⁺ T lymphocytes, mature CD8⁺ T lymphocytes reside as naive cells in secondary lymphoid organs. After encountering self-antigen [364] activated CD8⁺ T cell differentiate into effectors cells. Activated CD8⁺ T cells destroy β -cells either through a perforin-dependent pathway or alternatively by the Fas/FasL pathway [346, 365]. The pore-forming protein perforin and the granzyme B are key constituents of cytolytic granules. After

conjugate formation, perforin and granzyme are released toward the target cell membrane where they synergize to cause apoptotic cell death [366]. INF- γ is reported to be crucial in activating the Fas/FasL pathway in islets. [365]. Therefore, INF- γ and granzyme B levels are markers of CD8+ T cell-induced β -cell damage. Similar to CD4+ cells, the activity of CD8+ cells can be tuned at the genetic level. T-bet, known to regulate Th1 cell differentiation, also controls the differentiation of the CD8+ cytotoxic effector cell [367].

Recently, it was reported that the activity of Th17 cell, which is a newly discovered subpopulation of CD4+ T lymphocytes secreting IL-17, is associated with autoimmune conditions in a variety of autoimmune diseases including rheumatoid arthritis [368], inflammatory bowel disease [369], and multiple sclerosis [370]. Studies have shown that Th17 cells may up-regulate IFN- γ and also extinguish IL-17 in response to IL-12 or IL-23 in the absence of TGF- β *in vitro* and depreciated to a Th17/1 (IL-17+IFN- γ +) or Th1 phenotype [371, 372]. Although, the relative contribution of Th17 cells in T1D is not well defined yet, studies had shown that high level of the IL-17 transcripts have been found within insulitic lesions in NOD mice [373]. And increasing levels of serum IL-17 is associated with accelerated disease progression in a T cell receptor transgenic NOD model [373]. More recently, the protective effect of therapeutic intervention with an antigen-specific agent is associated with a decrease in Th17 population [374]. However, the specific contribution of this subpopulation of CD4+ T lymphocytes to the natural progression of T1D remains to be fully characterized.

3. The pathogenesis of T2D

T2D is a result of chronic *insulin resistance and loss of β -cell mass and function* [258]. Both in experimental animals and people, obesity is a leading pathogenic factor for developing

insulin resistance, which is always associated with the impairment in energy metabolism, causing increased intracellular fat content in skeletal muscle, liver, fat, as well as pancreatic islets. Constant insulin resistance will progress to T2D when β -cells are unable to secrete adequate amount of insulin to compensate for decreased insulin sensitivity, which is largely due to insulin secretory dysfunction and significant loss of functional β -cells [258-261, 375, 376]. Indeed, those individuals with T2D always manifest increased β -cell apoptosis and reduced β -cell mass [260, 261, 377]. Progression to full-blown T2D involves insulin resistance leading to β -cell dysfunction [378-380]. Insulin resistance is observed in a variety of patient conditions including gestational diabetes, obesity, impaired glucose tolerance (IGT) and polycystic ovarian syndrome [381, 382]. Although obesity is associated with T2D, most obese people don't develop the disease and increased insulin secretion due to enhanced function of pre-existing β -cells or expansion of β -cell mass compensates and restores blood glucose levels [383]. Enhanced functionality involves increased nutrient signals stimulating increased growth factor signaling in β -cells [379]. In particular, increased nutrient load in the gut can enhance GLP-1 production leading to growth-promoting effects on β -cells [384, 385]. In "susceptible" individuals compensation becomes insufficient and cell dysfunction ensues. Generally, diagnosis of T2D is associated with an approximate 50 % reduction in islet function and this is thought to manifest itself at least 10-12 yr prior to diagnosis, a condition exacerbated by elevated fasting blood glucose [386]. Obese non-diabetic humans show increased relative β -cell volume in islets while obese and non-obese patients with impaired fasting glucose and T2D show at least a 40 % reduction in β -cell volume compared with respective non-diabetic patients [377]. Apoptosis of β -cells was substantially increased in all diabetic patients and was implicated as the primary mechanism underlying the decrease in β -cell mass in T2D individuals although β -cell mass is

controlled by several factors including cell size, rate of cell renewal from proliferation of pre-existing cells or neogenesis (differentiation from other precursor cells) and rate of apoptosis. As the number of β -cells per islet declines in T2D patients, islet space becomes dominated by amyloid plaque deposits although the role of islet amyloid deposits in β -cell dysfunction is unclear [387]. The factors leading to a change in β -cell function (decreased insulin expression and secretion) and mass are central to the pathology of T2D.

The prevailing theories for causes of β -cell failure during the progression to T2D involve chronic exposure of the β -cell to glucose and fatty acids, also known as “glucotoxicity” and “lipotoxicity”, respectively [388-393]. It is known that transient exposure of islets to free fatty acids (e.g., hours) can augment GSIS whereas longterm exposure (e.g., days) decreases insulin secretion. In general, it is accepted that hyperglycemia precedes conditions for lipotoxicity while glucotoxicity can occur independently of lipotoxicity [284, 394]. Taking this idea a step further, the combination of these factors is known as “glucolipotoxicity”. We define “glucolipotoxicity” as chronic exposure of the islets to greater-than-physiological concentrations of glucose and fatty acids, leading to β -cell damage [286]. The following sections will dissect the meaning of these terms and some of the cellular and molecular mechanisms by which these phenomena alter β -cell function, with a particular emphasis on insulin synthesis and secretion. Bear in mind that differences in model system (in vitro vs. in vivo, primary vs. clonal cell lines (MIN6, INS1, HIT-T15, BetaTC-6), rodent vs. human, genetic vs. nongenetic), age of animal, concentrations of substrates (fatty acids, glucose), length of incubation or exposure (minutes vs. hours vs. days), etc. can all influence the outcome, making it very difficult to draw a definitive picture of β -cell pathology and diabetes mellitus. Studies described in this section involve primary cultured rat,

human and mouse islet cells, as well as clonal β -cell clones and tissues harvested from humans and rodents.

3.1 Glutotoxicity - glycation and reactive oxygen species (ROS) production in the β -cells

While exact mechanisms are debatable, the general consensus is that long-term elevated levels of glucose have deleterious consequences for β -cells, which rely on signals of energy status to control metabolism [393]. Abundant expression of the low-affinity high-capacity GLUT2 in β -cells coupled to the role of glucose in stimulating insulin synthesis and secretion leads to excessive glucose concentrations in β -cells and effects on β -cell metabolic pathways. Glucotoxicity also down-regulates GLUT4 levels in insulin-responsive cells [378]. Chronic exposure to glucose leads to increases in cytosolic calcium that induce β -cell destruction [395]. It also leads to increased production of IL-1 β , subsequent NF- κ B activation, increases in FAS, DNA fragmentation and damaged β -cell function [396, 397].

Hyperglycemia leads to glycation reactions and production of ROS [398]. Glycation occurs non-enzymatically and can alter the function of a variety of molecules, and advanced glycation endproducts (AGEs) are implicated in cellular damage [399]. The antioxidant and glycation-inhibitor aminoguanidine, which prevents formation of AGEs and ROS, is able to partly ameliorate the effects of those damaging compounds on β -cell function [399], similar to the beneficial effect of the hydrogen peroxide-scavenging *N*-acetyl-L-cysteine on insulin expression and secretion in *db/db* mice or Zucker rat β -cells subjected to oxidative stress [400, 401]. The importance of ROS in β -cell pathology is supported by the observation that 8-hydroxy-2'-deoxyguanosine (8-OHdG) an oxidative stress marker, is elevated in β -cells from diabetic Goto-Kakizaki (GK) rats [402] and that the insulin [403] and glucokinase [398]

promoters are sensitive to glycation and the presence of ROS (superoxide, hydrogen peroxide, nitric oxide, hydroxyl radicals). Furthermore, there are elevated levels of oxidative stress markers in the blood and urine of T2D patients, and reduced glutathione (GSH) in blood cells [286]. Fructose, D-ribose and 2-deoxy-D-ribose have a greater reducing capacity than glucose [404].

The process of aging, which involves long-term exposure to ROS, increases in body weight, a sedentary lifestyle, and reduced functionality of β -cells, partly accounts for prevalence of this disease in middle-aged to older adults [378]. Counter intuitively perhaps, the β -cell expresses relatively low levels of antioxidant enzymes, including CuZn superoxide dismutase (SOD), Mn-SOD, catalase, and glutathione peroxidase (GPx) [286, 405-408]. The Mn-SOD functions in the mitochondria, Cu/Zn-SOD in the cytosol, and both catalyze generation of hydrogen peroxide from the reaction of superoxide and hydrogen [286]. The GPx species reduce hydrogen peroxide to water with GSH, and also lipid peroxides to alcohols. Oxidized GSH (GSSG) can be converted back into GSH by GSH reductase using NADPH as a cofactor. Islet ROS levels were correlated with glucose concentration [408]. Blocking the existing GPx activity with buthionine sulfoximine, an indirect inhibitor of GSH synthesis, hampered the beneficial effect of N-acetylcysteine, an antioxidant, on ribose-induced decreases in insulin production [408]. Isolated islets transfected with GPx exhibited a six-fold increase in enzyme activity, which negated the detrimental effects of ribose.

Hydroxyl radicals are particularly dangerous in β -cells because of their ability to cross the nuclear membrane and exert a mutagenic effect [286]. The β -cells are particularly vulnerable to oxidative stress [286]. Oxidative phosphorylation generates ROS [409], as well as other pathways for glucose when glycolytic enzyme activity becomes saturated: glycosylation

(Schiff reactions) , autooxidation [410, 411], and the glucosamine pathway (O-linked glycosylation of proteins) [379, 412]. Activation of JNK and NF- κ B is also stress-induced [393, 396, 413]. The activated JNK phosphorylates the Ser³⁰⁷ residue of IRS-1, hence blunting IRS signaling leading to decreased nuclear PDX-1 [393]. The IRS proteins are intracellular tyrosine kinase substrates that are downstream of their receptor. The IRS-1 and IRS-2 are important for β -cell function and survival; their absence leads to insulin resistance [414-416]. The insulin-insulin receptor (IR)-IRS-PI3K-Akt signaling cascade is crucial for regulating islet cell differentiation and function. Decreased Akt signaling as a result of IRS-1 phosphorylation by JNK results in an increase in Foxo-1-dependent gene expression, which plays a role in mediating PDX-1 translocation from the nucleus [417].

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that controls anabolic cellular processes in response to a variety of environmental stimuli including amino acids, glucose and oxidative stress [418, 419]. The rapamycin-sensitive complex TORC1 phosphorylates S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1). The rapamycin-insensitive complex TORC2 phosphorylates Ser473 of Akt and PKC [420]. Continuous activation of mTOR by glucose/fatty acids leads to IRS-2 phosphorylation, tagging it for proteasomal degradation, resulting in decreased IRS-2 levels and increased β -cell apoptosis [421]. As discussed earlier, GLP-1 has a growth-promoting effect on β -cells and enhances proliferation and protects against apoptosis [384]. The GLP-1 receptor activation in β -cells leads to IRS-2 and PKB activation mediated by CREB and transactivation of EGFR [422]

Constant entry of glucose into the β -cell leads to a state of reversible insensitivity to glucose stimulation concomitant with an exhaustion of β -cell stores. When glucotoxicity occurs, β -cell damage leads to defects in insulin production such that chronic glucose exposure-induced

β -cell exhaustion becomes irreversible. Some of the explanations for glucose-induced β -cell damage include depressed rates of insulin synthesis [423], increased small heterodimer partner (SHP) nuclear receptor mRNA [424], downregulation of glucokinase mRNA [398], reduced PDX-1 mRNA, protein and insulin promoter binding activity [286, 423, 425], compromised mitochondrial function and induction of apoptosis. Upregulation of the SHP receptor is thought to prevent p300-mediated PDX-1 and BETA2 complex formation as a competitive inhibitor [424]. Repeated exposure to elevated glucose also reduces activator of RIPE3b1 [426, 427], while enhancing activity of insulin transcriptional repressor basic leucine zipper CCAAT/enhancer-binding protein β (C/EBP β) [428, 429]. Changes in PDX-1 expression and activity have a profound influence on insulin transcription. Supraphysiologic concentrations of glucose in the β -cell over an extended period of time are thought to affect posttranscriptional processing of PDX-1 mRNA [425]. The effect of short-term excess levels of glucose may be a reversible glucose desensitization whereas chronic, repeated exposure (long-term) causes less reversible effects on β -cell function, particularly in regards to insulin synthesis [423]. The effect on β -cells is most likely due to effects on function rather than simply apoptosis, per se [378]. For example, patients that undergo 60 % pancreatectomies do not always develop hyperglycemia and are able to compensate by enhanced functionality in remaining β -cells. Hence, initial pathology of T2D most likely involves an initial defect in β -cell responsiveness to glucose, leading to reductions in β -cell mass.

3.2 Lipotoxicity

The effect of fatty acids on β -cell apoptosis is complex and is most likely due to a multitude of factors including ceramide formation, oxidative stress, unfolded protein response,

and the inflammatory response as reviewed in [388, 389]. The effects of chronic exposure to fatty acids are a bit less clear-cut but appear to involve increased lipolysis in white adipose tissue as a result of insulin resistance, a process that is amplified with body weight gain and continuous accumulation of adipose tissue [378]. This destructive cycle, results in elevated circulating levels of free fatty acids that have a negative effect on GSIS and exacerbate insulin resistance in muscle and liver cells [378, 381]. Saturated fatty acids in particular, such as palmitate and oleate were shown to have inhibitory effects on GSIS [379]. Additionally, accumulation of adipose tissue leads to increased adipocyte secretion of cytokines and adipokines such as TNF- α , IL-6, leptin, resistin and adiponectin [430]. These cytokines can be cytotoxic to β -cells, especially TNF α [431]. Although elevated levels of free fatty acids can mediate cytotoxic effects in β -cells, it is likely that in the absence of hyperglycemia and/or pre-existing β -cell functional defects, these signals may play a role in adaptation to insulin resistance without necessarily having deleterious consequences to cell viability [379].

3.3 Glucolipotoxicity - Inhibition of β -oxidation, stimulation of complex lipid formation, and mitochondrial and ER stress

The effects of glucolipotoxicity may stem from the effects of glucose on fatty acid metabolism in β -cells [394, 432]. In the context of “glucolipotoxicity”, glucose metabolism in the β -cell leads to formation of citrate, a signal for formation of malonyl-CoA in the cytosol, which then inhibits carnitine palmitoyl transferase-1 (CPT-1) activity. This has the effect of blocking fatty acid oxidation since CPT-1 plays a key role in transporting fatty acids into the mitochondria, the site of β -oxidation. This leads to accumulation of long-chain acyl-CoA esters in the cytosol. Consequently, detoxification of fat is attenuated and free fatty acids are shunted

into pathways that lead to formation of cytotoxic complex lipids [120, 379, 432], through activation of AMP-activated kinase (AMPK), a metabolic sensor that drives energy metabolism [433-437]. Activity of AMPK is inversely correlated with glucose concentration and is enhanced by fatty acids in β -cells. Activation of AMPK leads to lipogenesis via transcription factor sterol-regulatory-element-binding-protein-1c (SREBP1c) [438]. Importantly, the form of lipid has a profound effect on β -cell function. Triglycerides (TGs) are relatively non-toxic, monounsaturated fatty acids are protective due to their propensity for esterification into TGs, HDLs are protective, while saturated fatty acids such as palmitate, as well as oxidized LDLs induce cell death [388, 439-441].

One of the reasons that it has been difficult to ascribe β -cell dysfunction to a single factor is that glucolipotoxic conditions lead to multiple metabolic pathways with various metabolites and intermediates, each having different effects on cellular metabolism [388]. The mechanism of action of fatty acids in β -cells is debatable due to the ability of β -cells to either directly cross the lipid-bilayer and act intracellularly or by their ability to activate the cell-surface receptor GPR40 [388]. In general, repeated exposure of β -cells to fatty acids dampens GSIS, reduces nuclear translocation of PDX-1 and expression of MafA, down-regulates insulin expression and induces apoptosis [53, 388, 442-445]. The mechanism by which chronic exposure of fatty acids to β -cells leads to impaired insulin secretion is unclear, but there are a variety of changes observed, including upregulation of the mitochondrial inner membrane protein uncoupling protein 2 (UCP2) [393, 446, 447], PLC- ϵ [448], and changes in insulin granule secretory machinery [449].

The increased flux of glucose and fatty acids places a tremendous burden on mitochondrial oxidation, leading to increased membrane potential as well as ROS production

[450]. As discussed earlier, the β -cell has a limited ability to cope with oxidative stress due to inherently low expression of antioxidant enzymes. The activation of UCP2 by ROS can dissipate the membrane potential by allowing protons to leak into the mitochondrial matrix, and couple the oxidation of fuel to heat rather than ATP [450]. In islets from human donors with T2D as well as *ob/ob* mice, UCP2 was up-regulated [451, 452]. Although this mechanism has a protective effect against generation of ROS, the reduction in ATP production and hence decreased ATP/ADP ratio leads to reduced insulin-secretory capacity [450, 453]. This places the mitochondria in a critical position for regulating cell function since glucose sensing requires production of ATP from oxidative phosphorylation [454]. Deletion of the UCP2 gene as well as reduction of endogenously produced superoxide in the mitochondria restored islet ATP levels and enhanced GSIS [452, 455].

Since apoptotic pathways converge in mitochondria with caspase-3 activation and cytochrome C export, function of this organelle may be key to the orchestration of events leading to cell death under conditions of glucolipotoxicity [456]. It was observed that C57BL/6J mice fed a high-fat diet for 12 wk showed a 60 % increase in mitochondria mass (despite no change in number of mitochondria) [456]. Strains of mice with a 5-exon deletion in nicotinamide nucleotide transhydrogenase (*nnt*) show impaired glucose clearance, and lack of GSIS [457, 458]. This enzyme is an important component of the respiratory chain, converting NADP^+ and NADH into NADPH and NAD^+ , respectively. It is suggested that ROS/aging-associated increases in mitochondrial DNA (mtDNA) mutations could lead to increased susceptibility of the β -cell to metabolic overload [459].

ER stress and the unfolded protein response (UPR) are also implicated in β -cell dysfunction [276]. The high demand for insulin secretion as a result of chronic glucose and fatty

acid-induced signaling places a tremendous metabolic burden on the ER in β -cells. The key players in the ER stress response include PERK, interferon response element (IRE)-1/X-box binding protein (XBP)-1 and activating transcription factor (ATF)-6 [460, 461]. The initial goal of the UPR is to activate chaperones such as Bip/GRP78 and GRP94 (heat shock protein 90; HSP90) and folding enzymes such as protein disulfide isomerase and peptidyl-prolyl *cis-trans* isomerase [461]. These proteins prevent aggregation of unfolded proteins and promote increased fidelity of protein folding. The ATF6 moves from the ER to Golgi where it is cleaved to release its bZIP domain, which then migrates into the nucleus and induces transcription of proteins involved in protein folding and ER-associated protein degradation [462]. To reduce the burden on the ER, protein translation is temporarily halted save for select proteins. The PERK, through phosphorylation of eIF2a mediates a reduction in ER load and an increase in translation of the bZIP transcription factor ATF4, leading to an increase in transcription of C/EBP homologous protein (CHOP; GADD153) and GADD34, which aid in cell recovery [463, 464]. The IRE1 is a kinase/endoribonuclease that splices XBP-1 mRNA, which is then translated into a bZIP transcription factor that induces transcription of protein-folding-related genes [463, 465]. Misfolded proteins are targeted for degradation by ubiquitination in the cytosol. In the event that this response is insufficient to attenuate the accumulation of misfolded proteins and ER function is compromised, apoptosis is induced. Thus, The PERK is responsible for the initial response of temporarily halting protein translation and entry into the ER to prevent overloading [463]. The IRE1 and ATF6 enhance transcription of genes encoding proteins that mediate protein folding, export and degradation.

To summarize the effects of glucolipotoxicity, high glucose levels prevent metabolism of fatty acids which results in funneling to pathways involving formation of toxic compounds (e.g.,

ceramide), which in turn down-regulate insulin, cause β -cell dysfunction and results in apoptosis [393]. The chronic exposure of cells to glucose and fatty acids places a tremendous metabolic burden on the mitochondria and ER. Production of ROS in the mitochondria, up-regulation of UCP2 leading to decreased ATP production, and induction of the unfolded protein response may all be central to the series of events leading to apoptosis.

4 Genistein and diabetes

Recently, phytochemical isoflavones have drawn wide attention for their potentially beneficial effects on some human degenerative diseases. Genistein, a isoflavone primarily present in legumes, has various biological actions including a weak estrogenic effect [466] by binding to estrogen receptors (ERs) [467] and inhibition of protein tyrosine kinase (PTK) [468]. It has well-known effects on cancer [469, 470] and osteoporosis [471, 472] and may also affect neurological end points [473, 474], though some of these reports remain controversial and the mechanisms of these effects are unclear.

Genistein may have anti-diabetic effect.

Genistein is widely used as a *dietary supplement* in the U.S. for various presumed health benefits [475-477], though the research evidence supporting the *beneficial effects* of genistein consumption on human health is not well established. Genistein has been previously investigated for its potential beneficial effects on cancer treatment, cognitive function, and cardiovascular and skeletal health, with a primary focus on exploring its potential hypolipidemic, anti-oxidative, and estrogenic effects [475-477]. Studies on whether genistein has an effect on diabetes are very limited. Recent studies performed in animals and humans have shown that ingestion of

isoflavones containing soy protein moderates hyperglycemia [478, 479]. However, it is not clear whether genistein primarily contributes to this beneficial effect. Emerging studies reported that administration of genistein lowered plasma glucose in diabetic animals [480, 481] and in humans [482-484]. However, the mechanism of this genistein action is unknown.

The mechanism of genistein action in diabetes is unknown.

Most published trials using isoflavones or genistein have focused largely on elucidating the effect of isoflavones on lipid profiles, and therefore data from recent studies suggest an anti-diabetic effect of genistein presumably by a hypolipidemic effect [481], thereby increasing insulin sensitivity. However, studies investigating the genistein effect on plasma lipid profiles have shown either only a moderate positive effect [485-488] or a neutral effect [489-492]. Indeed, a recent report by the American Heart Association summarizing data from 10 randomized trials indicated that soy isoflavones have no effect on human plasma lipids [476]. However, recent studies demonstrated that isoflavone administration still lowered plasma glucose even though lipid profile or insulin sensitivity was unaffected in obese and diabetic animals [480] and in humans [482]. These data therefore did not support that isoflavones exerts anti-diabetic effect through lowering plasma lipids, thereby increasing insulin sensitivity. Genistein is a relatively poor anti-oxidant and reactive oxygen species scavenger. There is increasing evidence showing that oxidative stress and reactive oxygen species play a potential role in the initiation of diabetes [493-496]. Genistein has been reported to exhibit anti-oxidant activity in aqueous phase systems [497, 498]. However, the antioxidant effect of genistein is achieved only at concentrations ranging from 25-100 μM , suggesting that genistein is not a physiologically effective antioxidant since the achievable levels of genistein through dietary

supplementation in the human circulation is no more than 5 μM [499, 500]. This result is further consolidated by a recent report indicating that isoflavones have no anti-oxidative effect in healthy postmenopausal women [501]. Consistently, isoflavones or genistein alone has been shown to be poor scavengers of reactive oxygen species [502, 503]. Therefore, the anti-diabetic mechanism of isoflavones is unlikely through scavenging reactive species. These studies suggest a possibility that isoflavones may have a direct effect on the management of diabetes by yet unrecognized mechanisms. Therefore, although these data suggest that isoflavones may have a protective role in diabetes, the mechanisms underlying these beneficial effects are still largely unknown. In addition, whether genistein is the primary isoflavone component that exerts this effect is unclear.

Genistein has a direct effect on pancreatic β -cells.

Although studies are limited and the results are inconsistent, the available data shows that genistein may have a direct effect on pancreatic β -cells. Several earlier studies demonstrated that genistein stimulates insulin secretion from a clonal pancreatic β -cell line [504] and cultured islets [505, 506] while other studies have found an inhibitory effect on insulin secretion [507, 508]. These discrepant data may be the result of variations in the experimental conditions and model used. Nevertheless, the concentrations ($>30 \mu\text{M}$) used in most of these studies are well above those physiologically achievable by dietary means. The serum concentrations of genistein are reported to be 0.16-0.89 μM [509] or 0.3-0.6 μM in Japanese men [510]. Humans consuming three meals per day containing soy milk or a single soy meal have peak serum genistein levels at 4.6 and 4.1 μM , respectively [499, 500]. Therefore, it is still unclear whether genistein at physiological doses can act directly on pancreatic β -cells to modulate cellular function. In

addition, studies elucidating the cellular or molecular mechanisms of the genistein action are lacking.

Genistein is a novel agonist of the cAMP signaling in β -cells.

We recently discovered for the first time that genistein directly activates the cAMP signaling system in β -cells and pancreatic islets, leading to enhanced GSIS [511]. The effect of genistein on the cAMP signaling system is rapid, which is not related to ER or PTK, but is dependent on activation of adenylate cyclase [511]. These effects of genistein are as potent as those of incretin hormone GLP-1, a potent insulinotropic hormone [512, 513]. In addition, our subsequent experiments demonstrated that genistein stimulates cell proliferation of INS1 cells and in mouse and human islets. Furthermore, we found that genistein improves the islet cells responsiveness to glucose and protects against cytokines- and STZ-induced cell dysfunction. Moreover, our initial animal studies clearly indicated an anti-diabetic effect of genistein. Despite these facts, the molecular mechanism of genistein action on β -cells is still unknown. Cyclic AMP is a central signaling molecule in a variety of cellular systems and has long been known to play an important role in GSIS both in primary and clonal β -cells. In addition, it has been recently shown that cAMP signaling has an array of beneficial effects on β -cells, including protection of cells from proinflammatory cytokine- and lipid-induced damage and apoptosis [514, 515], stimulation of β -cell survival and proliferation [422, 516], up-regulation of insulin [517] and IRS-2 gene expression in β -cells [518]. Furthermore, cAMP/PKA signaling also activates PDX-1 protein, a transcriptional factor with essential functions for pancreas development and islet formation [519]. Therefore we propose that genistein may be a novel, plant-derived agent that directly regulates β -cell function by targeting the cellular cAMP/PKA pathway and thereby

is capable of preventing or treating diabetes, an aspect that has not been previously recognized and thus warrants further investigation.

Abbreviations

AGEs advanced glycation endproducts
AMPK: AMP-activated kinase
ATF: activating transcription factor
CAMPS: cAMP sensor
ClC-3: chloride channel -3
CPT-1: carnitine palmitoyl transferase-1
CRE: c-AMP response element
CREB: cAMP response element binding protein
DAG: diacylglycerol
DHAP: dihydroxyacetone phosphate
DHP: dihydropyridines
ER: estrogen receptor
ER: endoplasmic reticulum
FFA: free fatty acid
FFAR-1: free fatty acid receptor -1
G6P: glucose-6-phosphate
GABA: Aminobutyric acid
GH: Growth hormone
GHR: growth hormone receptor
GIP: insulinotropic polypeptide
GISS: glucose-induced insulin release
GLP-1: glucagon-like peptide-1
GLUT2: Glucose transporter 2
Gly3P: glycerol-3-phosphate
GPx: glutathione peroxidase
GS-3: glycogen synthase kinase
GSH: glutathione
GSSG: Oxidized GSH
gSUR: granule SUR
HCSP: highly Ca²⁺-sensitive pool
HLH: a helix-loop-helix domain
HVA I: high voltage-activated
IAPP : islet amyloid polypeptide
IBMX: 3-isobutyl-1-methylxanthine
ICA512: islet cell autoantigen 512
IFN- γ : Interferon- γ
IGF-1: insulin-like growth factor-I
IL-1 β ,: interferon-1 β ,
iNOS: inducible nitric oxide synthesis

IP3: inositol 1,4,5-trisphosphate
IRE: interferon response element
IRS-2: receptor substrate
JNK: c-Jun NH2-terminal kinase
K_{ATP} channel: ATP-sensitive potassium channel
LVA: low voltage-activated
MAPKs: mitogen-activated protein kinases
MCP-1: monocyte chemoattractant protein-1
NF-κB: factor nuclear factor -κB
NO: Nitric oxide
OAA: oxaloacetate
ODNs: oligodeoxynucleotides
PDE: phosphodiesterase
PI3K: phosphatidylinositol 3-kinase
PIP2: phosphatidylinositol 4,5-bisphosphate
PKA: protein kinase A
PKB or Akt: protein kinase-B
PKG: cGMP/cGMP-dependent protein kinase
PP1: protein phosphatase 1
PTBPs: Polypyrimidine tract binding proteins
REM: Ras exchange motif
rER: rough endoplasmic reticulum
Rim2: Rab3-interacting molecule 2
RIPE3b: Rat insulin promoter element 3b
ROS: reactive oxygen species
RRP: readily releasable pool
SERCA-2b: sarcoendoplasmic reticulum Ca²⁺ ATPase type 2b
SHP: small heterodimer partner
SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOD: superoxide dismutase
SREBP1c: sterol-regulatory-element-binding-protein-1c
SRP: signal recognition particles
TGs: Triglycerides
TNF-α: tumor necrosis factor-α
UCP-2: uncoupling protein-2
XBP: X-box binding protein

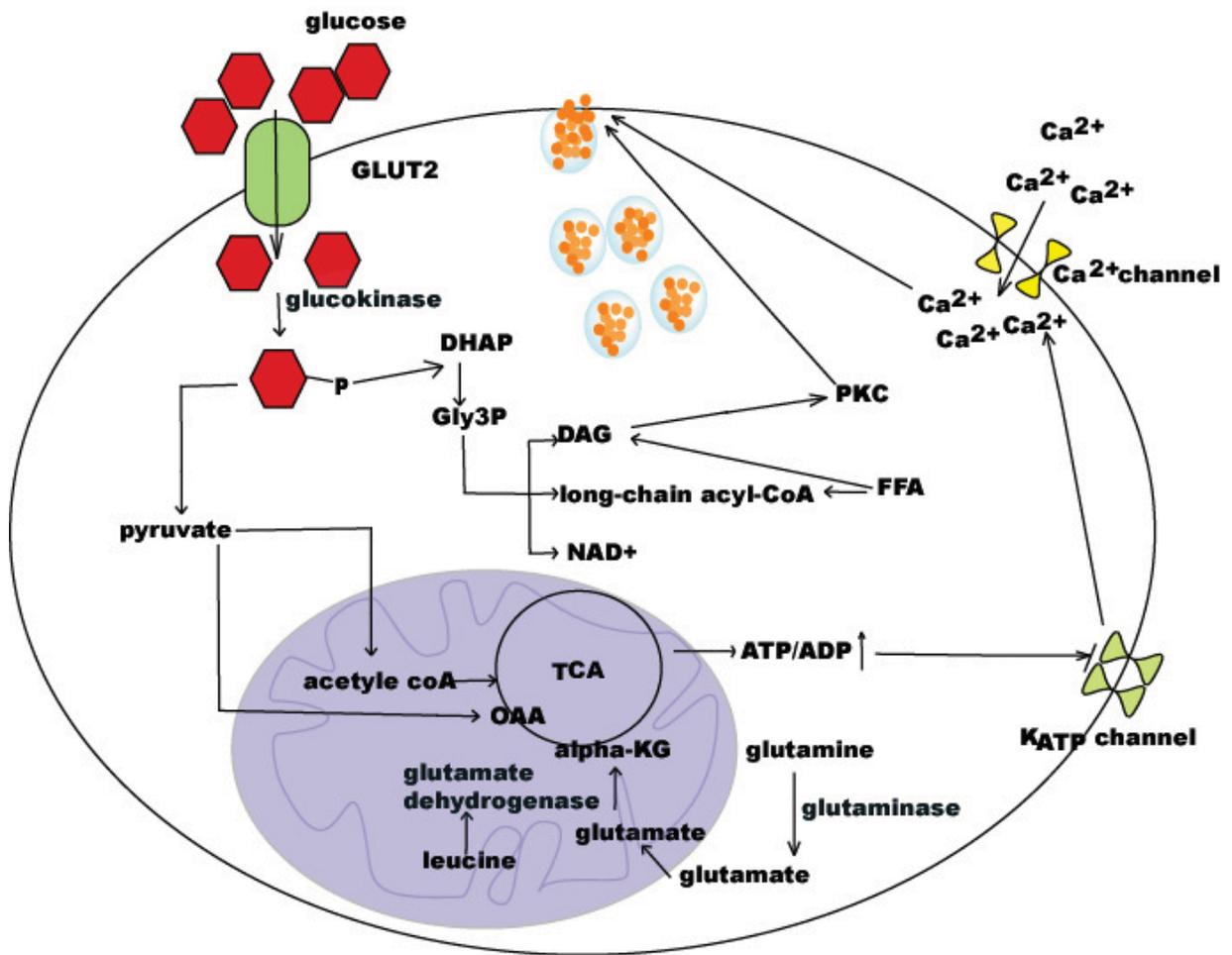


Figure. 1, Schematic illustration of nutrient-regulated insulin secretion.

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CHAPTER THREE

Chronic exposure to genistein improves insulin secretory function of pancreatic β -cells ¹

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Abstract

We recently found that genistein, a plant-derived natural compound, is a novel cAMP signaling agonist in pancreatic β -cells. In the present study, we further show that chronic exposure (48 hrs) of clonal insulin secreting (INS1E) cells to genistein at physiologically relevant concentration enhanced glucose-stimulated insulin secretion (GSIS), whereas insulin content was not altered, suggesting that genistein-enhanced GSIS is not due to a modulation of insulin synthesis. This genistein's effect is protein tyrosine kinase- and K_{ATP} channel-independent. In addition, genistein had no effect on glucose transporter-2 expression or cellular ATP production, but similarly augmented pyruvate-stimulated insulin secretion in INS1E cells, indicating that the improvement of insulin secretory function by long-term genistein exposure is not related to an alternation in glucose uptake or the glycolytic pathway. The enhanced insulin secretion by genistein was dependent on PKA and new protein synthesis as this effect was completely blocked by H89 or cycloheximide. Similarly, chronic genistein exposure also enhanced GSIS in freshly isolated mouse and human pancreatic islets, suggesting a non-species-specific and biologically relevant effect. These findings provide evidence that genistein may be a novel bioactive compound that has an anti-diabetic effect by improving insulin secretion from pancreatic β -cells.

Key words: β -cells; cAMP; genistein; glucose-stimulated insulin secretion; protein kinase A.

Introduction

Recently, phytochemical isoflavones have drawn wide attention for their potentially beneficial effects on some human degenerative diseases. Genistein, the primary isoflavone in legumes, has a well-known weak estrogenic effect by binding to estrogen receptors [377] and is widely used as a protein tyrosine kinase (PTK) inhibitor at pharmacological doses [378]. Study show that dietary intake of genistein can relief several symptoms in postmenopausal women [379, 380], and has beneficial effects on cardiovascular disease, cancer, hyperlipidemia, osteoporosis, and various forms of chronic renal disease [381-387], although some of these reports remain controversial and the mechanisms of these effects are unclear.

Studies on whether genistein has an effect on diabetes are very limited, and carefully controlled studies to determine this effect are lacking. Recent studies performed in animals and humans have shown that ingestion of isoflavones containing soy protein moderates hyperglycemia [388, 389]. However, it is not clear whether isoflavones primarily contributes to this beneficial effect. Emerging studies reported that administration of isoflavones lowered plasma glucose in diabetic animals [39, 40] and postmenopausal women [41] independent of its effect on food intake or weight gain, suggesting that genistein may be a novel plant-derived anti-diabetic agent, although the mechanism whereby genistein exerts such an beneficial effect on diabetes is unknown.

Most published trials using isoflavones or genistein have focused largely on elucidating the effect of isoflavones on lipid profiles, and therefore data from recent studies suggest an anti-diabetic effect of genistein presumably by a hypolipidemic effect [40], thereby increasing insulin sensitivity. However, studies investigating genistein's effect on plasma lipid profiles have shown

either only a moderate positive effect [42-45] or a neutral effect [46-49], consistent with the result of a recent meta-analysis by the American Heart Association showing that isoflavones have no significant effect on human plasma lipids [50]. Indeed, studies demonstrated that isoflavone administration lowered plasma glucose, but lipid profile or insulin sensitivity was unaffected in obese and diabetic animals [39] and humans [41]. These data therefore do not support the concept that isoflavones exerts an anti-diabetic effect through lowering plasma lipids or increasing insulin sensitivity.

There is increasing evidence showing that oxidative stress and reactive oxygen species play a potential role in the initiation of diabetes [390-393]. Genistein has been reported to exhibit anti-oxidant activity in aqueous phase systems [394, 395]. However, the antioxidant effect of genistein is achieved only at concentrations ranging from 25-100 μM , suggesting that genistein is not a physiologically effective antioxidant since the achievable levels of total plasma genistein in both humans [396, 397] and rodents [398, 399] through dietary supplementation are usually no more than 5 μM . This result is further consolidated by a recent report indicating that isoflavones have no anti-oxidative effect in healthy postmenopausal women [400]. Consistently, it has been shown that genistein is also a relatively poor reactive oxygen species (ROS) scavenger [401, 402].

Although studies are limited and the results are inconsistent, the available data suggest that genistein may have a direct effect on pancreatic β -cells. Several earlier studies demonstrated that genistein stimulates insulin secretion from a clonal β -cell line [59] and cultured islets [60, 376] while other studies have found an inhibitory effect on insulin secretion [61, 62]. These discrepant data may be the result of variations in the experimental conditions and model used. Nevertheless, the concentrations ($>30 \mu\text{M}$) used in most of these studies are well above those

physiologically achievable by dietary means ($<5\mu\text{M}$) as aforementioned. Therefore, it is still unclear whether genistein at physiological doses can act directly on pancreatic β -cells to modulate cellular function.

In contrast to these effects at high concentrations, We recently discovered for the first time that genistein at physiologically achievable concentrations (0.01-5.0 μM) acutely activates cAMP/protein kinase A (PKA) signaling by stimulation adenylyl cyclase activity, leading to rapid insulin secretion both in clonal β -cells and mouse islets [51]. These genistein effects are not related to estrogen receptors or PTK.

In the present study, we used clonal rat insulin secreting cell line (INS1E) and mouse and human islets to investigate the effect of relatively long-term genistein treatment on β -cell function. We found that chronic exposure to genistein enhanced glucose-stimulated insulin secretion (GSIS) both in clonal β -cells and pancreatic islets without affecting insulin content. The enhanced insulin secretory function of β -cells by genistein is not mediated through a change in glucose metabolism or K_{ATP} channel activity, but dependent on the cAMP/PKA pathway and new protein synthesis.

Materials and Methods

Reagents

RPMI-1640 media were purchased from Sigma-Aldrich (St. Louis, MO), CMRL-1066 media were from Mediatech Inc. (Herndon, VA), heat-inactivated fetal bovine serum (FBS) were obtained from HyClone (Logan, UT), and medium supplements from Invitrogen (Carlsbad, CA); insulin ELISA kit was obtained from Merckodia Inc. (Winston-Salem, NC); protein assay kit was

purchased from Bio-Rad (Hercules, CA); ATP assay kit was purchased from Promega (Madison, WI); glucose transporter-2 (Glut-2) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); all other reagents and chemicals were from Sigma-Aldrich (St. Louis, MO). Stock solution of genistein, at 20 mM dissolved in dimethyl sulfoxide (DMSO), was stored at -20 °C before use.

Cell and islet culture

INS1E cells (a generous gift from Dr. Pierre Maechler at University of Geneva, Switzerland) were cultured in RPMI-1640 medium containing 11.1 mM glucose and supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 units/ml penicillin, and 100 µg/ml streptomycin [403]. The medium was changed every other day until the cells became confluent. Mouse islets were isolated from male C57BL/6J mice by collagenase digestion as described [404] and maintained in RPMI-1640 at 37 °C and 5% CO₂ for 12 hrs before treatment. Human islets were isolated from cadaver organ donors in the Islet Cell Resource Centers at Southern California Resources Center & Southern California Islet Consortium at National Medical Center (Duarte, CA), Washington University (St. Louis, MO), University of Minnesota (St. Paul, MN), University of Miami (Miami, FL), University of Illinois (Chicago, IL), University of Wisconsin, University of Pennsylvania, University of Alabama (Birmingham, AL), and Joslin Diabetes Center (Boston, MA). The islet purity was 80-90% and viability was 80-97%. Before the experiment, the islets were maintained in CMRL-1066 medium containing 10% FBS.

Insulin secretion and content

Confluent INS1E cells or islets were cultured in RPMI-1640 containing 5.5 mM glucose and 2% FBS at 37 °C and 5% CO₂ in the presence of various concentrations of genistein or vehicle for 48 hrs. In some experiments, cells were co-incubated with genistein and PKA or translation inhibitor. Cells and islets were then washed in Krebs-Ringer bicarbonate buffer (KRBB: 129 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 5 mM NaHCO₃, 0.1% BSA, and 10 mM HEPES, pH 7.4), followed by incubation in KRBB containing either glucose, sodium pyruvate or potassium chloride for 30 min. Insulin secreted in supernatants was measured by an ELISA kit. For insulin content measurement, treated cells and islets were lysed in lysis buffer (50 mM HEPES, 0.1% (v/v) Triton X-100, 1 mM PMSF, 10 mM E-64, 10 mM pepstatin A, 10 mM TLCK, 100 mM leupeptin, pH 8.0). Proteins were extracted and harvested by sonication and centrifugation. Insulin and protein contents were measured by assay kits. Our data showed that exposure of the cells to genistein for 48 hrs had no effect on insulin or protein content. All insulin data in the present study were therefore expressed as optical density recorded by a plate reader.

ATP content

INS1E cells were treated with genistein or vehicle as described above for 48 hrs. Cells were washed in KRBB, and then either lysed for measuring basal ATP content, or stimulated with 20 mM glucose for 30 min at 37 °C before protein extraction. Cellular ATP levels were measured using an ATP luminescence assay kit according to the manufacturer's instruction.

Western blot analysis

After experimental treatments, INS1E cells were harvested in lysis buffer and performed immunoblot analysis as previous described [405]. Briefly, the tissues were sonicated and then centrifuged at $10,000 \times g$ for 5 min. Protein levels of the extracts were measured using a Bio-Rad assay kit. Equal amounts of protein from cell extracts were subjected to immunoblot. Membranes were probed with antibody against Glut-2. The immunoreactive proteins were detected by chemiluminescence. Nitrocellulose membranes were stripped and re-probed with β -actin. The protein bands were digitally imaged for densitometric quantitation with a software program (Gene tools, Synoptics Ltd. UK). Glut-2 protein level was normalized to β -actin expression from the same sample.

Statistics

Data were analyzed with one-way ANOVA, using the Proc Mixed procedure of SAS program, and are expressed as mean \pm standard error (SE). Treatment differences were subjected to Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$.

Results

Chronic exposure to genistein increases GSIS in INS1E cells.

To investigate whether relative long-term exposure of pancreatic β -cells to genistein can modulate insulin secretory function, we first incubated INS1E cells with various concentrations of genistein for 48 hrs, followed by GSIS assay without genistein. As shown in Fig. 1A, pre-treatment of the cells with genistein significantly augmented both 3 and 20 mM glucose-induced insulin secretion, with 1 μ M concentration already inducing a significant effect, although a maximal increase was observed at 5 μ M genistein. To assess whether the effect of genistein on

insulin secretion is due to its effect on insulin synthesis, we measured the total insulin content in control and genistein-treated cells. Data from these studies show that there was no significant difference in insulin content between the control and genistein-treated INS1E cells (Fig. 1B).

Chronic exposure to genistein improves insulin secretory function of pancreatic islets.

We also tested whether genistein has a similar effect on insulin secretion in pancreatic islets. As shown in Fig. 2, chronic exposure to genistein also dose dependently enhanced GSIS in both mouse (Fig. 2A) and human islets (Fig. 2B), suggesting physiological relevance of our *in vitro* findings. Consistent with the result seen in INS1E cells, genistein had no effect on insulin content in islets (Fig. 2C, 2D).

The effect of genistein on GSIS is independent of PTK.

Since genistein is often used as a PTK inhibitor in studies of PTK-mediated cellular events, and PTK may be involved in regulation of insulin secretion [62], we therefore evaluated whether genistein enhances GSIS by inhibition of PTK in INS1E cells. We compared the effect of genistein with that of daidzein, an analogue of genistein that is inactive for PTK inhibition, on GSIS. As expected, exposure of cells to daidzein at 5 μ M for 48 hrs also augmented GSIS (Fig. 3). Indeed, our recent study demonstrated that genistein, at the highest concentration used in the present study (10 μ M), did not inhibit PTK activity [51].

The effect of genistein on insulin secretion in INS1E cells is not due to a change in glucose metabolism or K_{ATP} channel sensitivity.

It is well characterized that glucose induces insulin secretion through glycolysis and oxidation, which increases intracellular ATP/ADP ratio, sequentially leading to closure of K_{ATP} channels, depolarization of voltage-gated L-type Ca^{2+} channels on the plasma membrane, Ca^{2+} influx, and activation of exocytosis of insulin-containing granules. To determine whether genistein enhances GSIS through regulation of glucose metabolism, thereby modulation of ATP generation, we first measured β -cell protein expression of Glut-2, which is essential for β -cell glucose uptake and subsequent GSIS. However, we did not observe any significant change in Glut-2 protein expression after genistein treatment (Fig. 4A). We then examined the effects of genistein on intracellular ATP levels under the conditions for insulin secretion assay. Data in Fig. 4B indicated no significant effects of chronic genistein exposure on basal or high glucose-stimulated ATP production. Further, we determined whether chronic exposure to genistein could augment sodium pyruvate-stimulated insulin secretion, which circumvents the key step enzyme of glycolysis. As shown in Fig. 4C, genistein also enhanced sodium pyruvate-stimulated insulin secretion. These data suggest that the long-term effect of genistein on insulin secretion is not due to the regulation of components in the glucose metabolism pathways. Moreover, we found that chronic genistein exposure elevated KCl-stimulated insulin secretion at a comparable degree to those induced by high glucose and pyruvate (Fig. 4D), suggesting that chronic effect of genistein on GSIS from INS1E cells is not mediated through modulating K_{ATP} channel activity.

Genistein-enhanced insulin secretion is mediated by the cAMP/PKA signaling pathway requiring protein synthesis.

Next, we determined whether the cAMP/PKA signaling pathway mediates genistein's effect. INS1E cells were incubated with genistein or vehicle in the presence or absence of PKA

inhibitor H89 (10 μ M) for 48 hrs. As shown in Fig. 5, H89 completely blocked the genistein-induced GSIS. This result suggests an important role of the cAMP/PKA pathway in enhanced insulin secretion from β -cells following chronic exposure to genistein. We further found that cycloheximide (CHX, 0.1 μ g/ml), an *inhibitor* of protein translation, also abolished the effect of genistein on GSIS (Fig. 5), suggesting that new protein synthesis is required for this genistein action in β -cells.

Discussion

Insulin is an important hormone required for normal metabolism. In healthy subjects, insulin is released in exquisitely exact amounts to meet the metabolic demand. Specifically, β -cells sense changes in plasma glucose concentration and respond by releasing corresponding amount of insulin [406]. Decrease in both sensing and secreting capacity of β -cells results in abnormal glucose homeostasis. While no pharmacological agent can restore the exact kinetics of insulin secretion in response to glucose [407], insulinotropic agents are still very important for effective glycemic control in diabetic patients. In the present study, we found that chronic exposure of β -cells to genistein, at physiologically relevant concentrations through dietary consumption [408], improves insulin secretory function of pancreatic β -cells. Genistein is a widely used dietary supplement in the US and has a relative long metabolic half-life in plasma [408]. This finding therefore may provide a basic mechanism underlying the physiological effects of genistein on diabetes.

In this study, we first found that exposure of INS1E cells to genistein for 48 hrs enhanced GSIS. A similar increase in GSIS was seen in both mouse and human pancreatic islets, showing that this effect of genistein is not species-specific and thus may be biologically relevant, given that the concentrations used in this study overlap with those of physiologically achievable

following dietary consumption of genistein products. Unlike free fatty acids, which acutely increase both basal and GSIS [409], but detrimentally reduce insulin synthesis after incubation with the cells for 48 hrs [410], genistein had no effect on insulin content, suggesting that its effect on insulin secretion is not due to a modulation of insulin synthesis or an adverse effect on the cells, such as apoptosis, as seen in β -cells chronically exposed to free fatty acids [409, 410]. Indeed, genistein had no effect on mitochondria metabolism as determined by ATP assay, further supporting that genistein-enhanced GSIS is not due to an abnormal effect on cellular function.

While genistein is a pharmacological inhibitor of PTK [378], we recently found that genistein at the concentrations used in the present study had no effect on basal or agonist-induced PTK activity in β -cells [51], which is consistent with previous reports that genistein only inhibits PTK at a much higher concentration [411], suggesting that genistein-improved insulin secretory function of β -cells is not related to PTK inhibition. Indeed, daidzein, an analogue of genistein that does not inhibit PTK [371, 412], also increased insulin secretion, an effect that is only slightly less potent than that of genistein, further supporting a PTK-independent effect of genistein.

It is well characterized that glucose induces insulin secretion through glycolysis and mitochondrial oxidation in the cells, which increase intracellular ATP/ADP ratio, sequentially leading to closure of K_{ATP} channels, depolarization of voltage-gated L-type Ca^{2+} channels on the plasma membrane, Ca^{2+} influx, and activation of exocytosis of insulin-containing granules [413-415]. Glut-2, the major glucose transporter expressed on the surface of pancreatic β -cells [416], primarily transports glucose into β -cells [417-419]. Following the entry into the cells, glucose is phosphorylated by the rate-limiting enzyme glucokinase and further hydrolyzed to generate pyruvate, which is oxidized through the tricarboxylic acid *cycle* by mitochondria in β -cells to

produce ATP. Therefore, Glut-2 and glucokinase are two critical proteins that control the rate of glucose metabolism and thus the rate of insulin secretion from β -cells [420-423]. However, we provide the following evidence suggesting that the effect of genistein on insulin secretion is not mediated through regulating these proteins or other components in glucose metabolic pathway. First, we didn't find that genistein-elevated insulin secretion was paralleled by increased Glut-2 protein level in β -cells. Second, genistein similarly enhanced insulin secretion stimulated by pyruvate, which bypassed glycolysis. Third, genistein exposure did not alter basal or glucose induced ATP generation in β -cells.

As aforementioned, K_{ATP} channels, which are present in the plasma membrane of pancreatic β -cells, play an integral role in mediating GSIS via regulation of cell membrane potential [413-415]. While genistein had no effect on intracellular ATP level, which determines the activity of K_{ATP} channels, thereby subsequent GSIS, it is still possible that genistein may directly inhibit K_{ATP} channel activity by binding to the sulfonylurea receptor 1 [424], thus resulting in closure of K_{ATP} channels, membrane depolarization, and insulin secretion. We considered this possibility and therefore examined insulin release elicited in the presence of KCl (50 mM), which directly causes membrane depolarization without altering K_{ATP} channel activity [425]. We observed that pre-treatment of β -cells with genistein for 48 hrs also potentiated potassium-stimulated insulin secretion, which closely resembled its effect on glucose- and pyruvate-evoked insulin secretion. This result suggests that genistein enhances insulin release through a K_{ATP} channel-independent mechanism.

Recent studies showed that activation of several protein kinases, including calmodulin-dependent protein kinase (CaMK), PKA, and protein kinase C (PKC), can facilitate insulin exocytosis through various mechanisms [426-428]. We recently discovered for the first time that

genistein at physiologically achievable concentrations rapidly activates cAMP/PKA signaling by stimulation adenylyl cyclase activity both in INS1E cells and mouse islets [371]. In the present study, we found that pharmacological inhibition of PKA activity completely abolished improved insulin secretion following chronic exposure to genistein, suggesting that chronic exposure to genistein improves insulin secretory function of β -cells through modulation of PKA.

It is well established that cAMP signaling plays an important role in incretin-stimulated insulin secretion in β -cells. Activation of PKA in response to elevated intracellular cAMP has an acute effect on insulin secretion through immediate interaction with L-type Ca^{2+} channel [429], increasing total number of insulin-containing secretory vesicles [38, 430], and sensitization of secretory machineries to Ca^{2+} [431]. While it is unclear how PKA is involved in the effect of chronic genistein exposure on insulin secretion, it is unlikely that the observed genistein action in the present study is mediated through a rapid activation of PKA, because genistein was removed from cell cultures during insulin secretion assay. In fact, our result showed that this genistein effect on insulin secretion is dependent on new protein synthesis, suggesting that chronic genistein exposure improves β -cell function via a genomic mechanism. PKA activates transcriptional factor cAMP response element binding protein, which might stimulate the expression of Glut-2 and glucokinase that plays a role in glucose sensing and thereby insulin secretion. However, while we didn't measure glucokinase in this study, we have not seen a significant effect of genistein on Glut-2 protein expression. Recent study demonstrated that hormone-sensitive lipase (HSL), the enzyme for acylglycerol hydrolysis that is expressed in β -cells [432], plays an important role in insulin secretion [432, 433]. HSL can be directly activated by PKA [434]. However, whether chronic exposure of β -cells to genistein improves insulin secretory function through increasing HSL activity or expression remains to be determined.

Another candidate protein that may be involved in mediating genistein-enhanced insulin secretion is synaptosomal-associated protein of 25 kDa (SNAP-25). Insulin is released from β -cells through regulated exocytosis, which requires transport and docking of insulin secretory granules to the plasma membrane and subsequent fusion. Studies showed that SNAP-25, a membrane bound protein, is involved in this process of insulin exocytosis from β -cells [426, 435], and its expression is up-regulated by PKA in oocytes and steroidogenic cells [436]. It is therefore tempting to speculate that genistein may enhance GSIS in β -cells through cAMP/PKA-mediated upregulation of SNAP-25 expression, an aspect that needs further investigation.

Acknowledgments

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Abbreviations

CaMK: calmodulin-dependent protein kinase

CHX: cycloheximide

ER: estrogen receptor

FBS: fetal bovine serum

GSIS: glucose-stimulated insulin secretion

HSL: hormone-sensitive lipase

INS1E: insulin secreting cell line

KRBB: Krebs-Ringer bicarbonate buffer

PKA: protein kinase A

PKC: protein kinase C

PTK: protein tyrosine kinase

ROS: reactive oxygen species

SNAP-25: synaptosomal-associated protein of 25 kDa

Figures

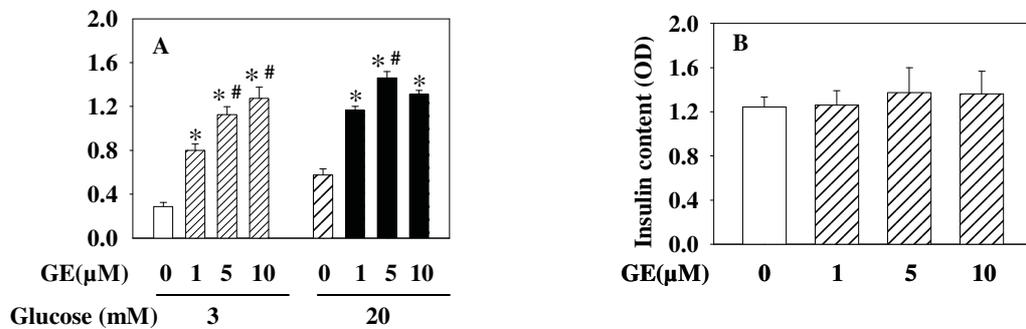


Figure 1. Chronic exposure to genistein potentiates GSIS in INS1E cells. INS1E cells were incubated in RPMI1640 medium containing various concentrations of genistein (GE) or vehicle for 48 hrs. Cells were then washed and further cultured in KRBB containing either 3 or 20 mM glucose for 30 min at 37°C. Insulin secreted into KRBB (A) and inside the cells (B) was measured by an ELISA kit. Values are means \pm SE derived from four to seven separate experiments. *, $p < 0.05$ vs. vehicle alone-treated cells; #, $p < 0.05$ vs. 1 μ M genistein-treated cells.

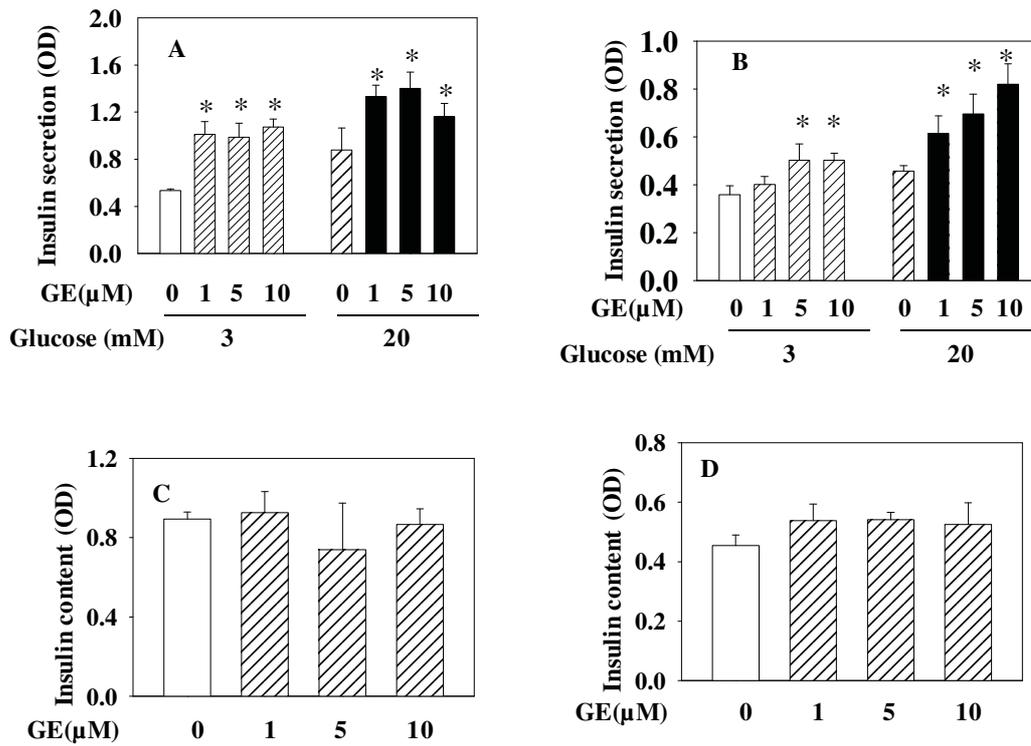


Figure 2. Chronic exposure to genistein potentiates GSIS in pancreatic islets. Mouse (A, C) and human (B, D) islets were incubated with genistein (GE) or vehicle for 48 hrs. GSIS and insulin assay protocols are the same as described in Fig. 1. Values are means \pm SE from four to seven experiments. *, $p < 0.05$ vs. vehicle alone-treated cells.

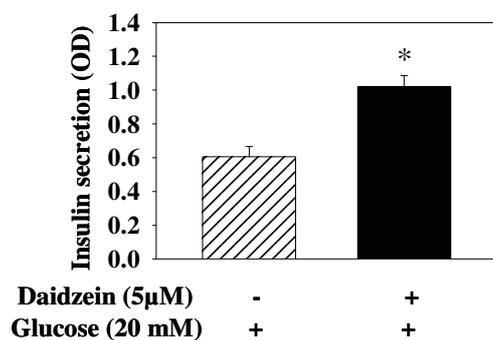


Figure 3. The effect of daidzein on GSIS in INS1E cells. INS1E cells were cultured with daidzein (5µM) or vehicle for 48 hrs. Cells were then incubated in KRBB containing 20 mM glucose for 30 min at 37°C. Insulin secreted into KRBB was determined by an EILSA kit. Values are means ± SE derived from seven experiments. *, p< 0.05 vs. vehicle alone-treated cells.

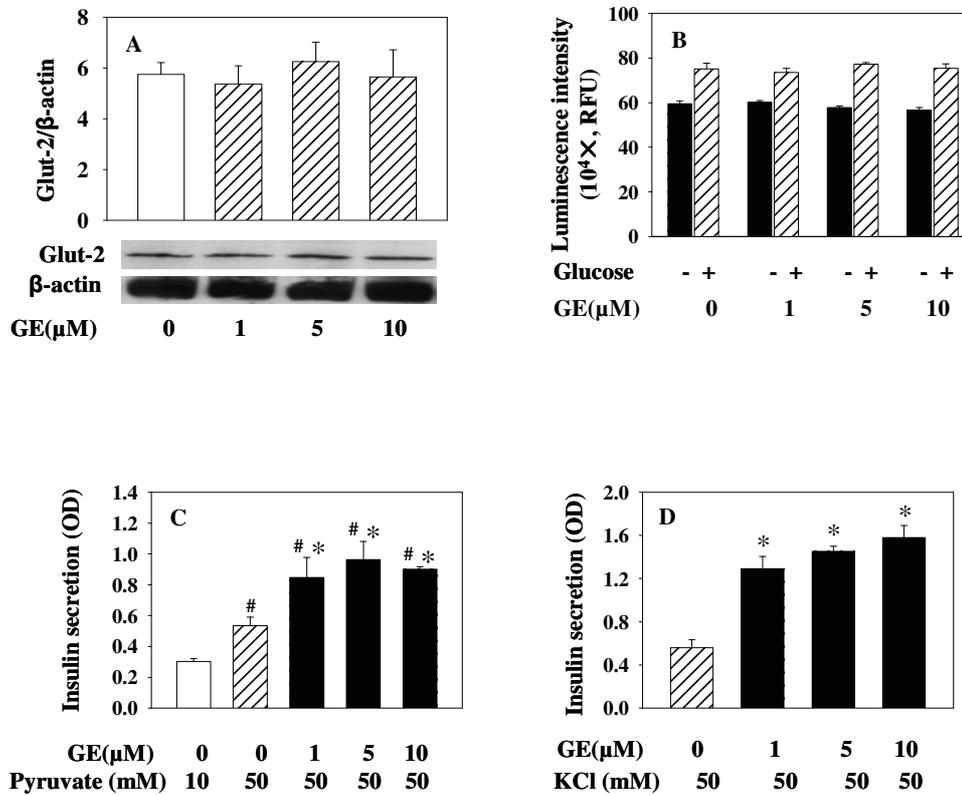


Figure 4. The effect of genistein on insulin secretion in INS1E cells is not due to a change in glucose metabolism or KATP channel sensitivity. INS1E cells were cultured in the presence of genistein (GE) at indicated concentrations for 48 hrs. Glut-2 protein was detected by Western blot using Gult-2 antibody, and normalized to β-actin (A). Basal and glucose (20 mM)-induced ATP production was determined as described in “Materials and Methods” (B). For insulin secretion assays, cells were washed and incubated with sodium pyruvate (C) or potassium chloride (D) in KRBB for 30 min at 37°C. Insulin secreted in KRBB was determined by an

EILSA kit. Values are means \pm SE from four to seven separate experiments. *, $p < 0.05$ vs. vehicle alone-treated cells; #, $p < 0.05$ vs. 10 mM pyruvate-stimulated cells.

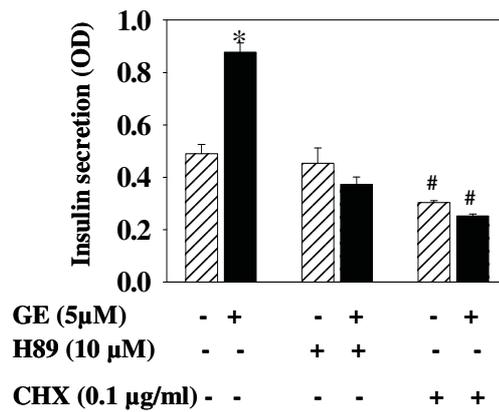


Figure 5. Genistein improves insulin secretory function through a mechanism involving PKA and new protein synthesis. INS1E cell were incubated with genistein (5 μM) in the presence or absence of PKA inhibitor H89 (10 μM), or translational inhibitor cycloheximide (CHX, 0.1 μg/ml), for 48 hrs. Cells were then incubated in KRBB containing 20 mM glucose for 30 min at 37°C. Insulin secreted into KRBB was determined by ELISA. Values are means ± SE derived from four independent experiments. *,#, p < 0.05 vs. vehicle alone-treated cells.

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CHAPTER FOUR

Genistein Induces Pancreatic β -Cell Proliferation through Activation of Multiple Signaling Pathways and Prevents Insulin-Deficient Diabetes in Mice¹

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Abstract

Genistein, a flavonoid in legumes and some herbal medicines, has various biological actions. However, Studies on whether genistein has an effect on pancreatic β -cell function are very limited. In the present study, we investigated the effect of genistein on β -cell proliferation and cellular signaling related to this effect and further determined its anti-diabetic potential in insulin-deficient diabetic mice. Genistein induced both INS1 and human islet β -cell proliferation following 24 h of incubation, with 5 μ M genistein inducing a maximal 27% increase. The effect of genistein on β -cell proliferation was neither dependent on estrogen receptors, nor shared by 17 β -estradiol or a host of structurally related flavonoid compounds. Pharmacological or molecular intervention of PKA or ERK1/2 completely abolished genistein-stimulated β -cell proliferation, suggesting that both molecules are essential for genistein action. Consistent with its effect on cell proliferation, genistein induced cAMP/PKA signaling and subsequent phosphorylation of ERK1/2 in both INS1 cells and human islets. Furthermore, genistein induced protein expression of cyclin D1, a major cell-cycle regulator essential for β -cell growth. Dietary intake of genistein significantly improved hyperglycemia, glucose tolerance, and blood insulin levels in STZ-induced diabetic mice, concomitant with improved islet β -cell proliferation, survival, and mass. These results demonstrate that genistein may be a natural anti-diabetic agent by directly modulating pancreatic β -cell function via activation of the cAMP/PKA-dependent ERK1/2 signaling pathway.

Key words: Genistein; β -cell; proliferation; cAMP; protein kinase A; ERK1/2, mouse.

Introduction

Genistein, an isoflavone in legumes and some Chinese herbal medicines, has well-known weak estrogenic effect and is a pharmacological inhibitor of tyrosine kinase. It has also been extensively explored for its potential hypolipidemic and antioxidative effects. Recent studies performed in animals [2] and humans [1] have shown that ingestion of isoflavones containing soy protein moderated hyperglycemia. However, it is not clear whether genistein primarily contributes to this beneficial effect. Emerging studies reported that administration of isoflavones or genistein lowered plasma glucose in diabetic animals [3-5] and in postmenopausal women [6], suggesting that genistein may be a plant-derived anti-diabetic agent. However, the mechanism of genistein action in diabetes is unknown. While data from one study showed that genistein intake exerted a hypolipidemic effect in obese diabetic rats [3], other studies demonstrated that genistein lowered plasma glucose without affecting lipid profile or insulin sensitivity in obese diabetic animals [7] and in humans [6]. There is a line of evidence showing that oxidative stress and reactive oxygen species play a potential role in the initiation of diabetes [8-11]. Genistein has been reported to exhibit anti-oxidant activity in aqueous phase systems [12, 13]. However, the antioxidant effect of genistein is achieved only at concentrations ranging from 25-100 μM , suggesting that genistein is not a physiologically effective antioxidant since the achievable levels of total plasma genistein in both humans [14, 15] and rodents [16, 17] through dietary supplementation is no more than 10 μM . Indeed, intake of isoflavones have no antioxidative effect in healthy postmenopausal women [18]. Consistently, it has been shown that genistein is a relatively poor reactive oxygen species (ROS) scavenger [19, 20].

Loss of β -cell mass and insulin secretory function, leading to the deterioration of glycemic control over time, is central to the development of both type 1 and type 2 diabetes (T2D) [21, 22]. Recent studies provide evidence that β -cells have the potential to regenerate by proliferation of pre-existing β -cells in both physiological and pathological conditions [23, 24]. As such, a strategy that induces β -cell proliferation, thus preserving functional β -cell mass, could be one of the essential strategies to prevent the onset of diabetes [21, 23, 25-28]. Several earlier studies reported that genistein directly acts on β -cells, leading to insulin secretion [29, 30], whereas other studies have found an inhibitory effect [31, 32]. We recently discovered that genistein is a cAMP signaling agonist by activation of adenylate cyclase in pancreatic β -cells [33]. It has been recently shown that several growth factors induce β -cell proliferation and exerts their anti-diabetic effects via activation of cAMP signaling [34, 35]. Given on this background, we investigated in the present study the effect of genistein on β -cell proliferation and cellular signaling related to this effect.

Materials and Methods

Cell and human islet culture

INS1 cells were cultured as we previously described [33]. Human pancreatic ductal cells (PANC1s), NIH3T3 preadipocytes (ATCC), human aortic endothelial cells (HAECs) and rat vascular smooth muscle cells (RVSMCs) (Lonza) were grown using standard methods. Human islets were obtained through The NIH-supported **Islet Cell Resource** Centers and the **Islet Distribution Program** at the Juvenile Diabetes Research Foundation. The islet purity was 80-90% and viability was 80-97%. Before the experiment, INS1 cells were synchronized in serum free, 3

mM glucose RPMI (Sigma) for 24 h and the islets were maintained in CMRL (Mediatech) containing 10% FBS (HyClone).

Cell proliferation assay

INS1 cells or human islets were incubated with various concentrations of genistein (Sigma) in RPMI at 37 °C. The culture medium contains 1 mM glucose for INS1 cells and 2.8 mM glucose for human islets. PANC1s, HAECs, and NIH3T3 and RVSMCs were incubated with genistein in RPMI, M199, and DMEM medium, respectively. 24 h later, the cultures were continued for an additional 4 h in the presence of 5-bromo-2-deoxyuridine (BrdU, 10 µM). In some experiments, INS1 cells or the islets were pre-incubated with PD 098,059 (PD), H89, ICI 182,780 (ICI) or vehicle (DMSO) for 30 min before addition of 1 µM genistein for 24 h. Cell proliferation was assessed by BrdU incorporation measurements with an ELISA kit (Roche).

In vitro free radical scavenging activity assay

Free radical scavenging activity of genistein was determined by oxygen radical absorbance assay as described [36].

Immunoblot analysis

Equal amounts of protein from cell extracts were resolved on 10% SDS-PAGE gels, blotted onto nitrocellulose membranes, and probed with anti-phospho-ERK1/2, anti-phospho-PKC-zeta, or anti-cyclin D1 (Cell Signaling) as we previously described [37]. Membranes were then stripped and re-probed with anti-ERK1/2 or anti-β-actin to monitor for equal sample loading.

Intracellular cAMP and PKA activity assays

Intracellular cAMP concentration and PKA activity in the lysates of human islets were measured as previously described [33].

Antibody transfection

Polyclonal antibodies against PKA C α plus C β or pre-immune IgG (Santa Cruz) were transfected into INS1 cells using a Chariot reagent according to the manufacturer's protocol (Active Motif). After 5 h in complete RPMI and 12 h in serum free medium containing 3.0 mM glucose following transfection, the cells were treated with 1 μ M genistein or vehicle for 24 h followed by cell proliferation assay.

Adenoviral PKA inhibitor gene construct and infection

Replication-deficient adenovirus containing the complete sequence of endogenous PKA inhibitor cDNA (AdPKI) was constructed as previously described [38]. For determining infection efficiency, human islets were exposed to purified adenovirus at 100-400 multiplicity of infection (MOI)/cells in RPMI medium for 1 h at 37 °C and then cultured in RPMI containing 5% FBS for 24 h at 37 °C. AdPKI null virus served as controls. For MOI calculation, it was assumed that each islet equivalent consists of 1000 cells. After infection, the islets were treated with 10 μ M forskolin and 0.2 mM IBMX for 15 min. The enzymatic activity of PKA in the lysates of islets was determined. For proliferation assay, islets were infected with AdPKI or AdPKI null virus at 200 MOI/cell, and then treated with 1 μ M genistein or vehicle for 24 h.

Animals and treatment with genistein

4-week old male C57BL/6J mice (Jackson Laboratory) were housed in a room maintained on a 12h light/dark cycle under constant temperature (22–25° C) with *ad libitum* access to food and water. The protocol of this study was approved by the Institutional Animal Care and Use Committee At Virginia Polytechnic Institute and State University. Mice were initially fed a modified AIN-93G rodent diet with corn oil substituted for soybean oil (Dyet, Inc.) for 2 weeks, and then were randomly divided into 3 groups with 12 mice per group and fed a diet containing either 0 g (groups, 1, 2), or 0.25 g (group 3) genistein/kg diet. This genistein dosage was used (approximately a human intake of 25-200 mg/day) because it is within the range that humans can realistically consume through taking supplements [39]. We confirmed by performing HPLC analysis that the basal diet is free of genistein. After 2 weeks, diabetic mice were induced with intraperitoneal injection (i.p.) of streptozotocin (STZ) dissolved in 0.1 mM cold sodium citrate buffer (pH 4.5) at 40 mg/kg daily for 5 consecutive days. Control mice received i.p. citrate buffer. After this procedure, mice were continually treated with the control or genistein diet. Body weight and feed intake were recorded weekly throughout the study. To confirm the results, we repeated this animal experiment using the same study protocols.

Plasma glucose, insulin and lipid measurements

At the beginning of the experiment, the fasting blood glucose levels in tail vein blood sample were measured using a glucometer (Roche) to assure that the mice were euglycemic. Following STZ injection, the levels of blood glucose were measured weekly to assess the onset of hyperglycemia (non-fasting blood glucose >250 mg/dl) [40]. Plasma insulin concentration was measured by ELISA (Mecodia) in mice fasted for 4 h. Fasting plasma total cholesterol and

triacylglycerides were measured in triplicate by enzymatic methods using a Pointer 180 Analyzer (Pointe Scientific) as described [41].

Plasma genistein measurements

Blood samples were drawn 30 min after food intake from the retrobulbar plexus through heparinized capillary tubes. Plasma was collected by centrifugation at 16,000 x g for 15 min. An aliquot of 250 μ L serum per sample was used for extraction of genistein using a previously described method [42]. Genistein in the extracted samples was determined by using the HPLC system (Waters2695) with a Luna Phenyl-hexyl column (5 μ C₁₈ 100 R) [43].

Glucose and insulin tolerance tests

For glucose tolerance tests, mice were fasted 4 h and injected i.p. with a single bolus of glucose (2 g/kg body wt). Glucose levels were measured with at time points of 0, 15, 30, 60, and 120 min after glucose administration. For insulin tolerances tests, mice were injected i.p. with insulin (0.75 units/kg body wt), and blood glucose levels were measured at 0, 15, 30, 60, and 120 min after insulin administration.

Hepatic antioxidant and other enzyme assays

The harvested livers were homogenized in ice-cold 50 mM phosphate buffer containing 2 mM EDTA on ice and then centrifuged at 10,000g for 15 min at 4 °C. The resulting supernatants were collected and kept on ice for measuring the level of glutathione (GSH) and activity of GSH reductase (GR), GSH peroxidase (GPx), GSH S-transferase (GST), catalase, superoxide dismutase (SOD) and NAD(P)H:quinone oxidoreductase 1 (NQO1) as previously described [44].

Immunohistochemistry and islet morphometry

Twelve mice per group were injected (i.p.) with BrdU (100 mg/kg body wt). Ten hours after injection, the pancreata were dissected, fixed in 4% (vol/vol) formaldehyde buffer (pH 7.2), and embedded in paraffin. A series of tissue sections (5- μ m thickness) were prepared, mounted on glass slides, and immunofluorescently stained for determining β -cell mass, proliferation, and apoptosis. The β -cell area was measured using images acquired from serial insulin-stained pancreatic sections (500 μ m interval). The β -cell mass were calculated by dividing the area of insulin-positive cells by the total area of pancreatic tissue, and multiplied by the pancreas weight [45]. Proliferating β -cells were identified by examining the incorporation of BrdU in β -cells, which was determined by sequential immunolabeling pancreatic sections with sheep anti-BrdU and guinea pig anti-insulin antibodies (Abcam) [46]. Apoptotic β -cells were detected by labeling the sections with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) kit (Roche) as described [47], followed by staining with a guinea pig anti-insulin antibody to visualize β -cells.

Statistical analysis

Data were analyzed with one-way ANOVA or Student's paired *t*-test when designated using SAS[®] program. Treatment differences were subjected to Tukey's test. A *p*-value < 0.05 was considered significant.

Results

Genistein stimulates β -cell proliferation

We first examined whether genistein has an effect on clonal β -cell proliferation. As shown in Fig.1A, genistein dose-dependently stimulated INS1 cell proliferation. The effect of genistein on β -cell proliferation was significant at 1 μ M concentration, with a maximal increase at 5 μ M genistein (27% over control, $p < 0.01$). We further evaluated the nuclear event that mediates genistein effect on cell proliferation, and found that exposure of INS1 cells to genistein for 3 h elicited a 61.6% increase ($p < 0.05$) in the expression of cyclin D1 protein (Fig. 1B), a major cell-cycle regulator essential for β -cell growth [48], suggesting that genistein may stimulate cell cycle progress involving cyclin D1 expression. We considered the possibility that the stimulation of genistein on β -cell proliferation might simply represent the repair of oxidation-induced damage to the DNA. Our studies excluded this possibility based on these observations. First, we directly counted the cells using a microscope cell counting chamber following genistein treatment. We observed that exposure of INS1 cells to genistein (1 μ M and 5 μ M) increased cell number by 28.9%-35.6 %. Second, we observed that exposure of genistein to β -cells for 24 h had no effect on cell viability (data not shown), suggesting that the increased cell proliferation by genistein is not due to a change in cell apoptosis. Third, while pharmacological dose of genistein (10-20 μ M) showed significant free radical scavenging activity, it has no effect at 1 μ M (data not shown). Fourth, as described below, a variety of flavonoids that are reportedly potent antioxidants, failed to stimulate β -cell proliferation (Fig. 2).

Genistein has no effect on the proliferation of PANC1, NIH3T3, HAECs and RVSMCs

Increased cell proliferation may not always be desirable for other tissues. We therefore tested the effects of genistein on proliferation of PANC1, NIH3T3, HAECs and RVSMCs. We

found that genistein at the same doses used for β -cell proliferation study, had no effect on proliferation of these cells (data not shown), suggesting that the stimulatory effect of genistein on cell proliferation may be restricted to β -cells.

The stimulatory effect of genistein on β -cell proliferation may be structure-specific

To determine the specificity of this genistein effect, we tested a host of structurally related flavonoid compounds in parallel to genistein. This analysis revealed the following data (Fig. 2): 1) isoflavones: genistin (a glycoside form of genistein) and biochanin A (a precursor of genistein), which are largely converted to genistein in the intestine [49], increased INS1 cell proliferation by 25% and 27%, respectively, a magnitude comparable to that of genistein (24%), whereas formononetin, glycerin, and equol had no effect; 2) flavones: quercetin and kaempferol, slightly decreased cell proliferation by 15.8% and 12.7%, respectively; 3) Flavanols: epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, had no effect; 4) resveratrol, a natural phytoalexin, was also inactive. These results demonstrated the unique effect of genistein on β -cell growth that is possibly not shared by other structurally related flavonoid compounds.

ER-independent effect of genistein on β -cell proliferation

As genistein has well-known weak estrogenic effects in some tissues by binding to ERs [50], we examined whether genistein-induced cell proliferation was mediated through ERs. As shown in Fig. 3A, genistein increased INS1 cell proliferation by about 30%. The ER antagonist ICI had no effect on genistein-induced cell proliferation. The activity of ICI used in this study was validated through blocking 17β -estradiol-induced Akt phosphorylation in endothelial cells [51]. In addition, exposure of INS1 cells to 17β -estradiol for 24 h failed to stimulate cell proliferation

(Fig. 3B). These results suggest that the effect of genistein on β -cell proliferation is independent of estrogen signaling mechanisms.

Stimulation of β -cell proliferation by genistein is mediated through PKA-dependent activation of ERK1/2 signaling

Recent studies suggested that the cAMP/PKA and ERK1/2-mediated pathways are two important signaling cascades mediating various stimuli-induced β -cell proliferation [52]. We have recently reported that genistein activates the cAMP/PKA signaling in clonal β -cells and mouse islets [33]. Here, we showed that incubation of INS1 cells in genistein for 15 min stimulated the phosphorylation of ERK1/2, with 5 μ M genistein inducing a maximal increase (Fig. 4A), a pattern that is consistent with its effect on cell proliferation. However, the expression of ERK1/2 protein was not changed in these studies (Fig. 4B).

Next, we determined if the activation of cAMP and ERK1/2 signaling is involved in genistein-induced β -cell proliferation. We showed that incubation of INS1 cells with the PKA inhibitor H89 or the MEK1/2 inhibitor PD completely abolished the genistein-stimulated proliferation of INS1 cells (Fig. 4C). Consistent with this result, inhibition of PKA or MEK1/2 blocked genistein-induced phosphorylation of ERK1/2 (Fig. 4D), suggesting that PKA acts upstream of MEK1/2 to mediate ERK1/2 phosphorylation and subsequent cell proliferation. To further confirm the role of PKA, we delivered PKA C α plus C β antibodies into INS1 cells [33]. Transfection of the cells with PKA antibodies significantly attenuated genistein-induced β -cell proliferation (Fig. 4E), whereas pre-immune IgG had no effect. These results indicate that activation of PKA is sufficient for genistein-induced β -cell proliferation.

Genistein activates cAMP and ERK1/2 signaling and subsequently stimulates human islet cell proliferation

We tested whether genistein has a similar effect on cell proliferation in human pancreatic islets. As shown in Fig. 5A, genistein significantly induced cell proliferation in human islets, with 5 μ M genistein inducing 44.5 % increase in cell proliferation over control. Since not all cells in the islets are β -cells, we performed an immunofluorescence study to determine whether cell proliferation in islets induced by genistein is β -cell specific. Islets treated with or without genistein were double immunostained with BrdU and insulin antibodies. The result showed that proliferative cells in human islets induced by genistein are β -cells (Fig. 5B).

To determine whether the same signaling pathways that mediate genistein effect on INS1 cell proliferation also operate in human islets, we first tested whether genistein elevates intracellular cAMP levels and activates PKA in human islets. The result showed that genistein also significantly elevated cAMP (Fig. 5C) and induced PKA activity (Fig. 5D and 5E), consistent with the dose-response pattern as that obtained in islet proliferation study.

To determine the role of PKA and ERK1/2 in the regulation of genistein effect in human islets, we incubated the islets with genistein in the presence or absence of PD or H89 for 24 h. In line with observations in INS1 cells, inhibition of MEK1/2 or PKA completely abolished the genistein-stimulated cell proliferation (Fig. 5F) and ERK 1/2 phosphorylation in human islets (Fig. 5G), further confirming a central role of PKA and a crosstalk between cAMP/PKA and MEK/ERK signaling pathways in mediating genistein action. To further confirm the role of PKA in mediating this genistein effect, human islets were infected with AdPKI. Treatment of islets with AdPKI greatly attenuated PKA activity (Fig. 5H) and genistein-induced cell proliferation (Fig. 5I), whereas control virus were inactive. Taken together, these results indicate that

genistein induces islet β -cell proliferation via activation of the cAMP/PKA/MEK/ERK signaling cascade.

Dietary genistein intake ameliorates hyperglycemia in STZ-induced diabetic mice

To investigate whether our *in vitro* and *ex vivo* findings are biologically relevant, we performed an animal study assessing whether genistein has potential to prevent diabetes. As expected, dietary supplementation of genistein significantly elevated plasma genistein levels. Under our experimental conditions, plasma genistein levels in STZ-induced diabetic mice fed basal or 0.25g/kg genistein diet were 0 and $6.84 \pm 0.59 \mu\text{M}$, respectively. Our data showed that genistein significantly ameliorated STZ-induced hyperglycemia in diabetic mice (Fig.6A). By the 28th day after STZ injection, 88% of STZ-alone-treated mice became hyperglycemic, whereas only 13% were diabetic in the genistein-treated group. Consistently, dietary genistein ingestion prevented body weight loss (Fig. 6B), whereas food intake was not affected in diabetic mice (data not shown). Mice fed genistein showed significantly higher blood insulin levels (Fig.6C) and improved glucose tolerance (Fig. 6D), whereas insulin tolerance was not altered by genistein treatment (data not shown), suggesting that genistein has no effect on insulin sensitivity. To confirm the results from this study, we conducted a second animal trial and the similar results were obtained.

The anti-diabetic effect of genistein is not due to modulating plasma lipid profiles or scavenging reactive oxygen species (ROS)

We first measured plasma lipid levels in mice treated or untreated with genistein. The results showed that genistein had no effect on plasma cholesterol (Fig. 7A) and triglyceride

concentrations (Fig. 7B). We have not observed significant differences in body weight and food intake between control and healthy mice fed a diet containing 0.25 g/kg genistein (data not shown). This result suggests that a moderate level of dietary genistein used in this study had no effect on appetite, energy expenditure, or lipogenesis, although it can reduce adipose deposition at higher doses (0.5-1.5 g/kg diet) in mice [17]. Next, we evaluated hepatic antioxidant defense system in these animals. Our data demonstrated that genistein intake had no significant effects on the activities of NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase that play important role in scavenging ROS (Fig. 7C), although the activities of some of these enzymes were significantly increased in diabetic mice compared to normal mice.

Dietary intake of genistein improves pancreatic β -cell proliferation, survival, and mass in diabetic mice

We evaluated pancreatic islet cell mass through immunohistochemical technique. We found that STZ administration severely decreased β -cell mass and disrupted the islet architecture (Fig. 8A and 8B), which is essential for normal islet function. However, dietary provision of genistein significantly improved islet β -cell mass. Next, we measured the proliferation and apoptosis of β -cells, which may represent the mechanisms by which genistein improves β -cell mass. STZ treatment increased residual β -cell proliferation, an observation consistent with previous findings that destruction of β -cells by STZ leads to subsequent regeneration of β -cell mass primarily from proliferation of pre-existing and surviving β -cells [53-55]. Dietary provision of genistein significantly enhanced islet β -cell proliferation (Fig. 8C and 8D). Furthermore, we performed in situ detection of DNA fragmentation by TUNEL assay to assess whether genistein

has an effect on apoptosis of mouse islets. In addition, the islets were co-stained for insulin to identify β -cells. Genistein significantly reduced STZ-induced β -cell apoptosis (Fig. 8E and 8F). This result shows that genistein exerts both mitogenic and anti-apoptotic effects on islet β -cells *in vivo*, thereby improving pancreatic β -cell mass in diabetic mice.

Discussion

Recent studies have shown that genistein may have anti-diabetic potential [3-7]. However, the mechanism of this effect is not understood. Here, we provide evidence that genistein stimulates both clonal and human islet β -cell proliferation through the cAMP/PKA- and the ERK1/2-dependent mechanisms. Recent studies demonstrated that loss of β -cell mass and function is central to the development of both T1D and T2D [21, 22]. Therefore, induction of β -cell proliferation are one of the essential strategies to prevent diabetes [21, 56]. Our data from animal studies showing that genistein can act as a growth factor for β -cells *in vivo* provide a novel mechanism for the observed anti-diabetic effect of this compound.

Genistein is a widely used dietary supplement. The reported plasma concentrations of genistein in both humans [57] and rodents [17] through dietary supplementation are usually within the range of 1 to 5 μ M. To consider the potential biological relevance of the observed effects of genistein on β -cell proliferation, we used genistein concentrations that are comparable to the physiologically achievable levels through dietary means. While there is a trend for genistein to stimulate β -cell proliferation at 0.1 μ M, significant effect was observed at 1 μ M, with the maximal effect achieved at 5 μ M genistein. The results observed in INS1 cells were confirmed with isolated human islets, suggesting that physiologically relevant concentrations of genistein may have anti-diabetic implications by directly acting as a β -cell growth factor. In

addition, these data may provide a molecular basis for some of the anti-diabetic effects of genistein observed recently in human and animal studies [3-5].

The result from comparing the effect of genistein on β -cell proliferation with a cohort of structurally related flavanoids demonstrated a highly specific genistein effect. Although the specific chemical structure responsible for the β -cell proliferative effect of genistein remains to be determined, the hydroxyl group at 5C position on the A ring may be crucial for the unique effect of genistein, because equol and 17 β -estradiol, which lack a hydroxyl group at C5, failed to induce β -cell proliferation. In addition, we showed that replacing the hydroxyl group at either 7C position with a glucose molecule (genistin) or at 4'C position with a methyl group (Biochanin A) had no significant effect on genistein activity, suggesting that these structure components may not be important for genistein action.

Genistein has well known weak estrogenic effects by binding to ERs. However, we provided evidence that the observed proliferative effect of genistein on β -cells is not related to its potential estrogenic effect. Interestingly, recent studies reported that genistein can activate an orphan G-protein coupled receptor 30 (GPR30) [58]. While the physiological role of GPR30 is still unknown, female GPR30 knockout mice display hyperglycemia and impaired glucose tolerance [59]. Therefore, it is intriguing to speculate that GPR30 may play a role mediating genistein effect.

Activated ERK 1/2 plays a pivotal role in environmentally stimulated cellular responses, including cellular proliferation, growth and differentiation. We observed that genistein induced a rapid ERK1/2 phosphorylation, which was sustained for at least 6 h. Such a prolonged ERK1/2 activation may be necessary for growth factors to drive β -cell proliferation [60]. ERK1/2 can be activated by various different mechanisms [34]. In this report, we found that genistein-stimulated

ERK1/2 phosphorylation was dependent on PKA activation in β -cells, suggesting that ERK1/2 is located downstream of PKA, which is generally cAMP-dependent. Cell growth and division are regulated by an array of D-type cyclins, cyclin-dependent protein kinases (Cdks) and inhibitors of Cdks. In the islets, cyclin D1 and D2 are essential for β -cell growth [48], and cyclin D1 alone can induce β -cell proliferation in rat and human islets [61]. While the mechanisms that link genistein to the cell cycle machinery are still unclear, we have found that genistein elevated cyclin D1 protein expression. Previous studies demonstrated that cyclin D1 is a nuclear target of activated ERK1/2 [62]. Besides, cAMP-responsive element sites (CREs) are present within cyclin D1 promoters which is trans-activated by CRE-binding protein (CREB) to regulate cyclin D1 gene expression [63]. In β -cells, both PKA and ERK1/2 can induce CREB phosphorylation at serine 133 which is required for CRE-regulated transcription [64]. Therefore, it is tempting to speculate that genistein may stimulate cell cycle progress at least partially through regulating cyclin D1 expression via activation of the PKA and ERK1/2 cascades, although we cannot exclude the possibility that genistein also modulates other nuclear molecules involved in β -cell proliferation.

Using a diabetic mouse model induced by multiple low doses of STZ, which causes mild to moderate level of diabetes mediated by a destruction of islet β -cells [40], we provided evidence that dietary supplementation of genistein could ameliorate diabetes and improve glucose tolerance, which is concomitant with enhanced β -cell proliferation and preserved β -cell mass, confirming the biological relevance of our *ex vitro* findings. Consistently, diabetic mice fed genistein displayed about 2-fold increase in circulating insulin levels compared with those in the control group, which however, could be partially due to improved insulin secretion from existing β -cells by genistein, as observed in our previous studies [33]. As blood lipids could be risk

factors for the pathogenesis of diabetes, the secondary action whereby genistein improves lipid profiles of diabetic animals may contribute to the overall anti-diabetic effects of this agent. We considered this possibility and measured plasma lipid levels. However, we did not find that genistein lowered plasma lipid levels or improved insulin sensitivity. Oxidative stress may play a potential role in the initiation of diabetes [8]. STZ may induce oxidative stress in diabetic mice, which may contribute to the diabetogenic effect of this agent. While genistein at physiologically relevant concentrations (<5 μ M) is a poor ROS scavenger [65], its biological effects are frequently attributed to a presumably antioxidant activity. We considered the possibility that the anti-diabetic effect of genistein might partially be due to a reduced tissue damage caused by STZ-induced oxidative stress, and therefore evaluated hepatic antioxidant defense system in mice treated or untreated with genistein. Although the activities of some of these enzymes were significantly increased in diabetic mice compared to normal mice, which may reflect an adaptive mechanism in response to elevated oxidative stress, genistein intake had no further effects on these enzymes, further confirming that genistein acts directly on pancreatic β -cells to improve glucose tolerance in diabetic mice.

The glucose analogue STZ is reported to be transported into β -cells by the glucose transporter 2 (GLUT2) for exerting its apoptotic effect [66]. While we did not study the effect of genistein on GLUT2 protein expression in mouse islets, our recent studies found no such an effect in cultured β -cells [67], suggesting that improvement of islet β -cell mass by genistein may not be due to modulation of GLUT2 expression, thereby preventing STZ influx in β -cells. Some studies showed that STZ increased peripheral lymphocytic infiltration into islets, thereby producing insulinitis [40, 68], which may contribute to STZ-induced β -cell apoptosis and diabetes. While it is presently still unknown if genistein affects apoptosis, which is an ongoing project in

this laboratory, we observed no effect of genistein on mononuclear cell infiltration into the islets. In fact, we hardly detected any infiltrated immune cells in these mice (data not shown), consistent with previous finding that this mouse strain is resistant to STZ-induced insulinitis [69].

In summary, we have identified for the first time that genistein may be a putative β -cell growth factor by targeting the cAMP and ERK1/2 signaling pathways. Our animal studies showed that genistein is capable of preserving islet β -cell mass and alleviating diabetes. Islet β -cell proliferation is a very important component of β -cell adaptation to increased apoptosis and insulin resistance and therefore holds promise as a treatment for both T1D and T2D. In this context, these findings may potentially lead to the development of novel, natural agents for diabetes prevention and treatment.

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Missouri, University of Pittsburgh, Pittsburgh, Pennsylvania, Massachusetts General Hospital, Boston, Massachusetts , and the Scharp/Lacy Research Institute, Irvine, CA.

Abbreviations:

BrdU: bromodeoxyuridine;

CMRL: CMRL-1066 medium;

CRE: cAMP-responsive element;

CREB: CRE-binding protein;

DMSO: dimethyl sulfoxide;

ER: estrogen receptor;

FBS: fetal bovine serum;

GLUT2: glucose transporter 2;

GPx: GSH peroxidase;

GR: GSH reductase;

GSH: glutathione;

GST: GSH S-transferase;

IBMX: 3-isobutyl-1-methylxanthine;

ICI: ICI 182: 780;

NQO1: NAD(P)H:quinone oxidoreductase 1;

PBS: phosphate-buffered saline;

PD: PD 098059;

PKA: protein kinase A;

ROS: reactive oxygen species;

RPMI: RPMI-1640 medium;

SOD: superoxide dismutase;

STZ: streptozotocin;

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Figures

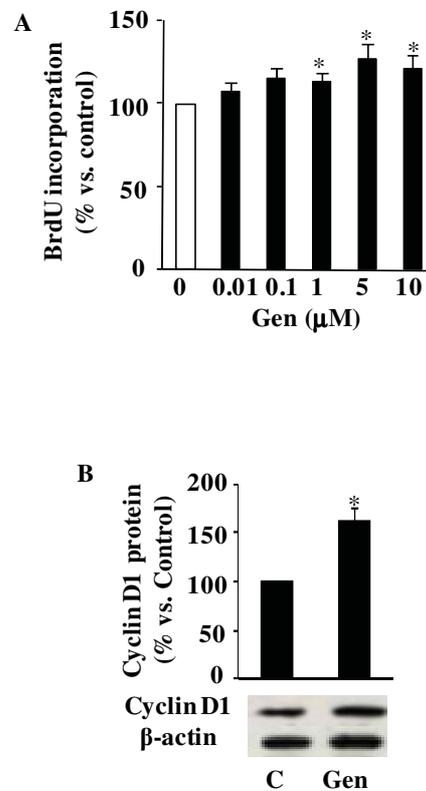


Figure 1 . Genistein stimulates proliferation of β -cells. A: INS1 cells were incubated with various concentrations of genistein (Gen) or vehicle in RPMI medium for 24 h: followed by addition of BrdU (10 μM) for 4 h. Cell proliferation were determined by measuring DNA synthesis using BrdU ELISA kit. Data were expressed as mean \pm SE of observations from four to five separate experiments: each performed in triplicate sample. B: INS1 cells were incubated with genistein (Gen: 1 μM) or vehicle (C) for 3 h. The level of cyclin D1 in the treated cell extracts was measured by Western blot and normalized to β -actin content from the same sample. The bar graphs (mean \pm SE) represent four independent experiments. *: $P < 0.05$ vs. vehicle alone-treated control.

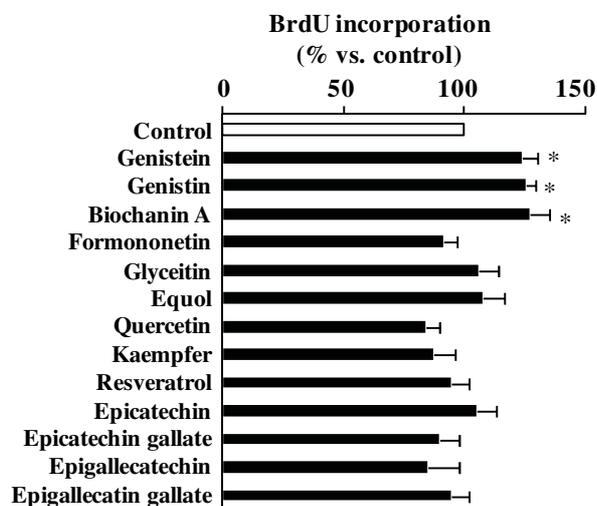


Figure 2. The stimulatory effect of genistein on β -cell growth is structure-specific. INS1 cells were cultured with various flavonoids (1 μ M) in RPMI containing 1 mM glucose for 24 h. Cell proliferation was determined using an ELISA kit. Data are expressed as mean \pm SE derived from three independent experiments performed in triplicate each. *: $P < 0.05$ vs. vehicle alone-treated cells.

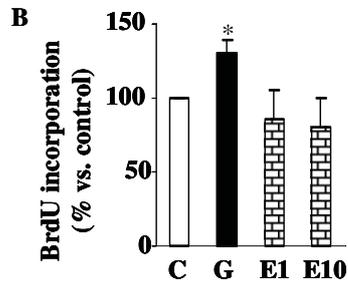
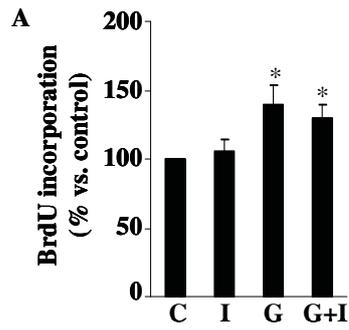


Figure 3. Genistein-induced β -cell proliferation is ER-independent. A: INS1 cells were pre-incubated with ICI (I: 2 μ M) or vehicle for 30 min: followed by addition of genistein (G: 1 μ M) or vehicle (C). B: INS1 cells were incubated with genistein (G: 1 μ M): 17 β -estradiol (E1: 1 nM: E10: 10 nM) or vehicle (C) in RPMI containing 1 mM glucose. 24 h later: cell proliferation was measured with ELISA. Data obtained from five separate experiments in triplicate determinations each are expressed as mean \pm SE. *: $P < 0.05$ vs. vehicle alone-treated cells.

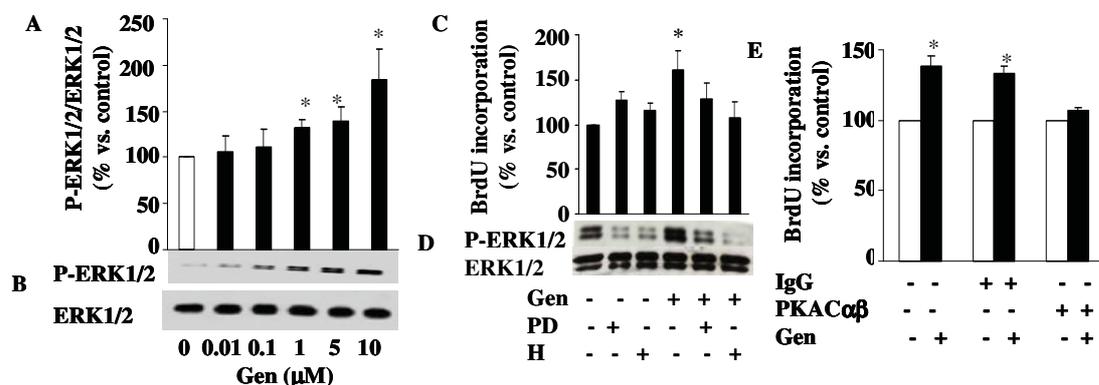


Figure 4. Genistein-stimulated β -cell proliferation is mediated through the PKA and ERK1/2 signaling mechanisms. A: INS1 cells were incubated in RPMI containing 1mM glucose with various concentrations of genistein (Gen) for 15 min at 37°C. Western analysis was performed per protocol described in “Materials and Methods” to detect phosphorylated ERK1/2 (P-ERK1/2): which was normalized to total ERK 1/2 (B) from the same sample. C & D: INS1 cells were pre-incubated with PD098059 (PD: 2 μM); H89 (H: 10 μM) or vehicle (C) for 30 min. Cells were then treated with genistein (Gen: 1 μM) or vehicle (C) in the continued presence or absence of inhibitors at 37 °C. Incubation was either terminated after 15 min to determine ERK1/2 phosphorylation by Western blotting (D) or continued for 24 h to determine cell proliferation (C). E: INS1 cells were transfected with antibodies against PKA C α plus C β or preimmune IgG (IgG) per protocol as described in “Materials and Methods”. Transfected cells were treated with genistein (Gen: 1 μM) or vehicle for 24 h followed by performing cell proliferation assay. Data are expressed as mean \pm SE of three to six experiments in triplicate each. *: $P < 0.05$ vs. vehicle alone or preimmune IgG alone-treated cells.

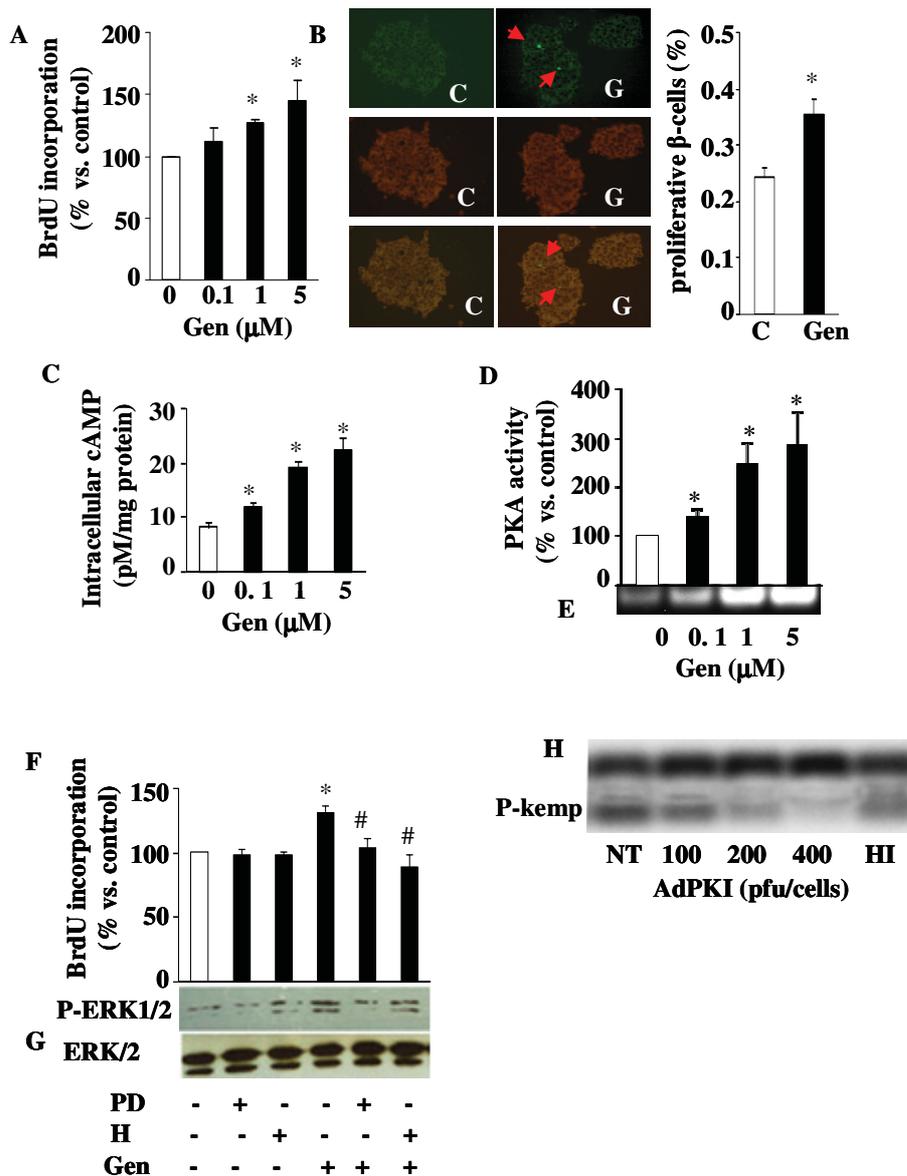


Figure 5. Genistein stimulates intracellular cAMP accumulation: activates PKA and ERK1/2 and subsequently stimulates cell proliferation in human islets. Human islets were incubated with various concentrations of genistein (A): or 1 μ M genistein (Gen: B) in RPMI containing 2.8 mM glucose for 24 h: followed by labeling with BrdU (10 μ M) for 4 h. Islet cell proliferation was either measured by ELISA (A) or visualized by double immunostaining for BrdU (Green) and insulin (Red) (B). The light green BrdU+ nuclei as identified by the arrows

from merged images (Brown) show that genistein increases β -cell proliferation. C & D: Human islets were stimulated with various concentrations of genistein (Gen) or vehicle in RPMI in the presence of 2.8 mM glucose at 37°C for 20 min. Intracellular cAMP concentration (C) and PKA activity (D) in cell extracts were measured: with a representative photograph of the agar gel used for PKA activity assay shown (E). E & G: Human islets were pre-incubated with PD098059 (PD: 10 μ M): H89 (H: 10 μ M) or vehicle (C) for 30 min. The islets were then stimulated with genistein (Gen: 1 μ M) or vehicle (C) in the continued presence or absence of inhibitors. Incubation was either terminated after 20 min to determine ERK1/2 phosphorylation by Western blotting (G) or continued for 24 h to determine cell proliferation by ELISA (F). H: Human islets were infected with 0: 100: 200: 400 MOI AdPKI/cell: or control virus (N-AdPKI): followed by incubation with 10 μ M Forskolin and 0.2 mM IBMX for 15min. PKA activity in the cell lysates was then determined by measuring phosphorylation of kemptide (P-kemp). I: Human islets were infected with 200 MOI AdPKI/cell: and then treated with genistein (Gen: 1 μ M) or vehicle for 24 h: followed by cell proliferation assay. Data are expressed as mean \pm SE obtained from three to four independent experiments in triplicate each. *: $P < 0.05$ vs. vehicle alone-treated cells; #: $P < 0.05$ vs. genistein-alone treated cells.

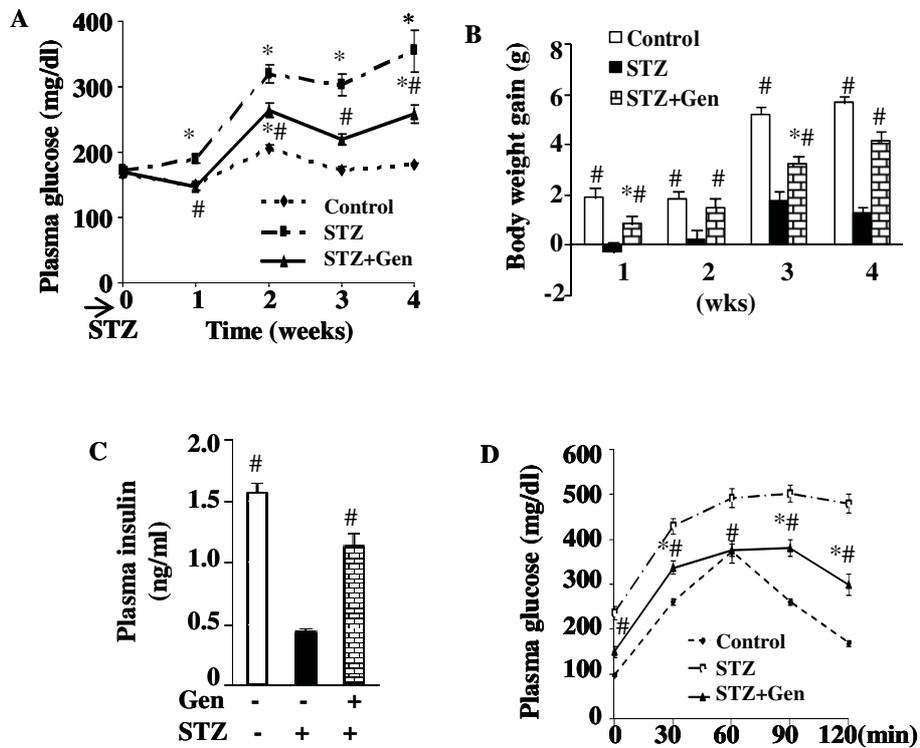


Figure 6. Dietary intake of genistein prevents STZ-induced diabetes in mice. Male C57B/6J mice (4 weeks old) were fed a control diet (C: STZ) or genistein (Gen) supplemented diet (0.25g/kg diet) for 2 weeks prior to administration of streptozotocin (STZ) (40 mg/kg body weight for 5 days) and continued on the same diet for 4 weeks. The mice in the control were injected with citrate buffer. A: Nonfasting blood glucose levels were measured before and 1: 2: 3 and 4 weeks after STZ administration; B: Body weight gain was measured weekly during the whole period of animal study; C: Plasma insulin levels in fasted mice were measured by ELISA; and D: glucose tolerance was determined. Data are expressed as mean \pm SE (n=12 mice). *: $P < 0.05$ vs. healthy control; #: $P < 0.05$ vs. STZ alone-treated mice.

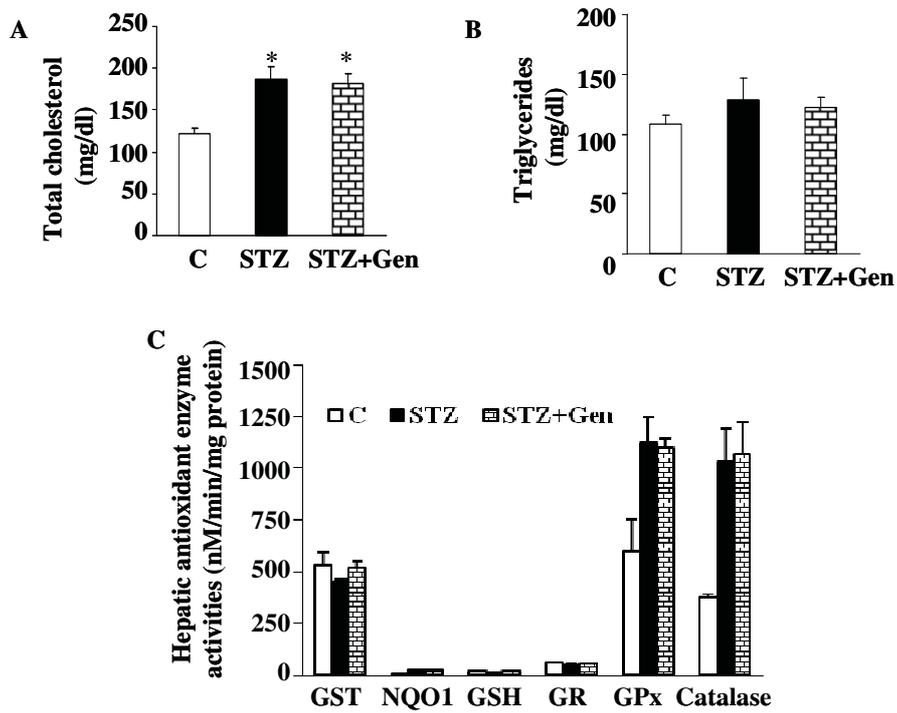


Figure 7. Genistein has no effect on plasma lipid profiles and hepatic antioxidant enzyme activities in STZ-induced diabetic mice. Diabetic mice were induced with STZ and fed a basal or genistein (Gen) diet as described in Fig. 6. Plasma total cholesterol (A); triglycerides (B); and hepatic activities of glutathione S-transferase (GST); NAD(P)H:quinone oxidoreductase 1 (NQO1); glutathione (GSH); glutathione reductase (GR); glutathione peroxidase (GPx) and catalase (C) were measured as described in “Materials and Methods”. Data are expressed as mean \pm SE (n=6-8 mice). *: $P < 0.05$ vs. healthy control (C).

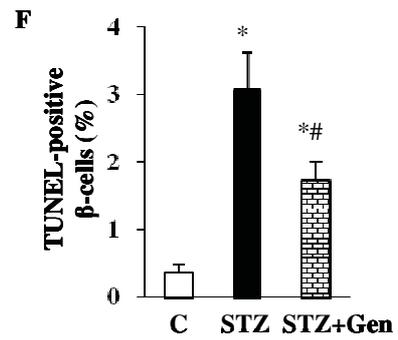
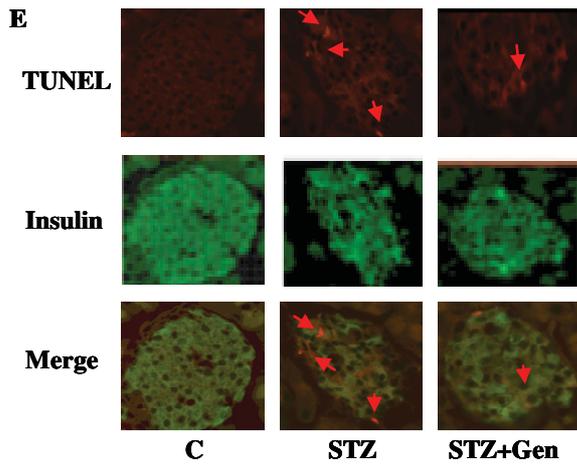
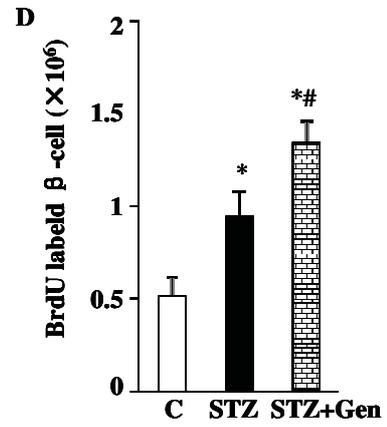
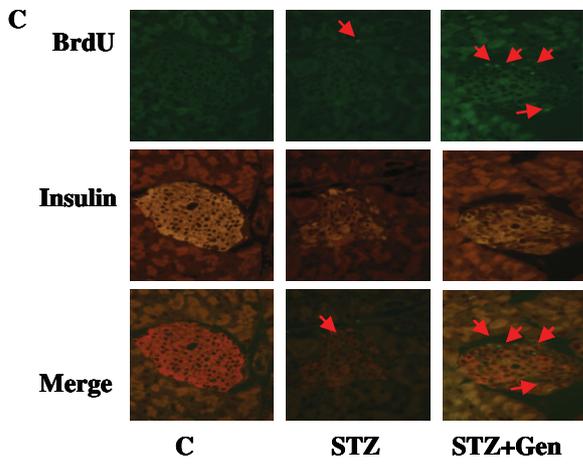
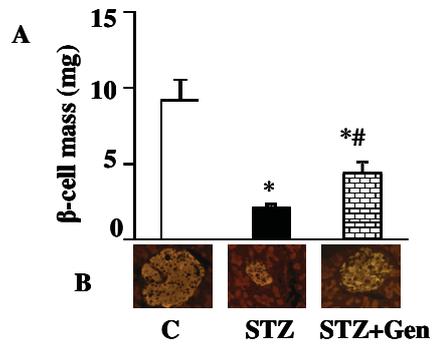


Figure 8. Dietary intake of genistein improves pancreatic β -cell proliferation: survival: and mass in STZ-induced diabetic mice. A and B: Pancreatic sections from control (C) or STZ diabetic mice fed control or genistein diet (Gen) were stained with an antibody against insulin. The β -cell mass was determined as described in “Materials and Methods”. C and D: Pancreatic sections were double-immunostained for BrdU and insulin. The number of BrdU- and insulin-positive cells as identified by the arrows from merged images was counted. E and F: Pancreatic sections were stained using TUNEL procedure and co-stained for insulin. TUNEL- and insulin-positive cells were identified by the arrows in merged sections (E). The number of apoptotic β -cells in each islet were counted and expressed as percentage of total insulin-positive cells (F). Data are expressed as mean \pm SE (n=12 mice). *: $P < 0.05$ vs. healthy control (C); #: $P < 0.05$ vs. STZ alone-treated mice.

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CHAPTER FIVE

Genistein ameliorates hyperglycemia in a mouse model of non-genetic type 2 diabetes

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Abstract

While peripheral insulin resistance is common during obesity and aging in mice and people, the progression to T2D is largely due to loss of β -cell mass and function through apoptosis. We recently reported that genistein, a soy derived isoflavone, can improve glycemic control and pancreatic β -cell function in insulin-deficient diabetic mice. However, whether it can prevent loss of pancreatic β -cell mass and diabetes in obese T2D mice is unknown. In the present study, we investigated the effect of dietary supplementation of genistein on glycemic control and pancreatic β -cell mass and function in non-genetic, middle-aged obese diabetic mice generated by high fat diet feeding and a low dose of streptozotocin (STZ) injection. Dietary intake of genistein (250mg/kg diet) improved hyperglycemia, glucose tolerance, and blood insulin levels in these obese diabetic mice, whereas it did not affect body weight gain, food intake, fat deposit, plasma lipid profile, and peripheral insulin sensitivity. Genistein increased the number of insulin-positive β -cells in islets, promoted islet β -cell survival, and preserved islet mass. These data demonstrate that dietary intake of genistein could prevent T2D via a direct protective action on pancreatic β -cells without alteration of periphery insulin sensitivity.

Key words: High fat diet; streptozotocin; β -cells; genistein; proliferation; apoptosis.

Introduction

It is estimated that at least 23.6 million or 7.8% of Americans presently suffer from diabetes, and 57 million people have pre-diabetes (1). While the availability of novel drugs, techniques, and surgical intervention has improved the survival rate of individuals with diabetes, the prevalence of diabetes is still rising in Americans, with the number of people with diabetes projected to double by 2025 (2). T2D is a result of chronic *insulin resistance and loss* of β -cell mass and function (3). Both in experimental animals and people, obesity is a leading pathogenic factor for developing insulin resistance, which is always associated with the impairment in energy metabolism, causing increased intracellular fat content in skeletal muscle, liver, fat, as well as pancreatic islets. Constant insulin resistance will progress to T2D when β -cells are unable to secrete adequate amount of insulin to compensate for decreased insulin sensitivity, which is largely due to insulin secretory dysfunction and significant loss of functional β -cells (3-8). Indeed, those individuals with T2D always manifest increased β -cell apoptosis and reduced β -cell mass (5-6, 9). As such, the search for novel agents that simultaneously promotes insulin sensitivity and β -cell survival may provide a more effective strategy to prevent the onset of diabetes (10).

Genistein is a flavonoid compound present in legumes and Chinese herb medicines *Genista tinctoria* Linn and *Sophora subprostrata* Chun et T.Chen. It is widely used as a dietary supplement in the U.S. for various presumed health benefits (11-13), although the research evidence supporting the *beneficial effects* of genistein consumption on human health is not well established. Genistein intake is considered safe as no toxic effects were observed in rats (14-16), mice (17), monkeys (18), and humans (19-20) following pharmacological administration, although genistein may have weak estrogenic or anti-estrogenic effects in some tissues by

primarily binding to estrogen receptor- β (21). Genistein has been previously investigated for its potential beneficial effects on cancer treatment, cognitive function, and cardiovascular and skeletal health, with a primary focus on exploring its potential hypolipidemic, anti-oxidative and estrogenic effects (11-13). While studies on whether genistein has an effect on diabetes are very limited, available data showed that administration of genistein moderately lowered plasma glucose in diabetic animals (22-23) and in humans (24-25) without affecting insulin sensitivity and fat metabolism. However, the mechanism of this genistein action is unknown. There is increasing evidence showing that oxidative stress and reactive oxygen species (ROS) play a potential role in the initiation of diabetes (26-29). Genistein has been reported to exhibit antioxidant activity (30-31). However, this effect of genistein is achieved only at concentrations ranging from 25-100 μM , suggesting that genistein is not a physiologically effective antioxidant because the achievable levels of plasma genistein in both humans (32-34) and rodents (13, 35-36) through dietary ingestion of genistein or soy-based diet is always less than 10 μM .

We recently discovered for the first time that genistein at physiologically achievable concentrations (0.1-5 μM) activated cAMP/PKA signaling by stimulating adenylate cyclase activity in β -cells and islets (37). We further found that dietary intake of genistein improved pancreatic β -cell proliferation and survival and prevented diabetes in insulin-deficient type 1 diabetic (T1D) mice (38). Because loss of functional β -cell mass and its progressive dysfunction are hallmarks in the pathogenesis of T2D (39-41), we tested in the present study the hypothesis that genistein can also protect pancreatic islets from apoptosis and thereby prevent T2D. In that regard, we gave genistein to non-genetic, middle-aged diabetic mice that were generated by high fat feeding and a low dose of STZ that did not cause diabetes in chow-fed mice. We show that dietary intake of genistein ameliorated hyperglycemia and improved β -cell mass in middle-aged

T2D mice, which were associated with improved pancreatic β -cell survival and circulating insulin levels.

Materials and Methods

Animals and study design

Ten months old male C57BL/6 mice (NCI, Frederick, MD) were housed individually on a 12/12 h light cycle with free access to food and water. Mice were fed either a standard diet (STD, AIN 76A with corn oil replacing soy oil) with 10% of calories derived from fat, a high fat diet (HF, AIN 76A with corn oil replacing soy oil and 60 kcal% fat are primarily from lard: Research Diets Inc., New Brunswick, NJ) with 60% of calories from fat, or HF diet containing 250 mg genistein/kg diet. After 4 weeks of dietary treatment, mice were intraperitoneally injected (i.p.) with a single dose of streptozotocin (90 mg/kg; Sigma Aldrich, St. Louis, MO) or vehicle. Fasting blood glucose was measured once every other week. Food intake and body weight were monitored weekly. Animal procedures performed in this study were reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Fasting blood glucose, glucose tolerance test (GTT), and insulin tolerance test (ITT)

Fasting blood glucose was measured after overnight fasting via tail vein bleeding using an ultra-sensitive hand-held glucometer (The Kroger Co., Cincinnati, OH). For glucose tolerance tests, mice were fasted overnight and injected i.p. with a single bolus of glucose (2 g/kg body wt). Glucose levels were measured at 0, 15, 30, 60, and 120 min after glucose administration. For ITT, mice were injected i.p. with insulin (0.75 units/kg body wt), and blood glucose levels were measured before and at 15, 30, 60, and 120 min after insulin administration as described previously (38).

Blood lipid profile and insulin

Blood total cholesterol, HDL-cholesterol, and triglycerides were measured using a CardioChek blood analyzer (Polymer Technology Systems, Indianapolis, IN). The LDL-cholesterol levels were calculated using the Friedewald equation: [LDL-Cholesterol = Total Cholesterol – (HDL-Cholesterol + Triglycerides/5)]. Insulin levels were measured using a mouse insulin ELISA kit (Mercodia, Inc., Uppsala, Sweden).

Pancreatic β -cell mass and apoptosis

Pancreata were collected and weighed from animals after euthanasia. Pancreas samples were embedded in paraffin and sectioned by AML Laboratories Inc (Baltimore, MD). A series of tissue sections (5- μ m thickness) were prepared, mounted on glass slides, and immunofluorescently stained with an insulin antibody and FITC-conjugated secondary antibody (Abcam, Cambridge, MA) for determining β -cell mass. Pancreatic β -cell area was measured using images acquired from five serial insulin-stained pancreatic sections sampled at 2.5 mm interval. Pancreatic β -cell mass was calculated by dividing the area of insulin-positive cells by the total area of pancreatic tissue and multiplied by the pancreas weight (38, 42). Apoptotic β -cells were labeled with an antibody against activated caspase-3 followed by detection with a streptavidin-biotin immunoenzymatic antigen system (Abcam, Cambridge, MA).

Statistical analysis

Data were analyzed with one-way ANOVA using the mixed models procedure of SAS (Cary, NC). The statistical model included the main effects of food and water intake, blood glucose, and body weight. Significant treatment differences were subjected to Tukey's test. A p-value < 0.05 was considered significant.

Results

Food intake, body and organ weights

Genistein supplementation did not alter food consumption pattern compared with HF diet-fed mice before or after STZ injection. The high fat diet decreased the accumulative average food intake, though it increased food consumption at the first week (Fig. 1A and B). Four weeks of consuming HF diet significantly increased body weight of mice. However, dietary intake of genistein at 250mg/kg diet had no effect on body weight gain before STZ injection. Injection of STZ resulted in a reduction in body weight. After STZ injection, mice fed genistein supplemented HF diet (HF-STZ-GE mice) were slightly heavier compared to mice on HF diet alone (Fig. 1C).

There was no change in heart, pancreas, and spleen absolute weight. A slight increase in liver and kidney weight was observed in mice on HF diet compared with mice on STD diet but the change was not significant. The abdominal fat pad was 50% greater in HF mice, while fat pads of mice in the HF-STZ group was 20% greater than that of STD mice. The fat pad weight of HF-STZ-GE mice was similar to that of HF-STZ mice (Fig. 1D).

Fasting blood glucose

After 4 weeks of HF diet consumption, mice displayed significantly elevated fasting blood glucose concentrations compared with animals that consumed STD diet. However genistein did not affect HF diet-induced rise in blood glucose. One week after injection of STZ, fasting blood glucose started to rise sharply in HF-STZ mice. In HF-STZ-GE mice blood glucose levels were significantly lower (30 % reduction) at this point than in HF-STZ mice (Fig. 2A). Although in the following weeks, fasting blood glucose of animals that received STZ injection continued to rise, blood glucose levels in HF-STZ-GE mice continued to be significantly lower.

GTT and ITT

Two weeks post STZ-injection, we performed a GTT, and the data showed that blood glucose levels in HF-STZ-GE mice was significantly lower at baseline and 15 minutes after glucose injection than those in HF-STZ mice; but followed the same pattern after 30 minutes as HF-STZ mice (Fig. 2B). The results from GTT demonstrated that blood glucose levels of HF-STZ mice at all time points were almost twice that of HF animals ($p < 0.05$), which were the result of STZ administration. HF-STZ-GE mice had a similar insulin response pattern as HF-STZ mice except a significantly lower ($p < 0.05$) baseline blood glucose level (Fig. 2C).

Blood lipid profile and insulin levels

Whole blood was collected from overnight-fasted animals for measurement of total cholesterol, HDL-cholesterol, triglyceride, plasma insulin, and then calculation of LDL-cholesterol. HF mice had slightly higher total cholesterol but not significant and significant higher LDL-cholesterol. HF-STZ and HF-STZ-GE mice had similar levels of total cholesterol, HDL-cholesterol, triglyceride, and LDL-cholesterol as STD mice. The plasma insulin levels in HF mice was almost twice as high as mice that received a STD diet ($p < 0.05$). HF-STZ mice displayed the lowest plasma insulin levels, while HF-STZ-GE mice had plasma insulin concentrations close to those of STD mice and significantly higher than those in HF-STZ mice ($p < 0.05$) (Fig. 3).

Pancreatic β -cell mass and apoptosis

We evaluated pancreatic islet β -cell mass through immunohistochemical technique. We found that consumption of the HF diet dramatically increased β -cell mass, while STZ administration severely decreased β -cell mass compared with mice on STD diet. However, dietary provision of genistein significantly improved islet β -cell mass compared with HF-STZ

mice (Fig. 4). In both STD and HF mice, apoptosis was rare. However almost 30% of β -cells were caspase-3 positive in pancreatic islets of HF-STZ mice. HF-STZ-GE mice have significantly lower ($p<0.05$) β -cell apoptosis as compared to that in islets from HF-STZ mice ($29\pm 5.6\%$ vs. $17\pm 6.1\%$). (Fig. 5).

Discussion

In humans, insulin resistance is associated with both obesity and T2D (43). However, most of the individuals with insulin resistance do not develop diabetes, because of the compensatory insulin secretion that overcomes the reduced insulin sensitivity in peripheral tissues (44-46). Therefore, those individuals with constant insulin resistance will progress to T2D only when extensive β -cell destruction occurs and residual β -cells are unable to meet the demands of the increased insulin requirement (3-8). Indeed, even though compensatory insulin secretion is capable of maintaining blood glucose homeostasis, β -cell damage reportedly already exists in the individuals at high risk for developing T2D, and those individuals with T2D always manifest increased β -cell apoptosis and reduced β -cell mass (5-6, 9). As such, the search for novel agents that promote β -cell survival and thereby preserve functional β -cell mass may provide an effective strategy to prevent the onset of diabetes (10). In the present study, we determined whether genistein has a protective effect on β -cells in a rodent T2D model.

We show that genistein (250 mg/kg diet) significantly prevented the development of diabetes and improved pancreatic islet mass in a non-genetic mouse model of T2D, which were generated by HF feeding and a mild dose of STZ administration that did not cause diabetes in chow-fed mice (data not shown). We used C57BL6 mice near one year old, the mouse equivalent of middle age in humans, because T2D usually occurs at middle and older age in humans. This

mouse model shares the metabolic characteristics of human T2D with peripheral insulin resistance and reduced β -cell mass and function (47). The use of a low dose of STZ also minimizes the variability of diet-induced diabetes development and thus provides better experimental controls for evaluating the anti-diabetic effects of this compound. The anti-diabetic effect of genistein observed in this study might be relevant to humans, because this dose of genistein used in the present study (equivalent to the human intake of 75-100 mg/day (48)) is within the dose range typically consumed by humans (49-51).

One study reported an improved lipid profile after genistein consumption in obese Zucker rats (23), which could result in an improvement in insulin sensitivity (52). Thus, we might expect that supplemental genistein in a HF diet may ameliorate lipid profile and therefore influence insulin sensitivity lower HF diet induced-hyperglycemia. However, insulin sensitivity was not modulated by the presence of genistein in the diet for 4 weeks. At the fourth week of consuming HF diet, we did observe a significant increase in fasting plasma cholesterol, blood glucose, and insulin levels in HF mice, which implies the existence of insulin resistance, consistent with the higher body weight observed in these mice. Excess fat deposit in adipocytes plays critical roles in the development of insulin resistance (53-54). In this study the majority of weight gain in HF diet-fed mice came from adipose tissue as shown by the organ fat pad weight data. However, genistein supplemented in HF diet did not cause any change either in body weight gain, fat mass, or fasting blood glucose after 4 weeks of genistein supplementation. In addition, identical food intake between HF mice and HF-STZ-GE mice suggested unchanged appetite and metabolic rate. This experimental results provide further evidence that genistein had no effect on energy metabolism and insulin sensitivity, which are in line with our recent finding in insulin-deficient diabetic mice (38).

We used STZ to induce diabetes by directly causing destruction of β -cells. As expected, STZ injected mice had a marked increase in fasting blood glucose and started to lose body weight the week after STZ administration. Genistein supplementation significantly lowered fasting blood glucose compared with HF-STZ treated mice. The results from ITT showed that genistein supplemented animals had significantly lower baseline blood glucose, while during the time after insulin injection fell to similar levels as HF-STZ mice. These data further confirm that t genistein had no significant effect on insulin sensitivity in HF-STZ mice.

To further confirm the effect of genistein on lipid profiles, we measured total cholesterol, triglyceride, LDL-cholesterol, and calculated LDL-cholesterol after mice were sacrificed. All the lipid parameters remained the same between HF-STZ and HF-STZ-GE mice. While mice consumed HF diet had significantly higher LDL-cholesterol and slightly higher total cholesterol, these numbers fell back in all of the mice that received STZ administration regardless of genistein supplementation, which could result from the reduction in fat mass and insulin caused by STZ injection (55).

Fasting blood glucose is derived primarily from hepatic gluconeogenesis. Thus, excessive hepatic glucose output is an important factor contributing to fasting hyperglycemia (56). A recent study demonstrated that dietary provision of genistein elevated blood insulin levels and suppressed hepatic gluconeogenic enzyme activities in non-obese diabetic (NOD) mice (57), suggesting that genistein may improve hyperglycemia partially through inhibition of hepatic gluconeogenesis. However, the effect of genistein on hepatic gluconeogenic enzymes could be due to a secondary action whereby genistein induces or preserves pancreatic β -cell insulin secretion (37-38), given that insulin is required for regulating hepatic gluconeogenesis (58). In the present study, our results suggest that the improvement in fasting blood glucose by genistein

treatment is primarily due to the improved pancreatic insulin secretion as demonstrated by significantly higher levels of circulating insulin levels in genistein-treated diabetic mice, because STZ induces diabetes by directly causing β -destruction and insulin deficiency. Indeed, we further showed that dietary supplementation of genistein β -cell apoptosis and preserved β -cell mass in the pancreas of diabetic mice.

The glucose analogue STZ is reported to be transported into β -cells by the glucose transporter 2 (GLUT2) for exerting its apoptotic effect (59). While we did not study the effect of genistein on GLUT2 protein expression in mouse islets, our recent studies found that genistein had no such an effect in cultured β -cells (60), suggesting that improvement of islet β -cell mass and survival by genistein may not be due to modulation of GLUT2 expression, thereby preventing STZ influx in β -cells. Some studies showed that STZ increased peripheral lymphocytic infiltration into islets by stimulating the production of several pro-inflammatory cytokines, thereby producing insulinitis (61-62), which may contribute to STZ-induced β -cell apoptosis and diabetes. However, we showed that genistein had no effect on mononuclear cell infiltration into the islets (38). Indeed, the mouse strain used in this study is reportedly resistant to STZ-induced insulinitis as STZ-caused infiltration of immune cells into islets is very rare in these mice (63)

We recently discovered for the first time that genistein at physiologically achievable concentrations (0.1-5 μ M) activated cAMP/PKA signaling by stimulating adenylate cyclase activity in β -cells and islets (37). Several factors protect pancreatic β -cells from apoptosis by activating the cAMP/PKA pathway (64-66). While we did not measure whether dietary intake of genistein affects the cAMP signaling pathway in the islets in vivo because it is difficult to isolate

adequate amount of islets for this study in STZ-induced diabetic mice, it is tempting to speculate that genistein might protect islets from apoptosis through activation of this signaling pathway.

In summary, using a middle-aged T2D mouse model, we provide evidence that genistein as the form of dietary supplement ameliorates hyperglycemia. This anti-diabetic action of genistein is not mediated through improving insulin sensitivity, but rather was due to protecting pancreatic β -cell from apoptosis and preserving functional β -cell mass. Loss of functional β -cell mass is the key for deterioration of glycemic control in both T1D and t2D. In this context, genistein may be a naturally occurring low-cost agent that can be used as an alternative or complementary treatment for diabetes. However, more studies are needed to further characterize the potential anti-diabetic effect of this compound and to define the cellular and molecular mechanisms underlying this effect.

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Abbreviations

GLUT2: glucose transporter 2

GTT: glucose tolerance test

ITT: insulin tolerance test

NOD: none-obese diabetic

ROS : reactive oxygen species

STZ : streptozotocin

T1D: type 1 diabetic

T2D: type 2 diabetic

Figures

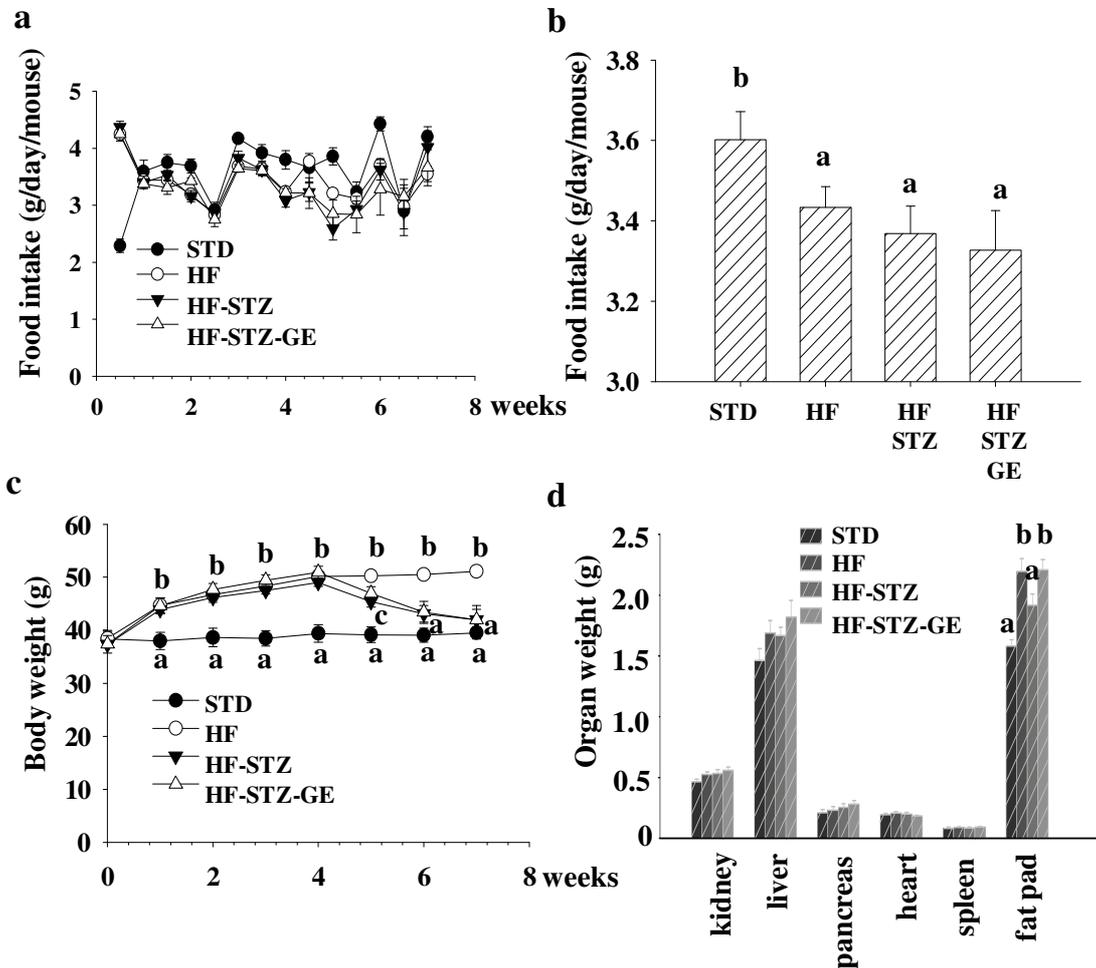


Figure 1. Genistein supplementation had no influence on food consumption, body weight, or major organ weight. Food intake was recorded every 3 or 4 days (a). Cumulative daily food consumption was calculated at the termination of the experiment (b). Body weight was monitored every week (c). Major organs were weighed after mice were sacrificed (d). Data are shown as means \pm SE (n=8 mice/group. Groups that are identified by different letters are significantly different at $p < 0.05$). (STD: standard diet, HF: high fat diet, STZ: streptozotocin, GE: genistein).

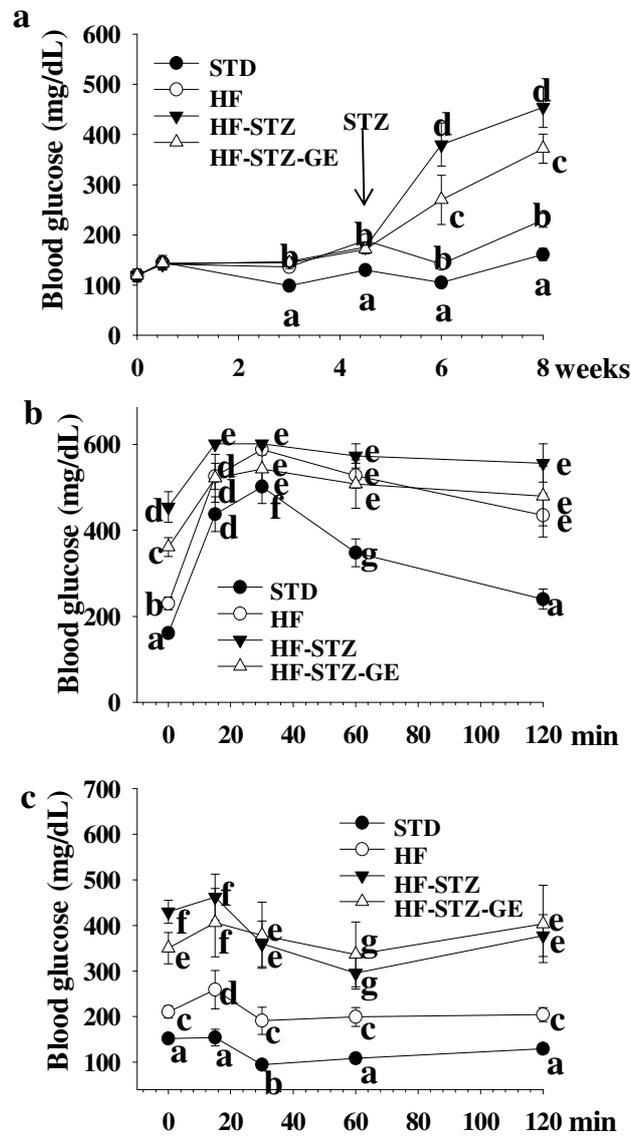


Figure 2. Genistein supplementation improved fasting blood glucose and glucose tolerance, but did not influence insulin tolerance. Blood glucose was monitored every other week. Arrow points to STZ injection (a). For glucose tolerance test (b) or insulin tolerance test (c), overnight-fasted mice were injected i.p. with a bolus of glucose (2g/kg body weight) or insulin (0.75 U/kg body weight) , followed by measurements of blood glucose at 0, 15, 30, 60, and 120 minutes after glucose injection. Data are means \pm SE (n=8 mice/group. Groups that are identified by different letters are significantly different at $p < 0.05$). STD: standard diet, HF: high fat diet, STZ: streptozotocin, GE: genistein.

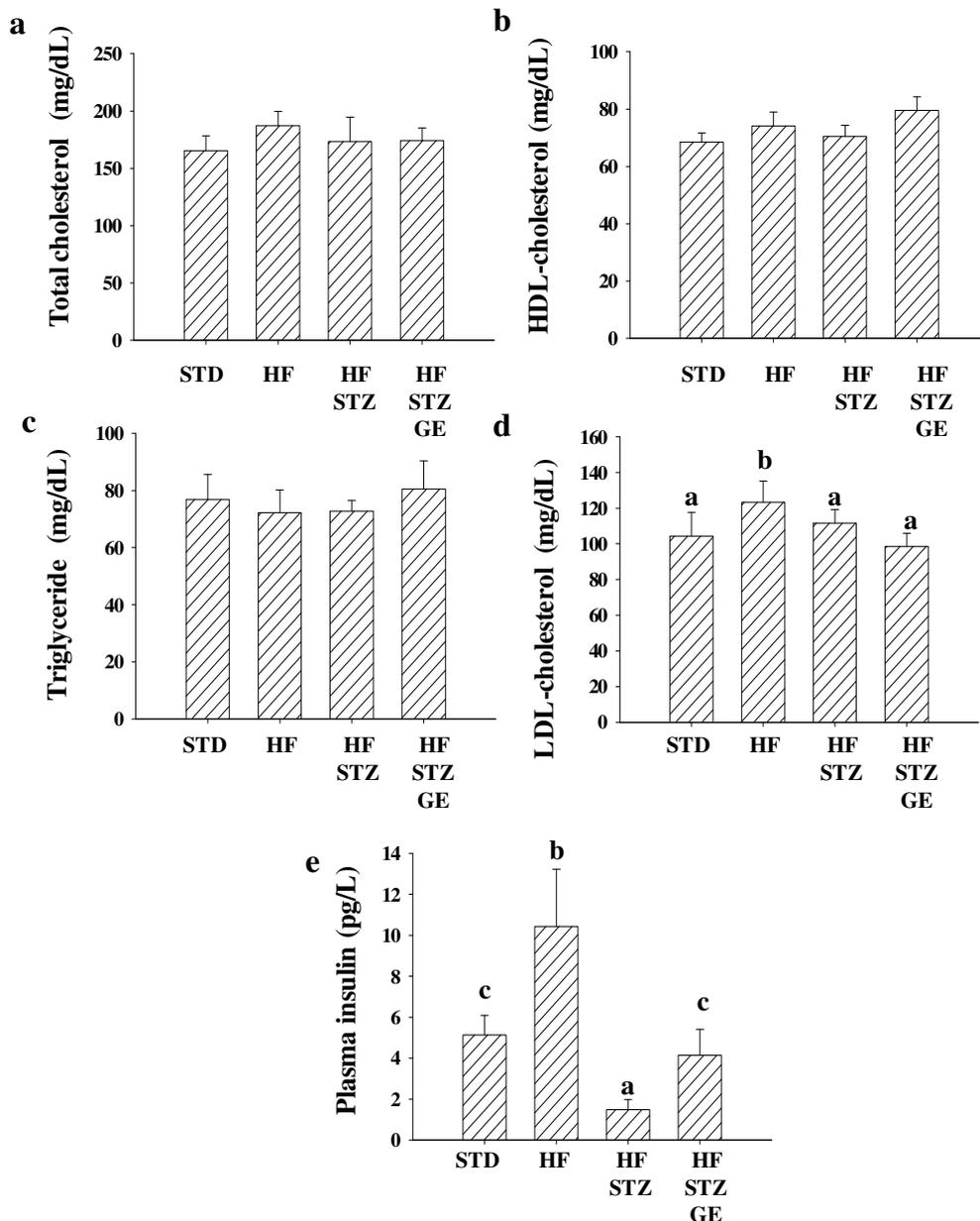


Figure 3. Genistein supplementation did not affect blood lipid profile, but increased plasma insulin levels. At the end of the eighth week of the experiment, fasting plasma total cholesterol (a), HDL-cholesterol (b), and triglyceride (c) were measured. Plasma insulin (e) was measured with an ELISA kit. LDL-cholesterol levels (d) were calculated based on total cholesterol, HDL-cholesterol, and triglyceride. Data are shown as means \pm SE (n=8 mice/group). Groups that are identified by different letters are significantly different at $p < 0.05$. STD: standard diet, HF: high fat diet, STZ: streptozotocin, GE: genistein.

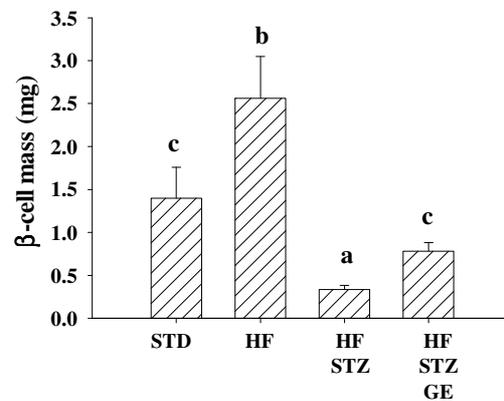
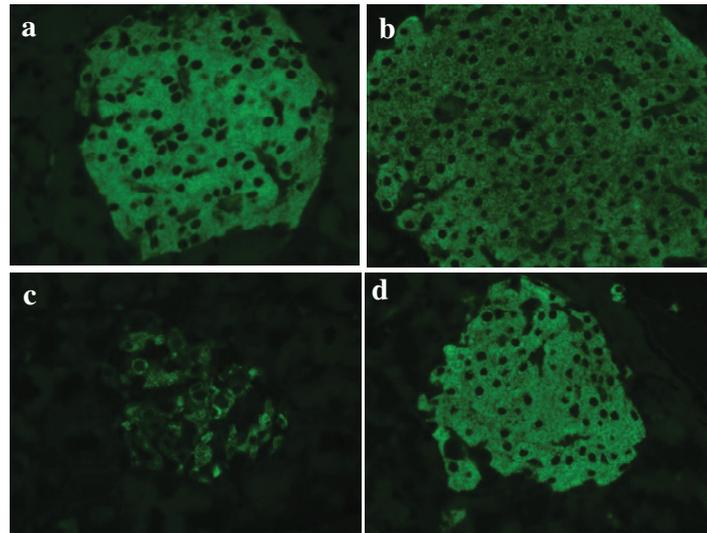


Figure 4. Genistein supplementation improved β -cell mass. A, B, C, and D represent pancreatic sections from mice receive a STD (a), HF (b), HF and STZ administration (c), and genistein supplemented HF and STZ administration (d), respectively. Pancreatic β -cells were stained with antibody against insulin and relative β -cell percentage was calculated. Pancreatic β -cell mass was determined as pancreas weight multiplied by the relative β -cell percentage. 5 sections were sampled from each mouse with 8 mice in each group. Data are shown as means \pm SEM. (n=8 mice/group. Groups that are identified by different letters are significantly different at $p < 0.05$). STD: standard diet, HF: high fat diet, STZ: streptozotocin, GE: genistein.

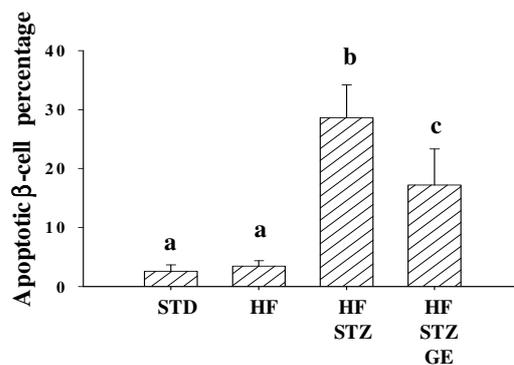
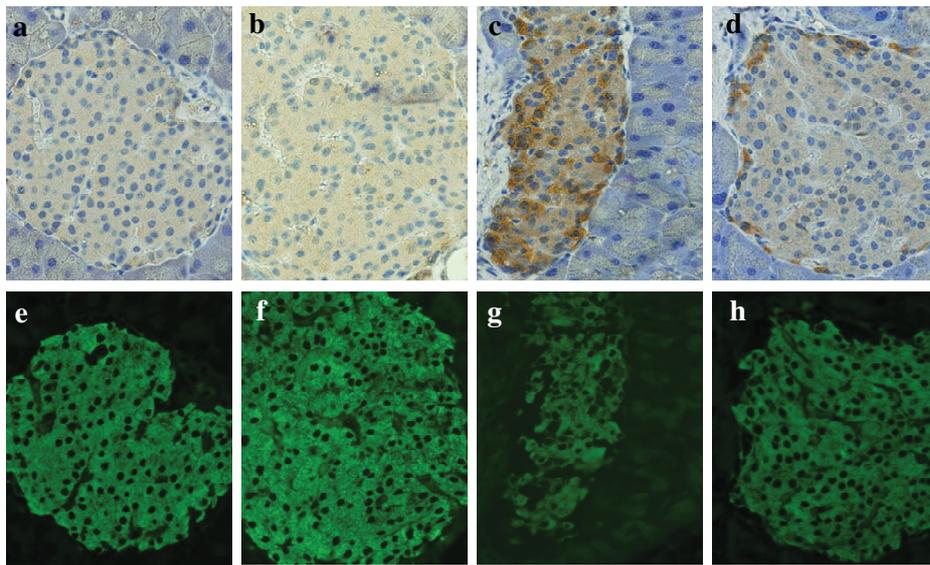


Figure 5. Genistein supplementation inhibited apoptosis of pancreatic β -cells. a, b, c, and d are representative pancreatic sections stained with activated caspase-3 from mice received a STD (a), HF (b), HF and STZ administration (c), and genistein supplemented HF and STZ administration (d); e, f, g, and h are the same sections fluorescently stained with insulin. Five sections of each mouse pancreas were co-stained with activated caspase-3 and insulin with 8 mice in each group. Data are means \pm SEM (n=8 mice/group. Groups that are identified by different letters are significantly different at $p < 0.05$). STD: standard diet, HF: high fat diet, STZ: streptozotocin, GE: genistein.

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CHAPTER SIX

Conclusions and future research

Conclusions

Genistein has been shown to exert an anti-diabetic effect in experimental animals and patients, but the mechanism underlying this action is unclear. In this dissertation research, I found for the first time to my knowledge that genistein is a novel insulinotropic agent and a putative β -cell growth factor by targeting the cAMP signaling pathway. My animal study work showed that genistein is capable of preserving islet β -cell mass, improving circulating insulin levels, and alleviating diabetes in both T1D and T2D mice. Loss of functional β -cell mass is the key for the development of both T1D and T2D. In this context, these findings may potentially lead to the development of novel, natural agents for diabetes prevention and treatment. In this context, genistein may be a naturally occurring low-cost agent that can be used as an alternative or complementary treatment for diabetes. However, more studies are needed to further characterize the potential anti-diabetic effect of this compound and to define the cellular and molecular mechanisms underlying this effect in T2D.

Future direction

While my research provided substantial new insight into how genistein prevents diabetes, the following aspects regarding the detailed molecular mechanisms underlying its various actions in β -cells still need to be defined.

1. Determine if genistein's potentiating effect on insulin secretion in β -cells is mediated by up-regulating hormone-sensitive lipase (HSL) and synaptosomal-associated protein of 25 kDa (SNAP-25). Recent study demonstrated that HSL, the enzyme for acylglycerol hydrolysis that is expressed in β -cells [1], plays an important role in insulin secretion [1, 2]. HSL can be directly activated by PKA [3]. However, whether chronic exposure of β -cells to genistein improves insulin secretory function through increasing HSL activity or expression remains to be determined. Another candidate protein that may be involved in genistein-enhanced insulin secretion is SNAP-25. Insulin is released from β -cells through regulated exocytosis, which requires transport and docking of insulin secretory granules to the plasma membrane and subsequent fusion. Studies showed that SNAP-25, a membrane bound protein, is involved in this process of insulin exocytosis from β -cells [4, 5], and its expression is up-regulated by PKA in oocytes and steroidogenic cells [6]. It is therefore tempting to speculate that genistein may enhance GSIS in β -cells through cAMP/PKA-mediated up-regulation of SNAP-25 expression, an aspect that needs further investigation.

2. Determine whether the effects of genistein on β -cells are mediated via activation of G-protein-coupled receptor-30 (GPR30). GPR30 is an orphan receptor. Recent studies reported that genistein can bind to GPR30 [7]. While the physiological role of GPR30 is still unknown, female GPR30 knockout mice displayed hyperglycemia and impaired glucose tolerance [8]. Therefore, it is intriguing to speculate that GPR30 may play a role in mediating genistein effect on β -cells.

3. Determine the mechanism by which genistein protects against β -cell apoptosis. Genistein's protective effect on islet cells from apoptosis is still unknown. It is well recognized that activation of nuclear factor κ B (NF- κ B) is a crucial step for β -cell dysfunction [9, 10]. NF- κ B-

mediated destruction of β -cells is executed at least partially through induction of its downstream gene inducible nitric oxide (NO) synthase (iNOS) and subsequent NO production [11, 12]. The critical role of iNOS-derived NO in the pathogenesis of T1D has been demonstrated in β -cell-specific iNOS knockout and transgenic animals [13-15]. It is a meaningful study to determine whether genistein at physiologically relevant doses can suppress the activated NF- κ B pathway, therefore preventing β -cells from destruction.

4. Investigate whether genistein has a synergetic or additive effect on diabetes prevention with insulin sensitizing compounds. T2D is a result of chronic insulin resistance and loss of β -cell mass and function. As such, it is intriguing to test whether a combination therapy of genistein with an insulin sensitizing compound that simultaneously promote insulin sensitivity and β -cell survival may provide a more effective strategy to prevent the onset of diabetes.

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