UNIVERSITAT ROVIRA I VIRGILI

THE EFFECTS OF GRAPE SEED PROCYANIDIN EXTRACT ON INSULIN SYNTHESIS AND SECRETION

Anna Castell Auvi

Dipòsit Legal: T. 271-2012

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Anna Castell Auví

The Effects of Grape Seed Procyanidin Extract on Insulin Synthesis and Secretion

DOCTORAL THESIS

directed by Dr. Anna Ardévol Grau

Departament de Bioquímica i Biotecnologia

UNIVERSITAT ROVIRA I VIRGILI

Tarragona 2012

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FAIG CONSTAR que aquest treball, titulat "**The Effects of Grape Seed Procyanidin Extract on Insulin Synthesis and Secretion"**, que presenta Anna Castell Auví per a l'obtenció del títol de Doctora, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que acompleix els requeriments per poder optar a Menció Europea.

Tarragona, 2 de març de 2012

La directora de la tesi doctoral:

Anna Ardévol Grau

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"La Vida És Una Oportunitat..."

La vida és un oportunitat	APROFITA-LA
La vida és maca	ADMIRA-LA
La vida és un somni	FES-LO REALITAT
La vida és un repte	AFRONTA'L
La vida és un deure	COMPLEIX-LO
La vida és un joc	JUGA-HI
La vida és preciosa	CUIDA-LA
La vida és riquesa	CONSERVA-LA
La vida és amor	GAUDEIX-NE
La vida és un misteri	DESCOBREIX-LO
La vida és promesa	COMPLEIX-LA
La vida és tristesa	SUPERA-LA
La vida és un himne	CANTA'L
La vida és un combat	ACCEPTA'L
La vida és una aventura	ENFRONTA-T'HI
La vida és felicitat	GUANYA-TE-LA
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Mare Teresa de Calcuta

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La cita anterior es refereix a la vida, però si us hi fixeu bé, es podria canviar la paraula Vida per Tesi en moltes frases i continuarien tenint sentit. Per exemple, "La Tesi és una oportunitat: Aprofita-la; La Tesi és un repte: Afronta'l; La Tesi és un misteri: Descobreix-lo; La Tesi és una aventura: Enfronta-t'hi; La Tesi és la tesi: Defensa-la...".

Us he de confessar que m'ha costat molt començar a fer aquest apartat, ja que significa que el moment de defensar la tesi és a tocar. Però ara que ja està tot pràcticament enllestit ha arribat el moment d'escriure els agraïments. Vull donar les gràcies a tota la gent que ha fet possible que aquesta tesi es fes realitat, sense el vostre suport i la vostra col·laboració aquest moment no hagués arribat mai.

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A LA MEVA FAMÍLIA

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I. INTRODUCTION

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A. Insulin as the main regulator of glucose disposal

Insulin is a hormone with two important functions related to overall metabolic homeostasis: to maintain adequate energy stores for development, growth and reproduction and to regulate plasma glucose levels [1]. The maintenance of normal glucose homeostasis requires complex and highly integrated interactions among the liver, muscle, adipocytes, kidneys, pancreas and neuroendocrine system [2] (Figure 1).

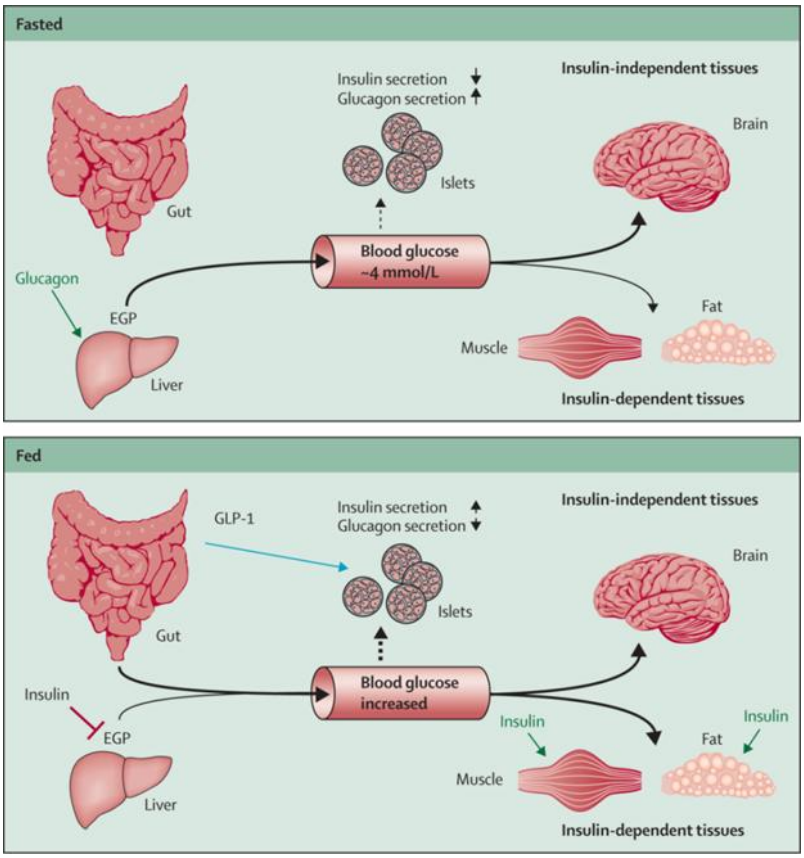


Figure 1. Overview of normal glucose homeostasis (from [3]).

In the fasting state, the blood glucose concentration is determined by the balance between endogenous glucose production (EGP) and its use by insulin-independent tissues [3]. The majority of EGP is derived from liver glycogenolysis and gluconeogenesis (approximately 85 %), and the remaining amount is produced by the kidney. Under basal conditions, approximately 50 % of all glucose disposal by insulin-independent tissues occurs in the brain, which becomes saturated at a plasma glucose concentration of about 2.22 mM (40 mg/dL). Another 25 % of glucose uptake occurs in the splanchnic area (liver plus gastrointestinal tissues) and is insulin independent. The remaining 25 % of glucose metabolism in the postabsorptive state occurs in insulin-dependent tissues, primarily muscles [4]. The delicate balance between EGP and tissue glucose uptake is disrupted in the fed state (glucose-containing meal). After ingestion, the glucose concentration in the blood rises because of absorption in the gut, which stimulates insulin secretion by islet β-cells and suppresses glucagon secretion from α-cells. EGP is suppressed and uptake into insulin-sensitive peripheral tissues, such as heart, skeletal muscle and adipose tissues is activated (which increases the rate of glucose disposal). The kidneys also play a central role in glucose homeostasis by reabsorbing all of the filtered glucose [2]. Neurohormonal processes include the release of the incretin hormones, such as glucagon-like peptide 1 (GLP1), which increase glucose-stimulated insulin secretion (GSIS) and glucose-suppression of glucagon secretion. Moreover, adipose tissue lipolysis is suppressed, and anabolic metabolism is promoted. Glucose concentrations become close to the fasting level within 2 h [3].

1. Insulin production and secretion

1.1 Insulin production

1.1.1 The islets of Langerhans

The pancreas is a multifunctional organ located next to the stomach, duodenum and bladder (Figure 2). Mammals, birds, reptiles and amphibians have pancreases with similar histologies and modes of development, whereas invertebrates do not have pancreases. The pancreas has two major functional units: the exocrine pancreas, which is responsible for the production of digestive enzymes to be secreted into the gut lumen, and the endocrine pancreas, which has a role in the synthesis of several hormones with key regulatory functions in food uptake and metabolism [5].

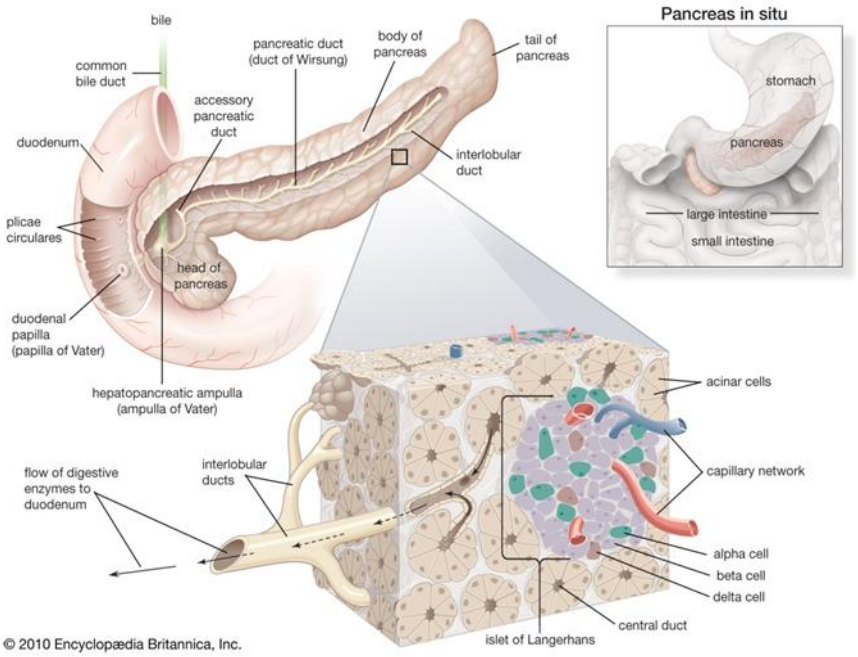


Figure 2. Structure of the pancreas. Acinar cells produce digestive enzymes, which are secreted into tiny ducts that feed into the pancreatic duct. Islets of Langerhans are clusters of cells that secrete hormones (from [6]).

The exocrine pancreas constitutes the majority of the mass of the organ and consists of two major cell types: acinar and ductal cells, which are organised in a lobular branched tissue architecture and secrete and transport digestive enzymes into the duodenum [7].

The endocrine pancreas represents less than 2 % of the organ and is made up of clusters (islets of Langerhans) of five major types of cells: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, pancreatic polypeptide (PP)-producing cells and ghrelin-producing ε-cells [8]. The islets also include nerves, dendritic cells, macrophages, fibroblasts and vascular cells, such as endothelial cells and pericytes (as reviewed in [9]).

The islet structure is highly diverse among vertebrates, from the principal islet in many species of fish to individual islets characteristic in mice and humans [10]. The arrangement of endocrine cell types in the islet has been suggested to have important physiological implications because the order of blood perfusion facilitates potential paracrine interactions between islet cells, such as those between α- and β-cells [11].

Murine islets are often described to have a highly ordered structure that is composed primarily of β-cells (60-80 % of islet cells) clustered in a central core, surrounded by smaller numbers of α-cells (15-20 %), δ-cells (<10 %), PP-cells (<1 %) and ε-cells in the periphery [10]. The size of mouse and rat islets can vary considerably, from ten or fewer cells to thousands of cells [12] (Figure 3).

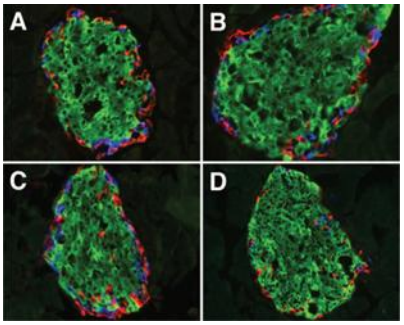


Figure 3. Histology of mouse pancreas. Micrographs of four different mouse islets show the typical core-mantle arrangement of β- and non-β-cells. (A-D) β-cells, green; α-cells, red; and δ-cells, blue (the minority islet's PP- and ε-cells are not represented). Magnification (A) x20, (B) x40, (C) x20, (D) x10 (from [13]).

The human islet architecture is distinct from that of rodents. Unlike the defined β-cell core and α-cell mantle characteristic of rodent islets, the α-, β- and δ-cells appear to be randomly distributed throughout human islets. The adult human islet is about ~50 % β-cells, ~40 % α-cells, ~10 % δ-cells and <1 % PP-cells and ε-cells. [12-14] (Figure 4).

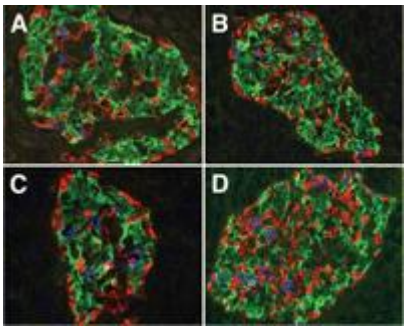


Figure 4. Histology of human pancreas. Micrographs of four different human islets show the intermingling of β- and non-β-cells. (A-D) β-cells, green; α-cells, red; and δ-cells, blue (the minority islet's PP- and ε-cells are not represented). Magnification x40 (from [13]).

Various studies have shown that the islet composition varies not only between species but also within species. Murine islets display a wide variety of morphologies under various physiological conditions, including pregnancy, obesity and diabetes. In *db/db* mice, which are obese and diabetic because of a mutation in the leptin receptor, the islet architecture and cellular composition resemble those of humans [10].

1.1.2 Insulin synthesis

Insulin gene expression begins early in embryonic development of the pancreas and remains tightly regulated throughout adult life. The mouse and rat genomes contain two insulin genes, insulin-1 and insulin-2 (INS1 and INS2). The INS2 gene has greater structural and functional similarity to other mammalian insulin genes whereas INS1 is considered to be a functional retroposon because it possesses only one of the two introns present in INS2 and other mammalian insulin genes [15,16]. Insulin is encoded on the short arm of chromosome 11 in humans. By contrast, the rat INS1 and INS2 genes are co-localised on chromosome 1, and the mouse genes are positioned on two different chromosomes, INS1 on chromosome 19 and INS2 on chromosome 7.

Similar to the human gene, rodent INS2, but not INS1, is expressed in the thymus, yolk sac, fetal liver and brain [17] as well as the pancreas. The role of insulin expression in non-β-cells is unclear. Hyperglycemia, with or without overt diabetes, has been reported to activate insulin gene transcription and proinsulin production in multiple extrapancreatic tissues, including the liver, spleen, adipose tissue, thymus and bone marrow [18].

Circulating and biologically active insulin is a monomer consisting of two peptide chains, A (with 21 amino acid residues) and B (with 30 residues), which are linked by two disulphide bonds (residues A7 to B7 and A20 to B19). The A chain has an additional internal disulphide bond between residues A6 and A11. The stability of disulphide bonds is important to the conformation of insulin. At micromolar concentrations, insulin dimerises, and in the presence of zinc, it further associates into a hexamer, which is the form of insulin stored in pancreatic β-cells. This inactive form has long-term stability and serves as a way to keep the highly reactive insulin protected, yet readily available [19].

The insulin gene encodes a much larger molecule than insulin, preproinsulin, which has 110 amino acids in humans (Figure 5). In addition to the mature protein, preproinsulin contains a predominantly hydrophobic 24 amino acid signal sequence at its N-terminus and a 35 amino acid connecting peptide linking the C-terminus of the B-chain to the N-terminus of the A-chain. The function of the signal peptide is to direct the protein into the secretory pathway by targeting it to the lumen of the endoplasmic reticulum (reviewed in [20]).

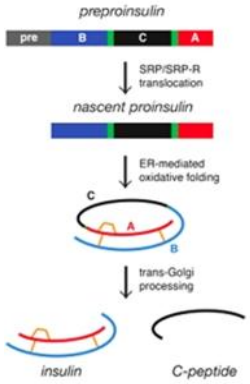


Figure 5. Pathway of insulin biosynthesis. Preproinsulin: signal peptide (grey), B-domain (blue), dibasic BC junction (green), C-domain (red), dibasic CA junction (green) and A-domain (red) (from [21]).

Insulin biogenesis is initiated with the synthesis of preproinsulin in the rough endoplasmic reticulum. Following the removal of a 24 residue signal sequence and packaging in the Golgi complex, insulin is stored as proinsulin in the immature secretory granules where conversion into its biologically active form is catalysed by the concerted activities of two endoproteases, prohormone convertase-1 and prohormone convertase-2, and exoprotease carboxypeptidase H to produce mature insulin and the C-peptide [22]. During the granule maturation process, insulin is crystallised with zinc and calcium in the form of dense-core granules and unwanted cargo and membrane proteins undergo selective retrograde trafficking to either the constitutive trafficking pathway for secretion or to degradative pathways [23]. Interestingly and for reasons that remain unclear, the last granules generated are most likely to undergo release when the β-cell is stimulated [22].

1.2 Insulin secretion

Secreting the right amount of insulin at the right moment is the crucial function of pancreatic β-cells that adjust insulin secretion to fluctuations of blood glucose levels. Although several nutrients can act as insulin secretagogues, glucose is the most potent secretagogue. Upon glucose stimulation, insulin secretion is biphasic, with a rapid transient (4-8 min) first phase and a second sustained phase that lasts as long as the glucose stimulation [24].

Two pathways interact in β-cells to ensure temporal and amplitude control of insulin secretion by glucose. The most well-known pathway is the triggering pathway where glucose triggers insulin secretion by increasing the concentration of cytosolic free calcium ($[Ca^{2+}]_c$) in β-cells. The pancreatic β-cells sense an elevated level of glucose in the plasma by glucokinase. Rapid entry of glucose through glucose transporters, GLUT2 (SLC2A2) for rodents and GLUT1 (SLC2A1) for humans, is followed by the phosphorylation of glucose by glucokinase, which increases the glycolytic flux, producing pyruvate as the terminal product of the pathway [25]. In the mitochondria, pyruvate is a substrate for both pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC). The latter forms oxaloacetate, providing anaplerotic input to the tricarboxylic acid (TCA) cycle [26]. Reducing equivalents generated by the TCA cycle activate the electron transport chain, resulting in hyperpolarisation of the mitochondrial membrane ($\Delta\Psi_m$) and formation of ATP. The increase in cytosolic ATP and/or the decrease in free ADP promotes the closure of the ATP-sensitive potassium channel (K_{ATP}) and depolarisation of the plasma membrane, which triggers the opening of voltage-dependent Ca^{2+} channels (VDCCs). The opening of VDCCs results in an influx of Ca^{2+} into the cell that triggers the exocytosis of insulin granules [25] (Figure 6).

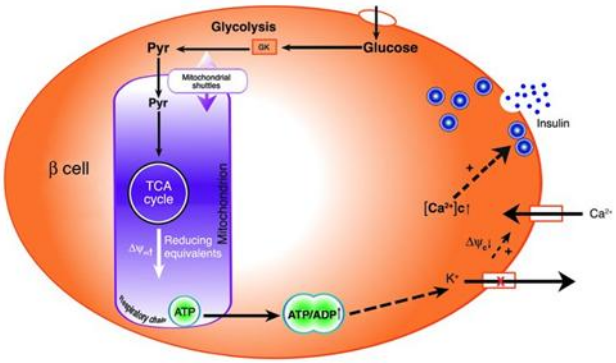


Figure 6. Model of GSIS in pancreatic β-cells (from [27]). GK: glucokinase; Pyr: pyruvate.

The K_{ATP} channels are complex structures composed of a pore made of Kir6.2 and a regulatory subunit made of sulfonylurea receptor 1 (SUR1). Although the central role of K_{ATP} channels in the production of the triggering Ca^{2+} signal by glucose is not disputed [28], the process described above is a simplification of the triggering pathway. Observations that glucose can control $[Ca^{2+}]_c$ in β-cells lacking K_{ATP} channels (because of a knockout of either SUR1 or Kir6.2 [24,29]) call for renewed attention to the possible influence of glucose metabolism on additional ion channels in the β-cell membrane. The relationship between the repletion of intracellular Ca^{2+} stores and voltage-independent Ca^{2+} channels in the plasma membrane should not be neglected (reviewed in [28]).

Numerous hormones and neurotransmitters bind to receptors in β-cell membranes and activate neurohormonal amplifying pathways that potentiate nutrient-induced insulin secretion. The transduction of their effects may involve changes in the triggering Ca^{2+} signal but mainly relies on amplifying the action of Ca^{2+} in exocytosis. Another mechanistically different pathway is set in operation by glucose. This amplifying pathway was identified during pharmacological bypass of K_{ATP} channels and clamping of the β-cell $[Ca^{2+}]_c$ at an elevated level. In one approach, K_{ATP} channels were held open by diazoxide, and high extracellular KCl was used to depolarise β-cells and steadily increase $[Ca^{2+}]_c$. In a second approach, all

K_{ATP} channels were closed by a high concentration of sulfonylurea, which depolarised β-cells and led to increased $[Ca^{2+}]_c$. Increasing the glucose concentration under these conditions augmented insulin secretion with hardly any effect on the already elevated $[Ca^{2+}]_c$. The effect of glucose also depends on sugar metabolism by β-cells and is mimicked by other metabolised secretagogues, but not by non-metabolised secretagogues [30]. These observations convincingly establish that glucose controls insulin secretion by a two-pronged mechanism [31]. Recent studies have shown that the pathway is operative during the stimulation of insulin secretion by glucose alone, when islet $[Ca^{2+}]_c$ is allowed to fluctuate freely in response to the stimulus [32]. Although it is clear that metabolic amplification is physiologically relevant and quantitatively important, its molecular and cellular mechanisms have not yet been identified completely [30]. In contrast to the transient secretion induced by Ca^{2+}-raising agents, sustained insulin release (amplifying signal) depends on the generation of metabolic factors.

In the presence of non-stimulatory concentrations of glucose, the rate of metabolism in β-cells is relatively low, and enough K_{ATP} channels are open in the plasma membrane to counteract depolarising currents and maintain the membrane potential at values more negative than the threshold for opening VDCCs. The influx of Ca^{2+} is minimal, $[Ca^{2+}]_c$ is low, and insulin secretion is basal [31].

The final steps in insulin granule exocytosis are governed by a series of proteins that mediate the correct docking, priming and fusion of insulin-containing secretory granules to the plasma membrane. In particular, a complex of proteins from both secretory granules and the plasma membrane is responsible for the efficiency of the fusion step. These proteins, known as soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, contain a signature sequence or SNARE motif with a high potential for coiled-coil formation [33]. In response to an increase in Ca^{2+} intracellular levels ($[Ca^{2+}]_i$), insulin granules fuse with the plasma membrane in a SNARE-dependent process [34]. The actin cytoskeleton is a highly dynamic and complex structure that is remodelled in response to various stimuli. The dynamics of insulin granules resulting from glucose stimulation could also be affected by actin remodelling [35].

Pancreatic β-cells contain at least two pools of insulin secretory granules that differ in release competence: a reserve pool (RP), which accounts for the vast majority of granules, and a readily releasable pool (RRP), which provides the remaining granules (less than 5%). The prevailing hypothesis is that the release of RRP granules accounts for the first phase of GSIS and that the mobilisation of a subsequent supply of new granules for release by mobilisation is responsible for the second phase [36]. More recently, Seino et al. proposed a model of insulin exocytosis in which both the first and second phases are caused by newly recruited granules without docking (restless newcomer) (Figure 7). Although both phases of insulin secretion are caused by these granules, the mechanisms of the first and second phases of insulin secretion are distinct, and the two phases are caused by granules from separate pools [37].

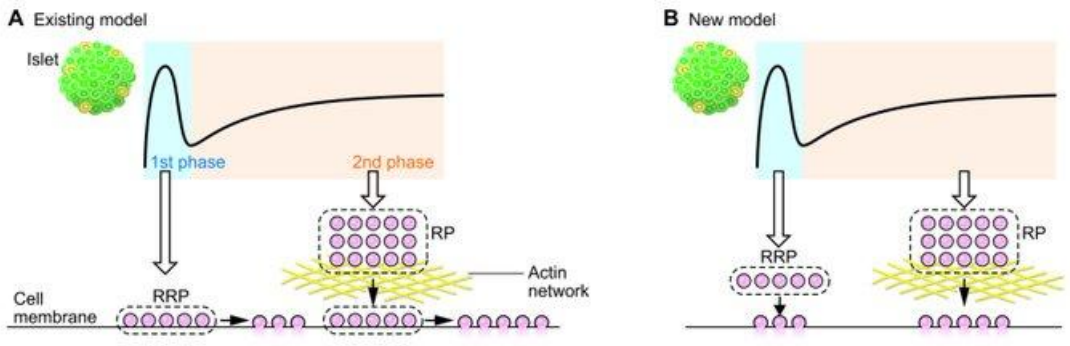


Figure 7. Existing and new models of GSIS in the normal state (from [37]).

2. Insulin signalling

2.1 β-cells

As described above, the pancreatic β-cell is a target for insulin feedback action. This autocrine feedback loop is important for proper β-cell function and survival and is involved in regulating gene expression, ion flux, insulin secretion, β-cell size and proliferation as well as β-cell survival (reviewed in [38]).

To execute its action and initiate a signalling cascade, insulin first has to bind to cell surface receptors, which are usually insulin receptor types A (INSRA) and B (INSRB). However, because pancreatic β-cells are exposed to insulin concentrations higher than those in the periphery, insulin-like growth factor 1 and 2 receptors (IGFR1/2), which have a lower affinity for insulin, cannot be excluded as targets for insulin binding [39]. Insulin binding to the receptors initiates activation of the intrinsic tyrosine kinase with subsequent autophosphorylation of these receptors followed by binding and tyrosine phosphorylation of so-called adapter proteins, such as insulin receptor substrate (IRS) proteins, SHC1 (Src homology 2 domain-containing transforming protein 1), GAB1 (GRB2-associated binding protein 1) and SH2B2 (SH2B adaptor protein 2). These adaptor proteins provide an interface between the activated receptors and the downstream-located effector molecules (reviewed in [40]). Data gathered over the past decade from both analytical (reverse-transcription polymerase chain reaction and western blotting) and functional studies (transgenic mice, knockout mice, expression of interfering protein variants and RNAi-mediated knockdown) have demonstrated the presence and function of various downstream effector proteins, such as isoforms of phosphoinositide-3-kinase (PI3K), isoforms of AKT1 (v-akt murine thymoma viral oncogene homolog 1), RPS6KB2 (ribosomal protein S6 kinase, 70 kDa, polypeptide 2), MAPK (mitogen-activated protein kinase) and PLCG (phospholipase C gamma). Published studies have shown that insulin activates both the mitogenic (MAPK ERK1/2 (MAPK3)) and metabolic branches of insulin signalling. The latter involves PI3K, AKT, MTORC1 (mechanistic target of rapamycin complex 1), RPS6KB2 and PLCG. All of these studies provide evidence for autocrine feedback of insulin at the molecular level but do not resolve whether insulin is a negative, positive, or complex (negative and positive) signal in β-cell function (reviewed in [38]).

2.2 Peripheral tissues

Insulin synthesised and secreted by pancreatic β-cells is released into the portal vein and carried mainly to the liver, skeletal muscle and adipose tissue.

Insulin mediates its physiological functions by binding to insulin receptors, a process that results in receptor autophosphorylation on tyrosine residues and tyrosine phosphorylation of IRS by the insulin receptor tyrosine kinase (Figure 8). IRS phosphorylation allows for association of IRS with the regulatory subunit of PI3K through its SH2 domains. Once activated, the catalytic subunit phosphorylates phosphoinositides at the 3' position of the inositol ring or proteins at serine residues. PI3K activates 3-phosphoinositide-dependent protein kinase 1 (PDPK1), which activates AKT. In turn, AKT deactivates glycogen synthase kinase 3 (GSK3), leading to activation of glycogen synthase and thus stimulating glycogen synthesis. The activation of AKT also results in translocation of glucose transporter 4 (GLUT4) vesicles from their intracellular pool to the plasma membrane, where they enable the uptake of glucose into the cell [41].

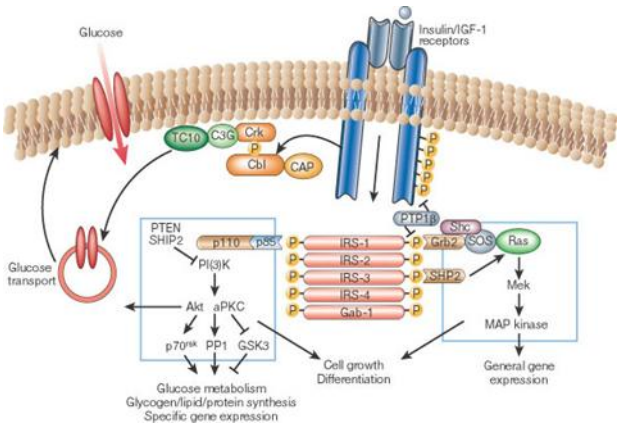


Figure 8. Signal transduction involving insulin (from [42]).

Other signal transduction proteins interact with IRS molecules, including growth factor receptor-bound protein 2 (GRB2) and a protein-tyrosine phosphatase containing SH2 domains (SHP2). GRB2, an adaptor protein, contains an SH3 (SRC homology domain 3), which allows constitutive association with the guanine nucleotide exchange factor mSOS. Moreover, GRB2 is part of a signalling cascade including RAS, RAF1 (v-raf-1 murine leukemia viral oncogene homolog 1) and MEKK (mitogen-activated protein kinase kinase) that leads to the activation of MAPK and mitogenic responses in the form of gene transcription stimulated by FOS (FBJ murine osteosarcoma viral oncogene homolog) and ELK1 (ELK1, member of ETS oncogene family). SHC (Src homology 2 domain-containing transforming protein 1) is another substrate of the insulin receptor that contains SH2 and phosphotyrosine-binding domains. When tyrosine is phosphorylated, SHC associates with GRB2 and can activate the RAS/MAPK pathway independently of IRS1 [41].

Signal transduction by the insulin receptor is not limited to its activation at the cell surface. The activated ligand-receptor complex, which is initially located at the cell surface, is internalised into endosomes, a process that is dependent on tyrosine autophosphorylation. Endocytosis of activated receptors has the dual effect of concentrating receptors within endosomes and allowing the insulin receptor tyrosine kinase to phosphorylate substrates that are spatially distinct from those accessible at the plasma membrane. Because of the presence of proton pumps, acidification of the endosomal lumen results in dissociation of insulin from its receptor. This loss of the ligand-receptor complex prevents any additional insulin-driven receptor re-phosphorylation events and leads to receptor dephosphorylation by extraluminal endosomally associated protein tyrosine phosphatases (PTPs) [41].

3. Regulation of insulin synthesis and secretion

3.1 Regulation of insulin synthesis

Insulin gene transcription is mainly controlled by a 340 bp promoter region upstream of the transcription start site of the insulin gene. The insulin promoter is organised in a complex arrangement of discrete cis-acting sequence motifs, which serve as binding sites for both ubiquitous and β-cell-specific transcription factors. The co-ordinated interaction between cis-elements and trans-acting factors at the promoter contributes to both β-cell-specific expression of the insulin gene and regulation of its expression in response to glucose, Ca^{2+} levels, nutrient availability and hormone signalling [43].

3.1.1 Regulatory elements within insulin promoters

Insulin promoters from the rat INS1 and INS2 genes and the human INS gene have been characterised in detail. These promoters all contain transcriptionally important E, A and C regulatory elements, as well as additional sequences that have more subtle regulatory effects (Table 1) [44].

Table 1. Regulatory elements within insulin promoters.

Regulatory element	Consensus sequence	Example of transcription factor that binds	References
A boxes	TAAT motif	PDX1, HNF1A, ISL1 and CDX3	[45-49]
GG boxes	GGAAAT-containing GG2 motif	PDX1	[50]
cAMP response elements	5'TGACGTCA	bZIP CREB/ATF family	[51,52]
C elements	5'TGCAGCCTCAGCC	MAFA, PAX6 and PAX4	[53-55]
E boxes	5′CANNTG	TCF3 and NEUROD1	[56,57]
Negative regulatory elements	5′GAGACATTTGCCCCCAGCTGT		[58]
Insulin-linked polymorphic regions	5'TCTGGGGAGAGGGG	PURA and MAZ	[59]
G1 boxes	5'GTAGGGGA	PURA and MAZ	[59]
Enhancer cores	5′TGTGGAAAG	CEBP	[60]
SP1 sites	5′CCGCCC	KLF11	[44]
Ink boxes	5′AG GTCCCCAGGTCATGCCCTC	Retinoic acid and thyroid hormone	[61]

3.1.2 Transcription factors that modulate insulin transcription

PDX1 (pancreatic duodenal homeobox 1) is the major regulator of glucose-stimulated insulin gene transcription and is essential for early pancreatic development and for pancreatic β-cell maturation and function [62]. PDX1 primarily acts in β-cells to up-regulate the transcription of several β-cell-specific genes, including insulin, GLUT2, glucokinase, somatostatin, islet amyloid polypeptide (IAPP) and MAFA as well as to auto-regulate its own expression. In addition, PDX1 has been reported to function as a transcriptional repressor for glucagon, cytokeratin K19 and MYC (v-myc myelocytomatosis viral oncogene homolog) [43].

MAFA (v-maf musculoaponeurotic fibrosarcoma oncogene homolog A) is a basic leucine zipper transcription factor belonging to the large MAF family of transcription factors. Beyond insulin expression, MAFA also appears to play a role in mediating the expression of a number of other genes such as GLUT2, NKX6-1 (NK6 homeobox 1) and PDX1. MAFA participates in insulin transcription by binding to and activating PDX1, NEUROD1 and NFAT (nuclear factor of activated T-cells) [43].

NEUROD1, also known as BETA2, belongs to the BHLH (basic helix–loop–helix) family of transcription factors and functions in a complex with the ubiquitously expressed TCF3 (transcription factor 3) protein. These two proteins associate with DNA as a heterodimer. The ability of NEUROD1 to activate transcription is enhanced by its interaction with the co-activator p300 or CREB (cAMP-responsive element-binding protein) [43], and NEUROD1's interaction with PDX1 is essential to activate insulin gene

transcription in β-cells [63]. NEUROD1 also binds to NR0B2 (nuclear receptor subfamily 0, group B, member 2), an orphan nuclear receptor that functions as a repressor of transcription [43].

Members of the ***CREB/ATF*** (activating transcription factor 1) family also play an important role in regulating the insulin gene. CREB/ATF proteins can exist as multiple isoforms that can be activated by cAMP and diacylglycerol (DAG) signalling pathways to form complexes with other transcription factors (such as NEUROD1, as explained above) [64]. ***ATF2*** is involved in the regulation of insulin gene expression through its binding to CRE-like elements in the promoter region, but it acquires binding capacity through complex formation with MAFA. Indeed, co-expression of ATF2, MAFA, PDX1 and NEUROD1 result in a synergistic activation of the insulin promoter [65].

NKX6-1 is a homeodomain transcription factor exclusively expressed in β-cells in mature islets and is required for normal GSIS. Although the specific function of NKX6-1 in GSIS of mature β-cells remains elusive, overexpression of NKX6-1 increases GSIS in rat islets. By contrast, islets isolated from type 2 diabetes mellitus (T2DM) patients have altered NKX6-1 expression [66].

HNF1A (hepatocyte nuclear factor 1 homeobox A) is the major transcription factor that is involved in most cases of maturity-onset diabetes of the young (MODY). Genes involved in β-cell regulation and metabolism, such as GLUT2, pyruvate kinase, insulin as well as the transcription factors PDX1, HNF4A (hepatocyte nuclear factor 4 homeobox A) and NEUROD1, are expressed abnormally in HNF1A-null mice. One of HNF1A's major regulators is NKX6-1 [66]. Rat and mouse insulin promoters contain a consensus binding site for HNF4A. The rat INS1 promoter has been shown to be activated directly by HNF4A, which can interact synergistically with PDX1 at the adjacent A1 site [67]. However, an HNF4A-binding site does not exist in the human insulin promoter [68]. Evidence suggests that HNF4A can modulate insulin gene expression directly or indirectly through a mechanism involving HNF1A, which is a regulator of HNF4A expression [69].

PAX6 (paired box 6) is a transcription factor essential for normal expression of insulin and GLUT2 [70]. The overexpression of PAX6 was recently reported to prevent activation of rat INS2, thus suppressing insulin synthesis and secretion. Indeed, PAX6 binds to A-boxes *in vitro*, thereby blocking binding of PDX1; at the same time, its paired domain interacts with NEUROD1 [71].

NKX2-2 (NK2 homeobox 2) may also be important in the maintenance of mature β-cell function. Transgenic mice, in which a repressor form of NKX2-2 was expressed in β-cells, exhibited reduced β-cell levels of MAFA, GLUT2 and insulin, and the islets displayed a disrupted architecture [72]. The effect of NKX2-2 on the insulin gene may be through binding sites in the promoter [73] or indirectly through its ability to activate MAFA expression [74].

FOXO1 (forkhead box O1) is a transcription factor of the forkhead family that negatively regulates PDX1 by modulating the PDX1 subcellular location or by suppressing PDX1 gene transcription [75]. In fact, these two transcription factors exhibit mutually exclusive patterns of nuclear localisations in β-cells, but the types of molecular events that occur between FOXO1 and PDX1 remain unclear [76]. Silencing of FOXO1 significantly elevated the expression of mouse INS2 but not INS1 mRNA; moreover, putative FOXO1-binding sites were identified in the distal promoter of rodent INS2 genes and direct binding of FOXO1 to the INS2 promoter has been demonstrated [77]. The transcription factor ***FOXA2*** (forkhead box A2) can bind to the PDX1 promoter and positively regulate PDX1 gene expression both *in vitro* and *in vivo* [78]. Indeed, Kitamura et al. reported that FOXO1 and FOXA2 share common DNA-binding sites in the PDX1 promoter, indicating that both transcription factors could regulate PDX1 expression through competition at the same binding site [79].

GLIS3 (GLIS family zinc finger 3) is a member of the Krüppel-like family and is highly expressed in islet β-cells. GLIS3 regulates insulin gene expression through two GLIS-binding sites in its proximal promoter [80]. Moreover, GLIS3 physically and functionally interacts with PDX1, MAFA and NEUROD1 to

modulate INS2 promoter activity. In addition, GLIS3 may indirectly affect insulin promoter activity through the upregulation of MAFA and downregulation of NKX6-1 [81].

PPARG (peroxisome proliferator-activated receptor gamma) is a key transcriptional and signalling regulator of events in β-cells. PPARG is a nuclear receptor that heterodimerises with retinoid X receptor (RXR) after binding to its ligand, translocates and binds peroxisome proliferator response elements (PPREs) in the promoter of target genes, including insulin. Indeed, PPARG regulates the expression of other genes that control functions important for the maintenance of secretory β-cell functions, such as GLUT2, glucokinase, PDX1 and IRS1 [82].

SRF (serum response factor) is a prototype of the MADS box domain-containing transcription factors. SRF binds to the serum response element (SRE) as a homodimer. Various ion channel proteins expressed in the pancreas, such as the Ca^{2+} pump ATP2A2 (ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2), the Na^{+}/Ca^{2+} exchanger pump SLC8A1 (solute carrier family 8 –sodium/calcium exchanger-, member 1) and the Cl^{-} pump CFTR (cystic fibrosis transmembrane conductance regulator), are regulated by SRF [83]. Sarkar et al. recently identified a conserved SRE in the insulin gene promoter and showed that SRF activates the rat INS2 promoter by binding to this SRE and interacting with PDX1 [83].

3.1.3 Signals that control insulin transcription

- *Insulin*

Evidence for negative feedback of insulin gene expression by insulin has been reported [84]. However, other studies have indicated that insulin exerts stimulatory effects on insulin production (reviewed in [85]). The general disruption of IRS2 in mice caused important β-cell defects [86], and the inactivation of the receptor resulted in a selective loss of first-phase secretion in response to glucose [87]. On the other hand, insulin regulates the gene expression of GLUT2 and glucokinase. In addition, the expression and action of several important transcription factors of β-cells, including PDX1, FOXO1 and FOXA2, are regulated by insulin (reviewed in [38]).

The mechanism by which insulin exerts stimulatory effects on its own transcription was suggested to involve IRS2/PI3K, RPS6KB2 and the calcium calmodulin kinase pathways [88]. In addition, the stimulation of insulin gene expression by insulin receptor signalling has been reported to be glucose dependent and to involve PI3K [89]. Therefore, the data indicate that glucose and insulin (involving PI3K) activate insulin gene transcription and that their effects can be additive in β-cells [90].

- *Glucose*

Glucose is the major physiological regulator of insulin gene expression and controls insulin synthesis at the transcriptional and post-transcriptional levels. Much of the glucose responsiveness inherent to the insulin promoter is conferred by the A3, E1 and C1 sites, which are bound by the transcription factors PDX1, NEUROD1 and MAFA, respectively. These three transcription factors have been demonstrated to play crucial roles in glucose induction of insulin gene transcription and pancreatic β-cell function (as described above). These transcription factors activate insulin gene expression in a co-ordinated and synergistic manner in response to increasing glucose levels. Changes in the glucose concentration modulate the functions of these β-cell transcription factors at multiple levels: changes in expression level, subcellular localisation, DNA-binding activity, transactivation capacity and interactions with other proteins [43].

One of the mechanisms that glucose uses to regulate insulin synthesis is through increasing O-linked N-acetylglucosamine (GlcNAc)-modified proteins. The O-GlcNAc modification is an important post-translational modification that modulates the function of many nuclear and cytoplasmic proteins. Proteins

are modified at serine or threonine residues by attachment of a single N-GlcNAc molecule, a reaction catalysed by O-linked N-acetylglucosaminyl transferase (OGT). UDP-GlcNAc, the substrate for OGT, is synthesised by the hexosamine biosynthetic pathway (HBP), which uses the glycolytic metabolite fructose-6-phosphate and glutamine; however, only a small fraction of glucose (2-5 %) enters the HBP as fructose 6-phosphate. The HBP together with the O-linked GlcNAc modification of proteins has been suggested to function as a nutrient sensor for the cell. Accordingly, exposure to high glucose levels leads to increased flux via the HBP and results in elevated levels of O-GlcNAc modified proteins. Many nuclear proteins, including transcription factors, are modified by O-linked GlcNAc [91].

Glucose modulates ***PDX1*** function in pancreatic β-cells by multiple mechanisms. Glucose regulates the interaction of PDX1 with various co-regulators in a phosphorylation-dependent manner in the mouse insulinoma cell line MIN6. Under low or normal glucose conditions, PDX1 is mainly associated with histone deacetylase 1 and 2 (HDAC1/2) to down-regulate insulin gene expression. An increase in glucose levels disrupts the interaction of PDX1 with HDACs and promotes its association with the histone acetyltransferase p300, which leads to the hyperacetylation of histone H4 and the induction of insulin gene transcription. This switch in the PDX1 interaction in response to high glucose requires a phosphorylation event that causes changes in PDX1 localisation. Several signalling pathways, including the p38/SAPK (stress-activated protein kinase), PI3K, atypical PRKC (protein kinase C) and MAPK pathways, as well as PASK (PAS domain containing serine/threonine kinase) have been implicated in PDX phosphorylation, nucleocytoplasmic shuttling, DNA binding and the transactivation potential [43]. Humphrey et al. recently showed that glucose regulates the steady-state levels of the PDX1 protein via a novel phosphorylation site. They observed that glucose stimulation of primary islets and cultured MIN6 β-cells decreases PDX1 phosphorylation; moreover, they described a novel C-terminal atypical non-primed GSK3 consensus site that regulates PDX1 protein stability in response to glucose [92].

Elevated concentrations of glucose lead to O-GlcNAc modification of PDX1 in pancreatic β-cells. The O-GlcNAc modification regulates PDX1 binding to the insulin promoter and thereby influences insulin secretion in MIN6. Moreover, the O-GlcNAc modification of PDX1 appears to be important for binding PDX1 to the insulin promoter and for activating insulin gene expression. However, the exact mechanism(s) by which O-GlcNAc modification enhances the DNA-binding activity PDX1; in addition, the PDX1 residues that are modified by O-GlcNAc remain to be identified [91]. Increased O-GlcNAc modification of PDX1 has also been observed in diabetic Goto-Kakizaki rats and was associated with decreased insulin secretion from pancreatic β-cells [93].

Glucose has been shown to regulate the nuclear localisation and transactivation capacity of ***NEUROD1*** via post-transcriptional modifications. While NEUROD1 is mainly localised in the cytoplasm under low or normal glucose conditions, exposure to high glucose causes NEUROD1 to translocate into the nucleus and thereby to activate insulin gene transcription in pancreatic β-cells. In the presence of high glucose, NEUROD1 becomes phosphorylated by ERK2 at multiple sites within its transactivation domain, which enhances its transactivation capacity [43]. Wong et al. demonstrated that the activation of the oestrogen receptor 1 (ESR1) potentiates the effect of glucose on NEUROD1 nuclear localisation and binding to the insulin promoter, thereby amplifying insulin gene transcription [94]. The nuclear translocation of NEUROD1 under high glucose conditions is also mediated by O-linked GlcNAc modification of NEUROD1 itself [95]. However, additional research is needed to map the O-GlcNAc-modified residues within NEUROD1 and to confirm their role in the regulation of the NEUROD1 subcellular localisation [91].

Both the ***MAFA*** mRNA and protein levels increase in response to high glucose [96]. Phosphorylation has been implicated in regulating MAFA protein levels and in modulating the binding of MAFA to the insulin promoter [97]. Current evidence suggest that enhanced production of MAFA under high-glucose conditions may regulate glucose-dependent insulin gene transcription, whereas decreased production and proteasomal degradation of MAFA probably enables the rapid inhibition of insulin transcription under

low-glucose conditions [43]. Indeed, the induction of MAFA expression by high glucose requires increased flux through the HBP and an O-linked GlcNAc modification event [96]. Knockdown of OGT using siRNA oligonucleotides abolishes the induction of MAFA expression by high glucose in pancreatic β-cells. Treatment of pancreatic β-cells with PUGNAc, an inhibitor of O-GlcNAcase, induces MAFA expression even in the absence of high glucose. Therefore, the induction of MAFA expression under high glucose may be mediated by an O-GlcNAc modification of an unknown transcriptional regulator(s) that is required to activate MAFfA expression [96].

- *GLP1*

GLP1 increases insulin gene transcription and biosynthesis by activating both protein kinase A (PRKAC, PKA)-dependent and PKA-independent signalling pathways. PDX1 is a key effector of the GLP1 receptor (GLP1R) signalling pathway, which is involved in insulin gene transcription and biosynthesis. GLP1 has been shown both *in vitro* and *in vivo* to be involved in regulating PDX1 by increasing its total protein levels and its translocation to the nucleus, followed by its binding to the A-box element and the GG2 element of the insulin promoter and the resultant increase in the activity of the insulin gene promoter in β-cells. The regulation of PDX1 by GLP1 mainly occurs via a cAMP/PKA-dependent signalling pathway. GLP1 triggers expression and nuclear localisation of PDX1 by a mechanism that involves the phosphorylation of FOXO1 via transactivation of the EGFR (epidermal growth factor receptor) and PI3K/AKT pathways, resulting in deactivation and nuclear exclusion of FOXO1 and consequent disinhibition of the FOXA2-dependent PDX1 gene promoter activity. The GLP1R signalling pathway also mediates insulin gene transcription via basic region-leucine zipper transcription factors that are related structurally to the transcription factor CREB and directly bind to CRE sites on the insulin gene promoter [98].

Similar to other hormones such as leptin, adiponectin, growth hormone, prolactin and oestrogens, GLP1 modulates insulin expression in pancreatic β-cells [99-104].

3.1.4 DNA methylation modulates insulin transcription

Like other mammalian gene promoters, the insulin gene promoter contains cytosine-guanosine dinucleotide (CpG) sites, which play a pivotal role in the control of gene expression. Methylation of the cytosine residues regulates transcription directly by inhibiting the binding of specific transcription factors and indirectly by recruiting methyl-CpG-binding proteins and their associated repressive chromatin-remodelling activities. These epigenetic changes are responsible for the modulation of developmentally regulated and tissue-specific gene expression [105]. Kuroda et al. found CpG sites in both the mouse INS2 and human INS promoters, and they observed that these sites are uniquely demethylated in insulin-producing β-cells. Methylation of these CpG sites suppressed insulin promoter-driven reporter gene activity by almost 90 %, and specific methylation of the CpG site in the CRE of the promoter alone suppressed insulin promoter activity by 50 %. Methylation did not directly inhibit transcription factor binding to the CRE *in vitro*, but inhibited ATF2 and CREB binding *in vivo* and increased binding to MECP2 (methyl CpG binding protein 2) [17]. Indeed, Yang et al. demonstrated that DNA methylation of the insulin promoter is increased in patients with T2DM [106].

3.1.5 Post-transcriptional control of insulin mRNA

Changes in mRNA abundance can be regulated post-transcriptionally, such as through differential effects upon mRNA splicing, nuclear export or stability. Following glucose stimulation, exocytotic events are responsible for the loss of granules from the storage pool. To replenish the pool and keep a balance between release and storage, β-cells should rapidly synthesise new granule proteins and produce new membranes in the endoplasmic reticulum [107]. Protein and mRNA analyses have suggested that the abundance of a large number of secretory granule-related genes is up-regulated by glucose in a coordinated manner [108]. As for increased protein synthesis in primary β-cells, this effect is achieved

primarily by a powerful acceleration of general translation. In primary β-cells, insulin mRNA is translated preferentially in comparison to non-insulin encoding mRNA. The increased translation of proinsulin mRNA results from the stimulation of translation initiation and elongation as well as from the reduced degradation of insulin mRNA [107]. This latter mechanism has been explored and involves the rapid nucleo-cytoplasmic translocation of polypyrimidine tract-binding protein (PTB) upon β-cell stimulation with glucose [108]. Activated PTB has been shown to increase the stability of insulin mRNA by binding to its 3′-untranslated region (UTR) [107]. The coordinated glucose-stimulated synthesis of insulin and other components of dense-core secretory granules can be explained (at least in part) by the fact that PTB also binds and stabilises mRNAs encoding different transcripts related to granule biogenesis, in particular prohormone convertases 1/3 or 2, pre-chromogranin A, secretogranin 2, synaptobrevin 2, synaptophysin and the tyrosine phosphatase-like molecule I-A2 [108]. More recently, PTB has been suggested to promote the stabilisation of mRNAs coding for not only insulin and other insulin granule proteins but also components comprising the entire secretory pathway [109].

3.1.6 MicroRNA

MicroRNAs (miRNAs) are a family of small non-coding RNAs that post-transcriptionally regulate gene expression [110]. Each miRNA gene encodes a mature miRNA approximately 22 nucleotides in length. MiRNAs play predominantly inhibitory regulatory roles by binding to *cis*-elements in the 3'-UTR of message-encoding RNAs. Regulation occurs by one of three mechanisms (which are not mutually exclusive): target cleavage, repression of target translation and message degradation in cytoplasmatic P-bodies [111].

Recent studies have demonstrated that miRNAs are required for pancreas development [112] and regulation of GSIS [113]. The most studied miRNA molecule that acts in the pancreas is miR-375. Published studies revealed the involvement of miR-375 in pancreatic islet cell viability and function, and its removal or overexpression profoundly affects glucose metabolism [114]. More recently, other miRNAs have been described as regulators of pancreas function. Some miRNAs modify insulin secretion by modulating the level of key components of the exocytosis process and insulin biosynthesis. Some miRNAs have also been related to β-cell apoptosis [115] (Table 2).

Table 2. MiRNA action on β-cells.

MicroRNA	Effects on β-cells	References
miR-375	Modify insulin secretion by modulating the level of key components of the exocytosis process	[114,116]
miR-9		[117]
miR-96		[117,118]
miR-124a		[118]
miR-130a		[119]
miR-200		[119]
miR-410		[119]
miR-30d	Modify insulin biosynthesis	[120]
miR-15a		[121]
miR-24		[122]
miR-26		[122]
miR-182		[122]
miR-19b		[123]
miR-148		[122]
miR-34a	Modify β-cell apoptosis	[115]
miR-146		[115]

MiRNAs are also involved in diabetes. Studies on Goto-Kakizaki rats, a model of spontaneous lean T2DM, showed differential expression of fifteen miRNAs in skeletal muscle compared with Wistar control rats [124]. In addition, Herrera et al. recently showed that the expression patterns of five miRNAs in insulin target tissues were modified by hyperglycemia, suggesting a role for these miRNAs in the pathophysiology of T2DM [125].

3.2 Key regulators of insulin secretion

The secretion of insulin by β-cells is a highly dynamic process regulated by complex mechanisms, including nutrient status, hormonal factors such as gastrointestinal hormones incretins and neural factors [37] (Figure 9).

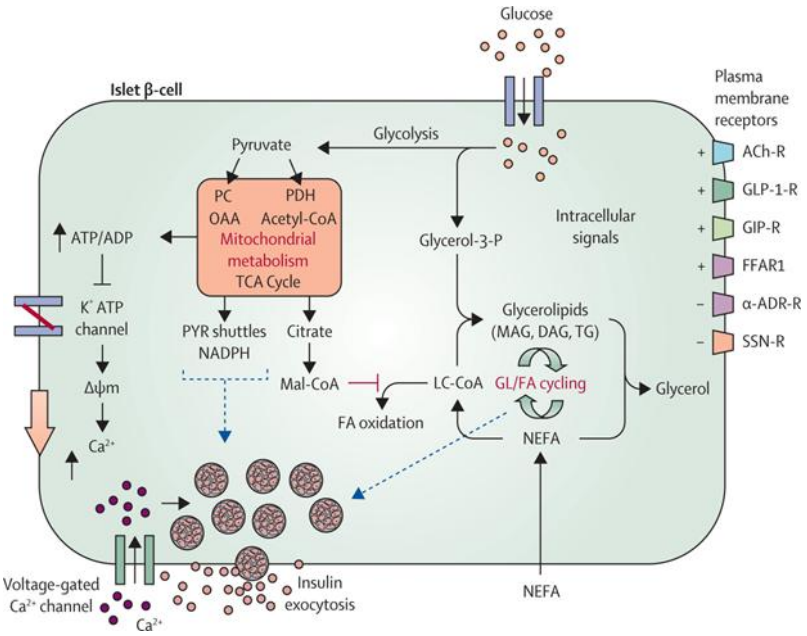


Figure 9. Regulation of insulin secretion process (from [3]).

3.2.1 Nutrients

- *Carbohydrates*

Glucose is the only nutrient secretagogue capable alone of promoting the release of insulin at concentrations within its physiological range *in vitro* [126]. Glucose stimulates insulin secretion through an oxidative metabolism (pathway also used by other carbohydrates) in which mitochondria are crucial elements.

In β-cells, mitochondria integrate and generate metabolic signals, ensuring efficient coupling of glucose recognition to insulin secretion [127]. Several mitochondrion-derived molecules distinct from ATP have been proposed to act as additive factors participating in the amplifying pathway of insulin secretion. In fact, mitochondrial defects in β-cells, such as mutations and reactive oxygen species (ROS) production, are associated with β-cell failure in diabetes [128]. These data suggest that this organelle plays an important role in insulin secretion.

Although mitochondria can be activated by three classes of fuels, amino acids, fatty acids and carbohydrates, the last class is most relevant for β-cells. Importantly, β-cells have negligible lactate dehydrogenase (LDH), so pyruvate from glycolysis is weakly metabolised into lactate, and the majority of pyruvate-derived carbons are essentially mitochondrial [129]. In fact, pyruvate, formed during glycolysis enters mitochondria where it can either be oxidised by PDH to form acetyl-CoA (oxidative pathway) or

carboxylated by PC to generate oxaloacetate, thereby ensuring anaplerosis to the TCA cycle (Figure 10). Therefore, these pathways result in the net synthesis of TCA cycle intermediates that act directly as or as precursors of important signals in insulin secretion. [25,129]

The anaplerotic enzyme PC participates directly or indirectly in several metabolic pathways that are important for GSIS, including the pyruvate/malate cycle, the pyruvate/citrate cycle, the pyruvate/isocitrate cycle and glutamate dehydrogenase (GDH)-catalysed α-ketoglutarate production. These four pathways enable 'shuttling' or 'recycling' of these intermediate(s) into and out of mitochondria, allowing continuous production of intracellular messenger(s) (reviewed in [25]).

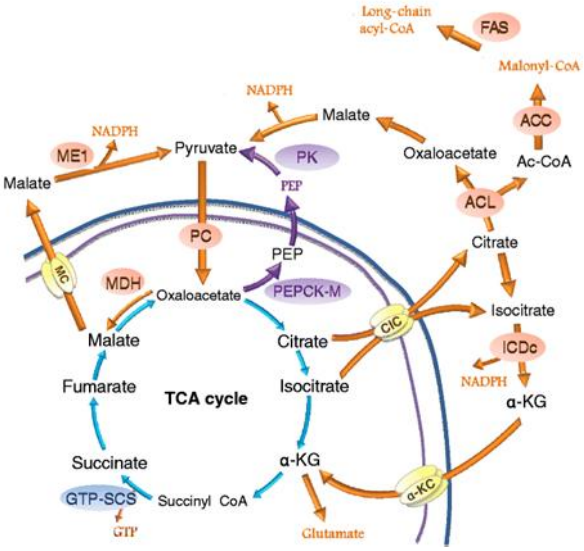


Figure 10. Mitochondrial biochemical pathways that are involved in the GSIS amplifying pathway (modified from [25]).

- *Amino acids*

Amino acids individually are poor insulin secretagogues but are able to actively promote secretion and augment GSIS in certain combinations [130]. The mechanisms by which amino acids induce insulin secretion are not well understood, but there is evidence that they involve both triggering and amplification processes [24]. The catabolism of amino acids involves pyruvate, acetyl-CoA and ketone bodies or intermediates of the TCA cycle. Amino acids can feed into glucose oxidation and anaplerosis pathways. Indeed, amino acids can also modify GSIS through direct depolarisation of the plasma membrane through the accumulation of positive charge and by co-transport with Na^+, resulting in membrane depolarisation and opening of VDCCs [131].

L-Arginine causes an elevation in $[Ca^{2+}]_i$ as a result of its electrogenic transport into β-cells via the amino acid transporter BCAT2 (branched chain amino-acid transaminase 2). Depolarisation of the plasma membrane triggers insulin secretion [132]. Alternatively, L-arginine metabolism in β-cells can give rise to glutamate production and thus can influence insulin secretion.

L-Alanine is cotransported with Na^+ and depolarises the cell membrane as a consequence of Na^+ transport, thereby inducing insulin secretion by activating VDCCs. Indeed, the metabolism of this amino acid results in partial oxidation and an increase in the cellular content of ATP, leading to closure of K_{ATP} channels and ultimately to insulin exocytosis [133].

L-Leucine stimulates insulin release in pancreatic β-cells by a process that involves increased mitochondrial metabolism by activation of GDH, an increase in ATP production by transamination of leucine to α-ketoisocaproate and subsequent entry into the TCA cycle via acetyl-CoA [134]. Other studies have shown that leucine and α-ketoisocaproate stimulate insulin release via distinct mechanisms. α-Ketoisocaproate was proposed to stimulate insulin release by a combination of mechanisms, including its own catabolism and transamination to leucine with production of α-ketoglutarate [135].

L-Glutamate is the most debated amino acid with respect to the possible molecular mechanism of its action on promotion of insulin secretion [130]. Glutamate has been suggested to act downstream of mitochondria and to participate in the amplifying pathway of the secretory response to glucose [136]. In permeabilised INS-1 β-cells, glutamate potentiates insulin secretion under conditions of clamped, permissive Ca^{2+} and ATP [136,137]. In patch clamp experiments, glutamate enhanced Ca^{2+}-induced insulin exocytosis from rat β-cells [137]. Therefore, potentiation of insulin secretion by glutamate depends on Ca^{2+} signals, which are initiated by the triggering pathway under physiological conditions. β-cells express the vesicular glutamate transporters SLC17A6 and SLC17A7 (solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6/7) [138]. SLC17A6 is upregulated by increased and decreased glucose in β-cells and α-cells, respectively, suggesting that exposure to high glucose concentrations stimulates glutamate uptake into secretory vesicles in β-cells [136-138]. Once inside the secretory granule, glutamate can induce pH changes, as reported in secretory vesicles from pancreatic β-cells, and/or activate GRM5 (glutamate receptor, metabotropic 5), which has been shown to be expressed in secretory granules, thereby mediating insulin release. Alternative mechanisms for glutamate action in β-cells include activation of acetyl-CoA carboxylase and inhibition of protein phosphatase enzymatic activities, as reversible protein phosphorylation-dephosphorylation cycles have been shown to play a role in the rate of insulin exocytosis [139].

L-Aspartate plays a key role in NADH shuttles, serving as an important mechanism for the maintenance of glucose metabolism [140].

Finally, ***dietary amino acids*** also influence insulin secretion by indirect mechanisms such as stimulating incretin release, modulating glucagon release and regulating lipid metabolism [130].

- *Fatty acids*

Non-esterified fatty acids (NEFAs) do not stimulate insulin secretion in the absence of glucose. However, there is substantial evidence that they are essential for GSIS to occur and that they can markedly augment GSIS. NEFAs regulate insulin secretion via three interdependent processes, which are referred to as the “trident model” of β-cell signalling [131].

Malonyl-CoA/Long-chain CoA signalling

In β-cells, the cytoplasmic NEFAs are converted to long-chain acyl-CoA (LC-CoA) by acyl-CoA synthase, thereby increasing the availability of LC-CoA, which is important for stimulating the insulin exocytotic machinery [141]. Under basal conditions, the LC-CoA molecules are transported into the mitochondria via CPT1A (carnitine palmitoyltransferase-1A), where β-oxidation occurs. However, high levels of glucose form acetyl-CoA and subsequently malonyl-CoA, which supplies the two carbon units for fatty acid synthesis. This process inhibits β-oxidation by CPT1A and induces a marked rise in the cytoplasmic content of LC-CoA. Thus, malonyl-CoA acts by switching β-cell metabolism from fatty acid oxidation to glucose oxidation. LC-CoA is responsible for triggering different mechanisms to release insulin. LC-CoA signalling includes (1) the activation of certain types of PRKC, which interacts with components of the NEFA microtubular/exocytotic machinery, (2) the modulation of ion channels directly or indirectly and (3) the protein acylation of GTP-binding proteins [142].

Triglyceride (TG)/NEFA cycling

In addition to fatty acid oxidation and esterification, lipolysis is the major pathway of intracellular fatty acid partitioning. Elevated glucose was demonstrated to increase lipolysis, thus increasing the levels of LC-CoA, DAG, phospholipids and NEFAs. DAG not only activates PRKC, which is implicated in insulin secretion, but also binds to the C1 domain of the synaptic vesicle priming protein UNC13 (unc-13 homolog A), which has recently been shown to be important for normal insulin secretion. TG/NEFA also affects membrane glycerophospholipid metabolism, which could influence secretion by changing the physicochemical properties of membranes [141]. The glycerophospholipids may also have more direct effects. The TG/NEFA cycle may be a means for targeting the delivery of NEFAs and perhaps specific NEFAs, such as arachidonic acid, to a particular subcellular site within β-cells [142].

Fatty acids and lipid receptor signalling

The finding that lipid molecules also activate islet G protein-coupled receptor (GPCR) signalling suggested that lipids may also stimulate insulin secretion through receptor-mediated mechanisms. Both GPR40 and GPR119 are known to be expressed in pancreatic β-cells and their activation stimulates insulin secretion in a glucose-dependent manner. GPR40 is highly expressed in β-cells and is activated by medium- to long-chained NEFAs to potentiate GSIS. C12–C22 fatty acids, such as linoleic acid, are most effective in activating GPR40, although the receptor does not appear to discriminate between different NEFAs. GPR40 is coupled to GNAQ (G protein, q polypeptide), which increases $[Ca^{2+}]_c$ concentrations, although mechanisms that involve the activation of PLC (phospholipase C) and increased formation of cAMP have also been proposed. In contrast to the stimulatory action of NEFAs on insulin secretion, long-term exposure of islets to NEFAs results in impaired GSIS through a lipotoxic action, which involves the progressive intracellular accumulation of lipid signalling molecules that inhibit insulin secretion. The activation of GPR119 augments GSIS through increased formation of cAMP, suggesting that the receptor mediates its actions through GNAS [143].

3.2.2 Hormones

- *Peptides and insular hormones*

β-cells

The role of ***insulin*** in insulin secretion is the most controversial topic related to insulin feedback and β-cell function. Published data have led to four possible outcomes, i.e., that insulin is (a) a negative regulator, (b) a positive regulator, (c) essential, or (d) not involved (reviewed in [38]). Insulin exocytosis was suggested to be inhibited by secreted insulin, as supported by several publications [144-146]. Although other older and more recent reports demonstrate no effects of insulin on its own secretion [144,147], other evidence suggests that secreted insulin may be essential for insulin exocytosis or that it may even have a positive effect on its own release [87,89,148]. Mice with a β-cell–specific insulin receptor knockout (βIRKO), a model of β-cell insulin resistance, manifest defective GSIS, progressive glucose intolerance and increased rates of diabetes [149]. Stimulation of β-cells with exogenous insulin leads to increased intracellular Ca^{2+}, suggesting that insulin also stimulates its own secretion by mobilising Ca^{2+} from the endoplasmic reticulum [150]. Furthermore, Park et al. more recently observed that changes in the phosphorylation of IRS1 and AMPK (PRKA, AMP-activated protein kinase) by glucose and extracellular insulin may be involved in this insulin feedback signalling pathway. The altered AMPK activity appears to modulate the activity of the K_{ATP} channel, thereby influencing $[Ca^{2+}]_c$ and insulin secretion [151]. Indeed, Bouche et al. recently demonstrated that insulin improves GSIS in healthy humans. However, the identity of the downstream transcription factors and/or repressors that mediate the effects of insulin and glucose remains unknown [152]. These data suggest that insulin's involvement in the regulation of its own secretion is complex. Whereas basal insulin may serve as a maintenance signal that primes β-cells to respond to the next glucose stimulus, insulin may inhibit further release at the peak

of the exocytotic event, i.e., at very high local insulin concentrations [38]. On the other hand, insulin also modifies its own secretion through transcriptional regulation of other genes, such as glucokinase [153].

Along with insulin, β-cells also secrete gamma-aminobutyric acid (***GABA***). Although GABA is a major neurotransmitter in the central nervous system (CNS), a large amount of GABA is also produced in β-cells. The stimulation of type A GABA receptors ($GABA_A$Rs) in β-cells induces membrane depolarisation, enhancing insulin secretion in the presence of physiological concentrations of glucose [154].

Pancreatic β-cells co-express and co-secrete amylin, also known as ***IAPP***, and insulin in response to several secretagogue stimuli. Different studies have demonstrated that IAPP can inhibit GSIS [155,156]. Recently, Soty et al. observed that β-cells overexpressing human IAPP showed a defect in insulin and IAPP secretion in response to glucose. The inhibition of hormone secretion occurs through altered K_{ATP} channels, and the increased mitochondrial metabolism observed is a compensatory response to counteract the secretory defect of the β-cells [157].

Serotonin and ***pancreastatin*** can be synthesised within β-cells and affect insulin secretion [158]. Serotonin mainly acts intracellularly. Serotonylation, the covalent coupling of serotonin to target proteins, regulates their activity and activates specific small GTPases, which in turn promote GSIS [159]. Otherwise, pancreastatin is a modulator of the early changes in insulin secretion after an increase in the glucose concentration within the physiological range. Pancreastatin has been reported to increase $[Ca^{2+}]_i$ in insulin-secreting RINm5F cells independent of the extracellular Ca^{2+} levels [160].

α-cells

Glucagon binds to glucagon receptors, which are expressed in several organs as well as in the β-cells, where glucagon-receptor signalling stimulates insulin release in a glucose-dependent manner [161]. The activation of glucagon receptor increases the cAMP levels and activates PLC. Glucagon stimulates insulin secretion in a glucose-dependent manner [162].

δ-cells

Somatostatin inhibits insulin secretion mainly via hyperpolarisation of the plasma membrane potential. The resultant inhibition of Ca^{2+}-dependent electrical activity reduces Ca^{2+} influx and insulin secretion. Hyperpolarisation by somatostatin occurs via the combined activation of K_{ATP} channels and G protein-coupled inward rectifiers, and this hormone can also act by directly blocking the VDCCs [163].

ε-cells

Ghrelin has been shown to inhibit GSIS in several experimental models [164-166]. Ghrelin has been suggested to attenuate membrane excitability through the activation of specific voltage-dependent K^+ channels (K_v channels), which in turn suppress Ca^{2+} uptake [164]. In addition, ghrelin directly interacts with β-cells to reduce glucose-induced cAMP production and PKA activation. Another study showed that ghrelin's inhibitory effect on insulin secretion is partly mediated by the AMPK-UCP2 (mitochondrial uncoupling protein 2) pathway [166].

PP-cells

Low concentrations of ***adrenomedullin*** inhibit insulin secretion. At higher concentrations, adrenomedullin induces a stimulatory component of insulin secretion, presumably via the elevation of cAMP, which cancels out the inhibitory component observed at lower concentrations. The inhibitory effect of adrenomedullin can be ascribed to the inhibition of insulin exocytosis through a PTX-sensitive G protein [167].

- *Gut hormones and peptides*

The gastrointestinal hormones, incretins, ***GLP1*** and ***GIP*** (glucose-dependent insulinotropic polypeptide), are released from gastrointestinal endocrine cells in response to the ingestion of nutrients [168]. Both of these hormones then potentiate GSIS by binding to their specific GPCRs in the pancreatic β-cell membrane and increasing cAMP production through activation of adenylate cyclase [169]. The modulatory action of incretins involves not only PKA but also RAPGEF4 (Rap guanine nucleotide exchange factor 4), which interacts directly with the SUR1 subunit of K_{ATP} channels. GLP1 also promotes Ca^{2+} influx through mobilising Ca^{2+} stored in intracellular organelles, which serves as a direct stimulus for exocytosis in β-cells. In addition, GLP1 exerts a direct action during the "late steps" of β-cell stimulus-secretion coupling in order to facilitate Ca^{2+}-dependent exocytosis of insulin [170].

The peptide ***neurotensin*** (NT) is secreted from neurons and gastrointestinal endocrine cells [171]. NT stimulates insulin secretion at low glucose levels and has a small inhibitory effect on stimulated insulin secretion from isolated islets or INS-1E cells. NT increases the Ca^{2+} influx through the opening of cation channels, and the NT-evoked Ca^{2+} regulation involves PRKC and the translocation of PRKCA and PRKCE (protein kinase C alpha/epsilon) to the plasma membrane. Part of the effects of NT appears to be mediated by PKA but not by the ERK pathway [172].

Xenin stimulates insulin secretion and exerts an additive effect on GIP-, GLP1- and NT-mediated insulin secretion in clonal β-cells. Xenin does not stimulate cellular cAMP production, alter the membrane potential or elevate $[Ca^{2+}]_i$. These data demonstrate that xenin may have significant metabolic effects on glucose control, but further studies are needed to identify the specific mechanism of action of xenin [173].

Cholecystokinin (CCK) enhances GSIS in mice [174] and humans [175] via the CCKA receptor (CCKAR). CCK-KO mice were reported to develop glucose intolerance despite increased insulin sensitivity associated with low insulin secretion [176]. Indeed, CCK is a potential therapeutic for T2DM because exogenous CCK treatment enhances insulin secretion in patients with T2DM [177].

- *Other hormones and peptides*

Leptin induces β-cell hyperpolarisation by opening the K_{ATP} channels [178], inhibiting insulin secretion and insulin mRNA levels in rat isolated pancreatic islets [179]. This hormone also inhibits insulin secretion by inhibiting the cAMP-PKA signalling pathway [180].

Adiponectin significantly stimulates insulin secretion and insulin gene expression in an AMPK-independent pathway [102].

Oestrogens, especially 17β-estradiol (E2) at physiological levels, are involved in maintaining normal insulin sensitivity for β-cell function. However, E2 levels above or below the physiological range may promote insulin resistance and T2DM. In synergy with a stimulatory glucose concentration, binding of E2 to a mER activates a guanylyl cyclase and consequently PRKG (protein kinase, cGMP-dependent), which closes K_{ATP} channels and triggers insulin release [103].

Melatonin is considered to be a main pineal product but may be also synthesised in the gastrointestinal tract. Melatonin inhibits insulin secretion through coupling of the receptor to inhibitory guanine nucleotide-binding proteins and subsequent downregulation of cAMP signalling [181].

3.2.3 Neurotransmitters

The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several different neurotransmitters including the classical neurotransmitters, acetylcholine and noradrenaline and several neuropeptides are stored within the terminals of these nerves. The neuropeptides, vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP) and gastrin-releasing peptide (GRP) are constituents of the parasympathetic nerves, whereas the neuropeptides galanin and neuropeptide Y (NPY) are localised at sympathetic nerve terminals [182]. Stimulation of the autonomic nerves and treatment with neurotransmitters affect islet hormone secretion. Thus, insulin secretion is stimulated by parasympathetic nerves or their neurotransmitters and is inhibited by sympathetic nerves or their neurotransmitters.

- *Parasympathetic nervous system*

Acetylcholine (Ach) exerts a pronounced stimulatory effect on pancreatic insulin release mediated by the M3R subtype [183]. In pancreatic β-cells, M3R-mediated activation of Gq-type G proteins stimulates the activity of different isoforms of PLCβ, resulting in the enzymatic breakdown of the membrane lipid phosphatidylinositol 4,5-bisphosphate and the generation of two second messengers, DAG and inositol 1,4,5-trisphosphate [143,182,184]. DAG activates PRKC, thus enhancing the effects of cytosolic Ca^{2+} on the exocytosis of insulin granules. In addition, stimulation of β-cell M3R can partially depolarise the plasma membrane via activation of a specific Na^{+} channel [184].

The neuropeptides ***VIP***, ***GRP*** and ***PACAP*** are released from the pancreas on electrical vagal activation and stimulate both insulin and glucagon secretion. [182]. Both VIP and PACAP stimulate insulin secretion in a glucose-dependent manner accompanied by increased action of adenylate cyclase with increased formation of cAMP [185]. PACAP also increases the cytoplasmic concentration of both Ca^{2+} and Na^{+} and has a distal effect on the exocytosis machinery, which may contribute to its potent insulinotropic action [143]. These effects have been suggested to be activated by cAMP-PKA signalling and to contribute to the increase in $[Ca^{2+}]_i$ and insulin secretion [185]. On the other hand, the islet action of GRP is related to changes in cytoplasmic Ca^{2+} through the formation of DAG and activation of PRKC [182].

- *Sympathetic nervous system*

Noradrenaline and ***adrenaline*** affect insulin secretion by acting as stimulators through β2-adrenoceptors and as inhibitors through α2-adrenoceptors in β-cells. β2-adrenoceptors are linked to activation of cAMP through Gs, whereas α2-adrenoceptors are linked to Gi and Go, which lead to the inhibition of cAMP production and opening of K^{+} channels. Selective knockout studies have shown that the α2A- and α2C-adrenoceptor subtypes mediate the inhibition of insulin secretion by catecholamines. Recently, a counteraction of GLP1 potentiation of GSIS by subthreshold α_2-adrenergic activation in the pancreatic β-cell was reported. The in-depth mechanisms whereby the α_2-adrenergic signalling system antagonises the GLP1 signalling system in pancreatic β-cells remain to be characterised [186].

The powerful inhibitory influence of ***galanin*** in rodent islets has been shown to be accompanied by a complex signalling mechanism, involving hyperpolarisation and reduction in $[Ca^{2+}]_c$, although reduced formation of cAMP and inhibition of a direct exocytotic mechanism could also contribute [187]. At least six different NPY receptors have been cloned, and there is evidence that the Y1 receptor subtype mediates the islet actions of ***NPY*** [188]. The mechanism underlying the inhibition of GSIS by NPY involves the inhibition of adenylate cyclase with reduced formation of cAMP. However, other mechanisms not involving the adenylate cyclase activity have also been suggested [189].

3.2.4 Nucleotides and metabolites involved in GSIS

Several metabolites and nucleotides (Figure 11) have been proposed to play a role in the amplifying pathway of insulin secretion, but neither the second messenger nor the cellular effector has been identified yet.

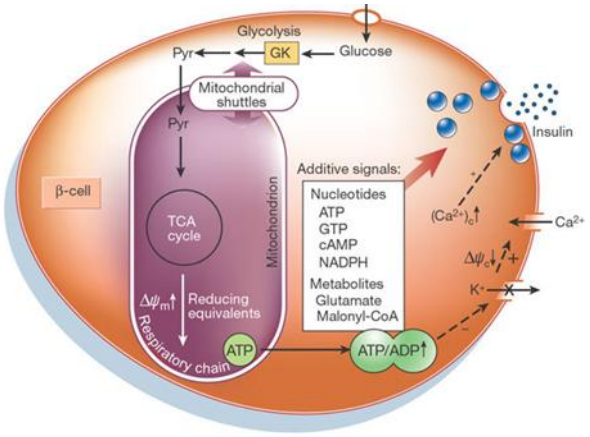


Figure 11. Nucleotides and metabolites involved in glucose-stimulated insulin secretion (from [190]).

A direct effect of ***NADPH***, generated by glucose metabolism via the pentose phosphate shunt and by mitochondrial shuttles [26], was reported in the release of insulin from isolated secretory granules [191]. In pancreatic β-cells, glucose acutely stimulates a sharp increase in the NADPH/$NADP^+$ ratio concomitant with insulin secretion. This effect is mediated by GLRX1 (glutaredoxin) and TXN1 (thioredoxin) [192]. GLRX1 was more recently demonstrated to mediate NADPH-dependent stimulation of Ca^{2+}-dependent insulin secretion in pancreatic β-cells by a local redox reaction that accelerates β-cell exocytosis and, in turn, insulin secretion [193].

Among the many products of the TCA cycle, attention has also focused on nucleotides formed by succinyl-CoA synthetase (SCS), which catalyses the synthesis of succinate from succinyl-CoA. Two isoforms SCS-ATP and SCS-GTP form ***ATP*** or ***GTP***, respectively, to preserve the energy that would otherwise be lost during hydrolysis of the thioester. Mitochondrial GTP synthesis correlates with TCA cycle activity, whereas TCA cycle-derived ATP makes only a minor contribution to the total mitochondrial ATP production [194]. Studies have shown how SCS-GTP-dependent GTP synthesis may link the TCA cycle activity to anaplerosis and to coupling factors that augment nutrient-dependent insulin secretion [195]. Down-regulation of SCS-GTP inhibits GSIS. Reduced expression of the SCS-ATP isoform diverts the reaction to SCS-GTP and markedly enhances insulin secretion [195]. Mitochondrial GTP, unlike ATP, is trapped within the mitochondrial matrix and therefore cannot stimulate exocytosis directly. Nevertheless, this signal was able to cause insulin secretion under conditions assessing the amplifying pathway [196].

cAMP serves as an almost universal signal that modulates or regulates exocytosis in various secretory systems including pancreatic β-cells [197,198], but the precise mechanisms of its actions are still unclear. In β-cells, specific G proteins can activate or inhibit adenylate cyclases that catalyse cAMP synthesis. In turn, cAMP signalling is attenuated by phosphodiesterase-catalysed degradation [199]. cAMP activates PKA, a multifunctional regulatory enzyme, and binds to guanyl exchange proteins [197,198]. Different effectors of PKA and guanyl exchange proteins regulate the potentiation of exocytosis in β-cells [198]. The combination of increased cAMP levels and high extracellular glucose potentiates GSIS [197,198]. In mouse β-cells, an elevation of intracellular cAMP potentiates secretion mainly by a direct effect on the exocytotic machinery [197] and to a lesser extent by increasing $[Ca^{2+}]_c$ [200]. Indeed, cAMP in the submembrane space has important effects on ion channels and exocytosis of insulin

granules [200,201]. As explained above, many neurotransmitters and hormones increase cAMP levels in β-cells by activating adenylate cyclase.

Malonyl-CoA can also act as a metabolic coupling factor in insulin secretion and as a messenger derived from citrate generated in the mitochondria. Malonyl-CoA inhibits CPT1A, which transports fatty acyl-CoA into mitochondria where it is oxidised and then causes an increase in long-chain acyl-CoAs in the cytosol. Glucose rapidly induces acetyl-CoA carboxylase production with a concomitant rapid rise in malonyl-CoA concentration preceding insulin secretion [25].

Other metabolites closely linked to glutamate, such as the amino acid ***glutamine*** or the TCA cycle intermediate ***α-ketoglutarate***, are also potential signalling molecules [202,203]. Glutamine by itself is not a secretagogue. Like glutamate, glutamine causes a pronounced increase of second phase insulin secretion following activation of the β-cell by suboptimal glucose concentrations [202]. Cytosolic Ca^{2+} signalling is required for the amplifying potential of glutamine [196].

Recent studies also suggest a role of reactive oxygen species such as H_2O_2 [204], granule translocation by the cytoskeleton [36] and ***AMPK*** [205]. AMPK acts as a cellular integration node for various nutrient and hormone signals, and subsequent changes in AMPK activity regulate multiple metabolic pathways of glucose metabolism [206]. The AMPK activator AICAR has been shown to enhances GSIS, an effect that can be attributed to increased electrical activity and $[Ca^{2+}]_c$ resulting from AICAR-induced inhibition of the K_{ATP} current. [207]. Indeed, RIPCreα2KO mice, which lack AMPKA2 in β-cells and a population of hypothalamic neurons, exhibited glucose intolerance and impaired GSIS. Moreover β-cells lacking AMPKA2 or expressing a kinase-dead AMPKA2 failed to hyperpolarise in response to low glucose even though the K_{ATP} channel function was intact [208].

3.2.5 Other regulators

Several reports recently demonstrated that some thermosensitive transient receptor potential (TRPM) channels are expressed in pancreatic β-cells. These channels can function as multimodal receptors and cause Ca^{2+} influx and membrane depolarisation at physiological body temperature. TRPM channels (TRPM2, TRPM4 and TRPM5) control insulin secretion levels by sensing an intracellular increase in Ca^{2+} or NAD metabolites or through hormone receptor activation [209].

4. Insulin degradation

Insulin clearance is a complex mechanism involving multiple organs and cells. Several steps are involved including binding to the insulin membrane receptor, its internalisation as an insulin-insulin receptor complex and degradation by the insulin-degrading enzyme (IDE) or by lysosomal enzymatic processes [210]. A major part of insulin is internalised by receptor-mediated processes, but internalisation can also occur by pinocytosis in cases of hyperinsulinaemia. Under normal conditions, almost all insulin is degraded intracellularly or at least by membrane processes. Some studies have suggested that significant amounts of insulin may be cleared and degraded extracellularly in wounds, which appears to be due primarily to IDE and may play a role in the wound-healing activity of insulin [211].

Insulin clearance includes both first-pass hepatic and peripheral insulin uptake. Degradation is a characteristic of all insulin-sensitive tissues. The liver, the primary site of insulin clearance, removes approximately 50 % of portal insulin, but this percentage varies widely under different conditions. Prolonged increases in portal insulin levels also result in reduced clearance because of receptor down-regulation. Removal of insulin from the circulation does not result in immediate destruction of the hormone. A significant amount of receptor-bound insulin is released from the cell and re-enters the circulation [210]. Nutrient intake alters insulin clearance. In general, glucose ingestion increases hepatic

insulin uptake and decreases hepatic fractional extraction. Increasing doses of glucose result in insulin secretion increase with simultaneous decreased hepatic extraction. Fatty acids also alter hepatic and splanchnic insulin uptake and degradation and may be involved in the changes associated with T2DM [211].

Because 50 % of insulin secreted by the pancreas is removed on first pass by the liver before reaching peripheral circulation, a reduction in hepatic insulin extraction would lead to substantial peripheral hyperinsulinaemia in insulin-resistant states caused by both hypersecretion and reduced hepatic extraction of insulin. The NEFA-mediated reduction and hepatic insulin extraction may be viewed as an adaptive mechanism to generate peripheral hyperinsulinaemia and thus to partially overcome the peripheral insulin resistance induced by NEFA, which could relieve the stress on pancreatic β-cells imposed by insulin resistance [210].

The kidney is the major site of insulin clearance from systemic circulation, removing approximately 50 % of peripheral insulin. In addition, the kidney removes 50 % of circulating proinsulin and 70 % of C-peptide by glomerular filtration. In general, insulin degradation by kidney cells is accomplished by the same processes as by the liver. Insulin is internalised into endosomes where degradation is initiated. Some insulin is released from the cell by retroendocytosis. As with the liver, isolated endosomes from the kidneys can degrade insulin, probably by IDE. Unlike the liver, lysosomes play a greater and earlier role in kidney insulin degradation, with most of the endosomal insulin and partially degraded insulin fragments delivered directly to lysosomes where degradation is completed [210]. The kidney plays an even greater role in insulin clearance in insulin-treated patients with diabetes than in normal subjects. Because insulin administered by subcutaneous injection escapes first-pass removal by the liver, the kidneys have increased importance in insulin removal in these patients [211].

Insulin not cleared by liver and kidneys is ultimately removed by other tissues. All insulin-sensitive cells remove and degrade the hormone, because they contain insulin receptors and internalisation mechanisms. After the liver and kidneys, muscles play a significant role in insulin removal with a mechanism that involves insulin binding to its receptor, internalisation and degradation as in other tissues. Insulin uptake and degradation also occur in adipocytes, fibroblasts, monocytes, lymphocytes, gastrointestinal cells and many other tissues [210].

In addition to IDE, other enzymes are involved in insulin degradation including protein disulphide isomerise (PDI), which acts after IDE cleavage of the B chain of receptor-bound insulin, catalysing disulphide cleavage and leading to production of intracellular fragments of insulin with potential biological activity [210]. During the last decade, evidence has emerged to reveal an important role of the transmembrane glycoprotein, CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1), in regulation of hepatic insulin clearance [212]. CEACAM1 is phosphorylated by the insulin receptor kinase after insulin binding and can subsequently bind to insulin receptor and be internalised [213]. CEACAM1 increases the rate of receptor-mediated insulin endocytosis and degradation to mediate insulin clearance in the liver [212]. Because CAECAM1-null mice or mice that express a dominant-negative CEACAM1 mutant both have impaired hepatic insulin clearance, a close relationship between CEACAM1 and the insulin receptor clearly has mechanistic importance [212,214].

4.1 Insulin-degrading enzyme (IDE)

Insulin-degrading enzyme was first described by Mirsky as “insulinase” on the basis of its ability to rapidly degrade insulin [215]. IDE, also referred as insulysin, is a zinc metallopeptidase enzyme that is predominantly present in the cytosol (~95 %) but is also found in small but significant amounts in endosomes, peroxisomes, mitochondria and extracellular space and at the cell surface [211,216-218]. In rats, Kuo et al. observed that IDE is highly expressed in adult rat testes, tongues and brains, moderately

expressed in the kidney, prostate, heart, muscle, liver, intestine and skin and lowly expressed in the spleen, lung, thymus and uterus [219]. The highest levels of IDE gene expression were found in germinal epithelium, suggesting an important role of IDE in the regulation of cellular growth and differentiation. In humans, IDE protein expression was found in normal tissues of the kidney, liver, lung, brain, breast and skeletal muscle as well as in breast and ovarian cancer tissues [220].

IDE is reported to cleave small proteins of diverse sequences, several of which have common abilities to form β-pleated sheet-rich amyloid fibrils, such as insulin, β-amyloid, amylin, glucagon, atrial natriuretic factor and calcitonin [210,221]. However, IDE is also responsible for the degradation of IGF1 and 2 [222] and transforming growth factor α (TGFA) [223].

IDE exists predominantly as a dimer in equilibrium with tetramers and to lesser extent monomers, with the dimer having the highest activity [224]. IDE is unique among the enzymes comprising the zinc metallopeptidase M16 family, which exhibit allosteric kinetic behaviour and show increased activity in the presence of substrate peptides [225]. In addition to certain peptide substrates, the activity of IDE is also influenced by other factors. For example, calcium-depleted muscle tissue has a decreased ability to degrade insulin and reduced IDE activity; however, the addition of calcium to the muscle restored insulin degradation. The catalytic activity of IDE *in vitro* was also observed to be inhibited by free long-chain fatty acids and acyl-CoA. These results suggest that elevated intracellular long-chain fatty acid concentrations may act directly on IDE to decrease insulin metabolism [226]. Moreover, insulin degradation by IDE is also affected by ATP through the triphosphate moiety [227]. Camberos et al. showed that ATP inhibits insulin degradation by IDE. This inhibition of insulin degradation appears to be strongly dependent on ATP concentration; indeed, other nucleotides, such as ADP and AMP, cannot induce the same inhibitory effect. ATP decreases insulin binding and degradation and diminishes dimer formation, suggesting that IDE undergoes conformational changes [227]. Thus, the energy status of the cell may serve as a feedback inhibition signal for insulin hydrolysis. Indeed, IDE is also capable of hydrolysing ATP [228]. Oxidative and nitrosative stress have also been proposed as other modulators of IDE activity [226]. Cordes et al. demonstrated the sensitivity of insulin degradation by IDE to the redox environment as oxidised glutathione inhibited IDE through glutathionylation and reduced glutathione had no effect on IDE [229].

On the other hand, IDE has been identified as a candidate gene for diabetes susceptibility in the Goto-Kakizaki rat, a genetic model of non-insulin-dependent diabetes. These animals exhibit elevated blood glucose and insulin levels due to a mutated form of IDE, which leads to reduced insulin degradation and causes symptoms typical of human T2DM [230]. The evidence for the putative influence of IDE on the pathogenesis of T2DM has been confirmed with human genetic studies that have linked polymorphisms in the IDE gene to an increased risk for insulin resistance and T2DM [231,232]. Furthermore, genome-wide association studies in humans have revealed that the IDE region of chromosome 10q contains a variant that confers risk for T2DM [233]. Lastly, Abdul-Hay et al. characterised IDE knockout (IDE-KO) mice at 2, 4 and 6 months of age. Consistent with a functional role for IDE in insulin clearance, fasting serum insulin levels in IDE-KO mice were found to be ~3-fold higher than those in wild-type controls at all ages examined. Six-month-old IDE-KO mice exhibited a severe diabetic phenotype, but 2-month-old IDE-KO mice showed multiple signs of improved glycemic control. These results indicate that the diabetic phenotype in IDE-KO mice is not a primary consequence of IDE deficiency but that it is an emergent compensatory response to chronic hyperinsulinemia resulting from complete deletion of IDE in all tissues throughout life [234].

IDE is also strongly linked both functionally and genetically to the pathogenesis of Alzheimer's disease (AD) [235]. Genetic evidence implicates variations in and around the IDE gene with the incidence and onset of AD [236]. In addition, several studies suggest that IDE is the principal protease responsible for the degradation of β-amyloid in the extracellular space [237].

5. Diseases linked to β-cell dysfunction

5.1 Diabetes mellitus

Diabetes mellitus is not a single disease but a syndrome of disordered metabolism with abnormally high blood glucose levels resulting from defects in insulin secretion, insulin action, or both. The estimated worldwide prevalence of diabetes among adults was 285 million (6.4 %) in 2010, and this value is predicted to increase to about 439 million (7.7 %) by 2030 (Table 3). The two most common forms of diabetes are type 1 diabetes (T1DM, diminished production of insulin) and T2DM (impaired response to insulin and β-cell dysfunction). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and the failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. The classical symptoms of diabetes include polyuria, polydipsia, weight loss, sometimes with polyphagia and blurred vision. Indeed, the impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia [238].

Table 3. Estimated numbers of adults aged 20-79 with any type of diabetes mellitus and prevalence, by region, in 2010 and 2030 (from [3]).

	2010		2030		
	Number of adults with diabetes (million)	**Prevalence***	**Number of adults with diabetes (million)**	**Prevalence***	**Percentage increase in number**
Africa	12.1	3.8 %	23.9	4.7 %	98.1 %
EMME	26.6	9.3 %	51.7	10.8 %	93.9 %
Europe	55.4	6.9 %	66.5	8.1 %	20.0 %
North America	37.4	10.2 %	53.2	12.1 %	42.4 %
South and Central America	18.0	6.6 %	29.6	7.8 %	65.1 %
Southeast Asia	58.7	7.6 %	101.0	9.1 %	72.1 %
West Pacific	76.7	4.7 %	112.8	5.7 %	47.0 %
Worldwide	284.8	6.4 %	438.7	7.7 %	54.1 %

*Values are standardised to world age distribution for that year for each region.

5.1.1 Type 1 Diabetes Mellitus

T1DM is a T-cell mediated autoimmune disease that is characterised by the destruction of insulin-producing β-cells of the pancreas because of the activation of pro-apoptotic signalling events. This metabolic disorder accounts for approximately 10% of diabetes mellitus cases. Autoimmune destruction of β-cells has multiple genetic predispositions and is related to environmental factors. Although patients are rarely obese when they are present with this type of diabetes, the presence of obesity is not incompatible with the diagnosis [238]. However, it is unclear whether the causes that trigger the development of this type of diabetes can be corrected with diet because genetic predisposition is a key factor.

5.1.2 Type 2 Diabetes Mellitus

T2DM is the most common type of diabetes. This metabolic disorder of fuel homeostasis is characterised by hyperglycemia and altered lipid metabolism caused by islet β-cells being unable to secrete adequate insulin in response to varying degrees of overnutrition, inactivity, consequential overweight or obesity and insulin resistance. The burden of this disorder is enormous because of its rapidly increasing global prevalence and the devastating damage it can do to many organs of the body [3].

Chronic fuel excess is the primary pathogenic event that drives the development of T2DM in genetically and epigenetically susceptible people [239,240]. However, many chronically overnourished and overweight or obese individuals do not develop diabetes at all or develop it very late in life. These individuals remain resistant to T2DM and safely partition excess calories to subcutaneous adipose tissue (SAT) rather than to the heart, skeletal muscle, liver and islet β-cells because of the following mechanisms (Figure 12): successful islet β-cell compensation; maintenance of near-normal blood nutrient concentrations; development of minimal insulin resistance; increased expansion of SAT relative to visceral adipose tissue (VAT); and limited increase in liver fat. In this way, key organs of the body avoid nutrient-induced damage [3].

In contrast, susceptible overnourished individuals develop T2DM because of the failure of these adaptive responses to safely dispose of the fuel surfeit (Figure 12). The following metabolic defects are crucial to the development of T2DM: inability of islet β-cells to compensate for the fuel excess; increased glucagon secretion and reduced incretin response; impaired expansion of SAT, hypoadiponectinaemia and inflammation of adipose tissue; increased endogenous glucose production; and development of peripheral insulin resistance [131,239-241], Importantly, the fuel surfeit is not safely deposited into SAT such that it has to be disposed of elsewhere, namely, less healthy VAT and “ectopic” storage in organs, such as the liver, heart, skeletal muscle and pancreas, which causes widespread tissue damage [241].

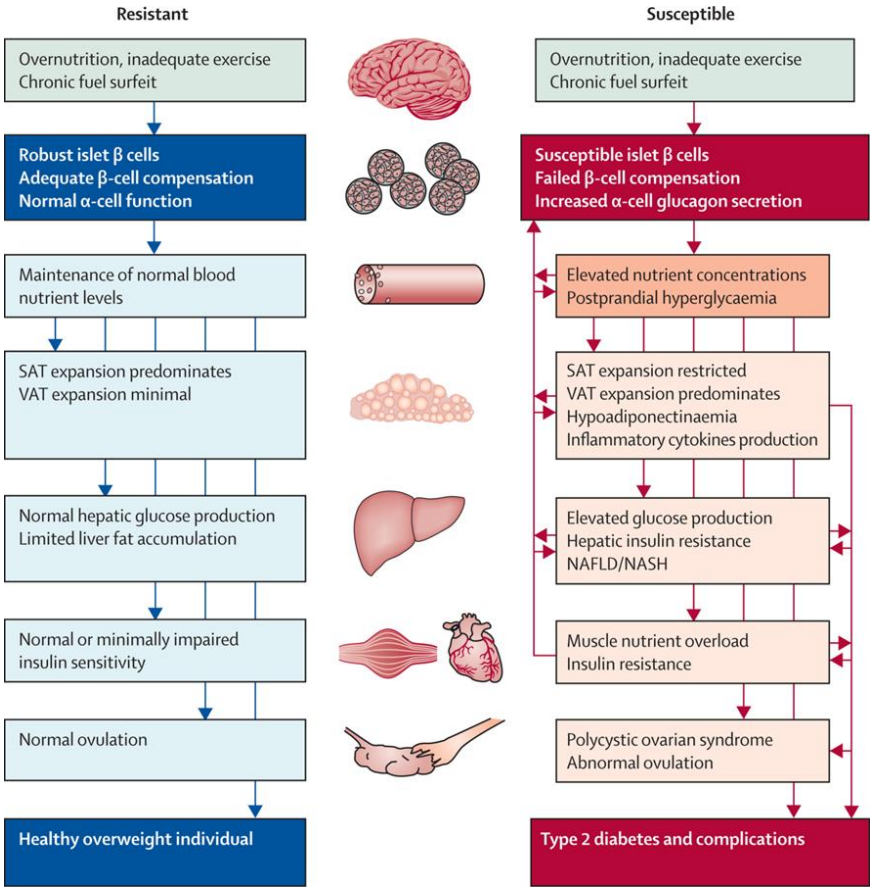


Figure 12. Pathway to T2DM and related complications (from [3]).

- *Pathogenesis of T2DM*

T2DM and impaired glucose tolerance (IGT) result from an interaction between genetic and environmental factors as described above. The genetic background causes insulin resistance and β-cell failure, whereas weight gain and physical inactivity exacerbate the inherited metabolic abnormalities.

Both insulin resistance and impaired insulin secretion are characteristic of T2DM [240]. Current evidence favours a two-step development of T2DM. During step one, normal glucose tolerant (NGT) individuals progress to IGT with insulin resistance as the primary determinant. Plasma insulin levels are elevated, but β-cell function is clearly impaired [240] (Figure 13). Thus, it is important to distinguish between the plasma insulin response and β-cell health. In step two, IGT advances to T2DM because of a progressive decline in β-cell function [242].

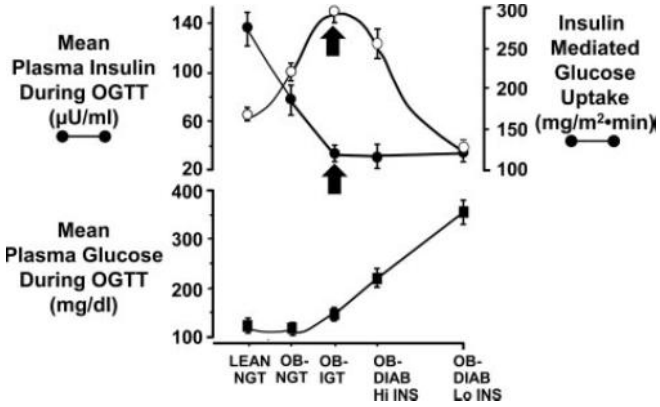


Figure 13. Natural history of T2DM. Progression from lean NGT to obese NGT to obese IGT (bottom) is associated with worsening insulin resistance and compensatory hyperinsulinemia (top). The development of overt diabetes (bottom) is associated with a progressive decline in insulin secretion with little further deterioration in insulin resistance (bottom) (40). OB, Obese; DIAB, diabetes; Hi INS, high insulin; Lo INS, low insulin (from [242]).

- *Insulin resistance*

By placing an increased demand on β-cells to hypersecrete insulin, insulin resistance also plays an important role in progressive β-cell failure in T2DM. Insulin resistance involves liver, muscle and adipose tissue and precedes development of glucose intolerance and overt T2DM (Figure 14) [240]. NGT first-degree relatives of T2DM individuals and people with IGT are markedly resistant to insulin and manifest compensatory hyperinsulinaemia. Much evidence supports a genetic component of insulin resistance, which is aggravated by weight gain, physical inactivity and aging. Not surprisingly, interventions that enhance insulin sensitivity and reduce the insulin secretory demand have been shown to effectively prevent/delay IGT progression to T2DM [242].

- *β-cell dysfunction and impaired insulin secretion*

Although insulin resistance is an important pathogenic factor, β-cell failure is ultimately responsible for progression of IGT to T2DM [240,243]. Insulin resistance was well established early in the natural history of T2DM; however, glucose tolerance remains normal because of a compensatory increase in insulin secretion [244]. The pancreas in persons with normal-functioning β-cells is able to “read” the severity of insulin resistance and adjust its secretion of insulin to maintain normal glucose tolerance.

In patients with T2DM, the fasting plasma insulin concentration invariably has been found to be normal or increased [245], and basal insulin secretion, as measured from C-peptide kinetics, is elevated [246]. The progressive rise in the fasting plasma insulin level can be viewed as an adaptive response of the pancreas to offset the progressive deterioration in glucose homeostasis. However, when the fasting

plasma glucose concentration exceeds 7.78 mM (140 mg/dL), β-cells no longer can maintain their elevated rate of insulin secretion and the fasting insulin concentration declines precipitously [4] (Figure 14).

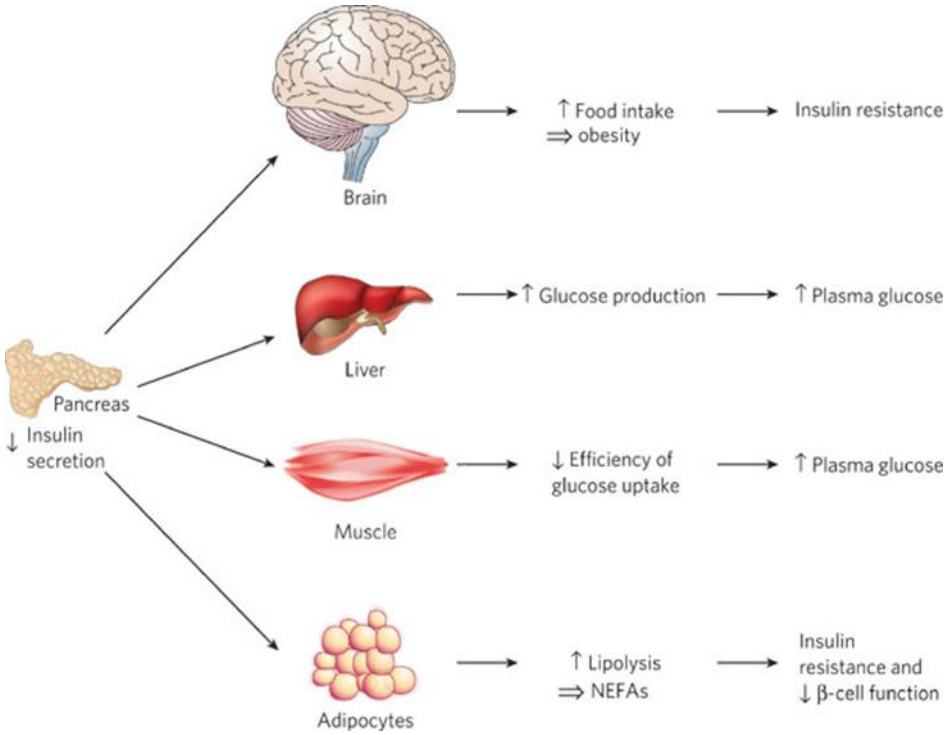


Figure 14. Impaired insulin secretion results in decreased insulin levels and decreased signalling in the hypothalamus, leading to increased food intake and weight gain, decreased inhibition of hepatic glucose production, reduced efficiency of glucose uptake in muscle and increased lipolysis in the adipocyte, resulting in increased plasma NEFA levels. The increase in body weight and NEFAs contribute to insulin resistance, and the increased NEFAs also suppress the β-cell's adaptive response to insulin resistance. The increased glucose levels together with the elevated NEFA levels collectively can adversely affect β-cell health and insulin action and is often referred to as glucolipotoxicity (from [247]).

Currently, the defect in insulin secretion in T2DM is thought be a combination of two components: reduced functional β-cell mass and intrinsic β-cell dysfunction [248,249]. T2DM patients have disrupted pulsatile insulin secretion, abnormal potentiation of non-glucose secretagogues and a reduced maximal secretory capacity to glucose and arginine. Moreover, the conversion of pro-insulin to insulin is reduced in T2DM leading to elevated pro-insulin levels. Finally, interactions of glucagon-producing α-cells within the islet are altered, leading to increased plasma glucagon levels, which contribute to hyperglycemia [250]. In addition to intrinsic functional defects in the insulin secretory machinery, anatomical abnormalities are also present in diabetic patients. Autopsy studies demonstrated a 63 % reduction in islet mass of T2DM patients compared with matched normoglycemic controls. In the same study, subjects with impaired fasting glucose (IFG) were shown to have a 40 % reduction of relative β-cell volume, suggesting that the loss of β-cell mass is present in the early stages of the disease [251]. The reduced β-cell mass is not due to the reduced formation of new islets but is caused by increased rates of apoptosis in islets [251]. The deficit in β-mass was correlated with fasting plasma glucose levels (FPG) levels, suggesting that the number of (functional) β-cells may play a role in physiological regulation [252]. Despite a vast body of research, the molecular mechanisms underlying β-cell dysfunction and apoptosis in the pathophysiology of T2DM remain unclear. However, increasing evidence suggests that hyperglycemia and hyperlipidemia in susceptible individuals further deteriorate β-cell function by inducing a cascade of processes referred to as glucotoxicity and lipotoxicity, respectively [253].

Glucotoxicity

The term glucotoxicity refers to the slow and irreversible detrimental effects of chronically elevated glucose levels on β-cell function, characterised by decreased insulin synthesis caused by reduced insulin gene expression. A number of mechanisms have been proposed for how chronically elevated glucose levels may impair β-cell function and increase β-cell apoptosis rates. Mechanisms related to glucotoxicity include endoplasmic reticulum (ER) stress, oxidative stress, mitochondrial dysfunction and islet inflammation [253].

Sustained increased demand for insulin due to chronic hyperglycemia may impose a burden or "stress" on the ER, characterised by an accumulation of misfolded proteins inside the organelle. The so-called unfolded protein response (UPR) is initiated, which aims to restore ER homeostasis by decreasing the ER protein load and by increasing the folding capacity. When the UPR fails to alleviate ER stress, the UPR triggers apoptosis [254]. In cultured rat islets, high glucose levels were shown to induce both components of the ER stress response and to trigger apoptosis. In β-cells from pancreatic sections obtained from T2DM patients, distended ER was found using electron microscopy, and markers for both ER stress and apoptosis were increased compared with non-diabetic patients [254,255].

Elevated levels of glucose lead to increased generation of ROS in islet cells, which induce oxidative stress. ROS are produced following oxidative phosphorylation of glucose in mitochondria but may also be generated through alternative metabolic pathways. Because β-cells have very low levels of antioxidant enzymes, they are particularly vulnerable to oxidative stress [256]. ROS mostly exert their detrimental effects on β-cells by impairing the function of mitochondria [257], which play a crucial role in GSIS (as described above) and in the regulation of β-cell mass. In rat islets and INS-1E cells, hyperglycemia-induced ROS production was shown to damage mitochondrial DNA and mitochondrial membrane proteins and to decrease the ability of mitochondria to produce ATP, resulting in reduced GSIS [258]. In isolated islets of pancreas from T2DM patients, increased markers of oxidative stress were observed compared with healthy controls, and oxidative stress correlated with the degree of impairment in insulin secretion [259]. Moreover, treatment of Zucker diabetic fatty (ZDF) rats with antioxidants led to decreased markers for oxidative stress and improved insulin gene expression and glycemic control [256]. ROS may impair mitochondrial dysfunction through an additional pathway related to uncoupling. Increased glucose fluxes through oxidative phosphorylation enhance ROS production. Increased glucose and ROS levels activate UCP2, which reduces the mitochondrial membrane potential, and thus, ATP synthesis is necessary for insulin secretion [239].

β-cell dysfunction observed during the course of T2DM is partially due to β-cell destruction through apoptosis and inflammation. Similarly as in T1DM, the pro-inflammatory cytokines interleukin IL1B and nuclear factor NFKB (nuclear factor of kappa light polypeptide gene enhancer in B-cells) in T2DM may orchestrate an autoimmune reaction leading to β-cell apoptosis. Some studies have proposed that the pro-apoptotic signals following chronic hyperglycemia are mainly derived from mechanisms related to the ER and oxidative stress [260]. Additionally, β-cell apoptosis in humans may also be triggered by pro-inflammatory signals from other organs, such as adipose tissue, which is associated with the production of numerous pro-inflammatory adipocytokines [253].

Lipotoxicity

Although slightly elevated levels of fatty acids may play an important role in sustaining basal insulin secretion and in maintaining a normal insulin secretory response to glucose, several studies have suggested that prolonged exposure to pharmacological levels of fatty acids impairs β-cell function. In line with *in vitro* findings, prospective studies in subjects at risk for T2DM have shown that the development of abdominal obesity is correlated with a loss of β-cell function [260] and that increased NEFA concentrations are a risk factor for the development of T2DM, independently of its effects on insulin

sensitivity [261]. Whether these fatty acids are derived from adipose tissue lipolysis or from hydrolysis of TG-rich lipoproteins in the plasma remains unclear [262]. The negative effects of elevated plasma fatty acids concentrations on β-cell function are referred to as lipotoxicity.

Fatty acids were shown to impair GSIS and insulin gene expression and to increase β-cell apoptosis and necrosis *in vitro* both in cell lines and in isolated (human) islets [263,264]. In addition, a 48 h infusion of intralipids or oleate in Wistar rats impaired GSIS [265]. In another *in vivo* study in wild-type rats, insulin gene expression was decreased by intralipid infusion [256].

A number of mechanisms have been proposed for how fatty acids impair β-cell function. First, fatty acids induce ER stress, most likely as a consequence of the overstimulation of NEFA esterification, which reduces the capacity of the ER for other processes [266]. Second, NEFAs have been shown to induce oxidative stress both *in vitro* and *in vivo* [265,267]. As such, treatment with anti-oxidants prevented oleate-induced β-cell dysfunction in Wistar rats. Third, fatty acids and especially the saturated NEFA palmitate may serve as substrates for the formation of ceramide and other metabolites, which have been shown to be pro-apoptotic and to reduce insulin gene expression [267].

More recently, the term glucolipotoxicity has been used because the alterations in intracellular lipid partitioning underlying the mechanisms of lipotoxicity are dependent upon elevated glucose levels. Consequently, hyperglycemia is a prerequisite for lipotoxicity to occur. Therefore, the term glucolipotoxicity rather than lipotoxicity is more appropriate to describe the deleterious effects of lipids on β-cell function [256].

In T2DM, overnutrition is a primary pathogenic event that triggers the development of T2DM, although it does not develop the illness genetically or epigenetically. In other types of diabetes, including MODY, the genetic factor is more decisive. The people suffering from this type of diabetes have genetic defects in β-cell function [268,269]. The different forms of MODY are all due to ineffective insulin production or release by pancreatic β-cells. Maternally inherited diabetes with deafness (MIDD) is another genetic caused type of diabetes. Impaired pancreatic β-cell insulin secretion is the major pathophysiological mechanism of MIDD, which is caused by mutations in the mitochondrial genome (most frequently the A3243G tRNALeu substitution) [270]. Many other mutations in mitochondrial DNA have been linked with the diabetic phenotype. MIDD is characterised by variability in clinical presentation, as it may mimic T1DM and T2DM. The disease is usually diagnosed in early adulthood; however, the age range of onset is very wide [270,271].

B. Flavonoids in insulin metabolism

1. Flavonoids

Plants contain more than 100,000 secondary metabolites, ranging from structurally simple alkaloids to more complex phytosterols and polyphenolic molecules. These 'phytochemicals' are not recognised as essential dietary components because they are not associated with a specific deficiency condition. Nevertheless, many of these non-nutritive compounds exert biological activities in mammalian systems that may affect health and disease risks. Indeed, some of these phytochemicals have been used therapeutically since ancient times, and their molecular structures are the basis of many modern pharmaceuticals. As such, bioactive compounds are likely to be present in a wide range of plant-based foods, and there is growing interest in their potential role in health and disease prevention in nutritionally relevant amounts. Phenolic compounds or polyphenols constitute one of the most numerous and ubiquitously distributed groups of plant secondary metabolites, and more than 8000 phenolic structures are currently known. These molecules are widespread in plant foods and are synthetic precursors and catabolic products of many more complex phytochemicals [272].

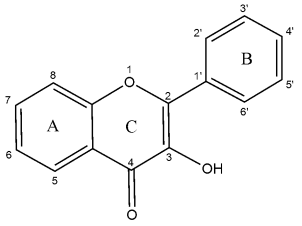
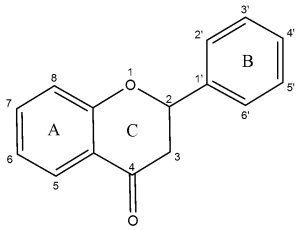
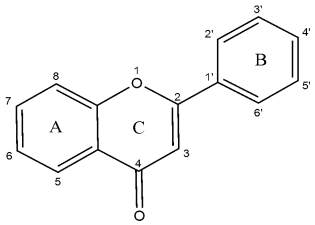
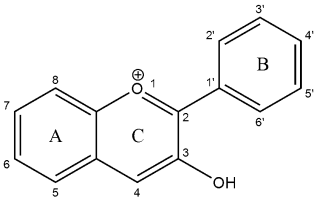
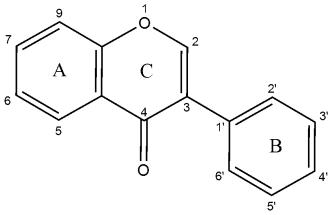
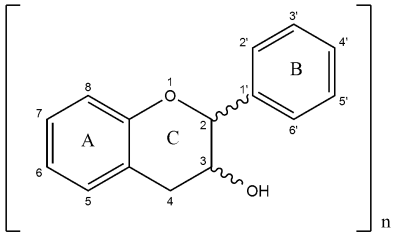
Polyphenols can be classified into different groups according to the number of phenolic groups that they contain or the structural elements that bind the rings to one another. The main classes of polyphenols are phenolic acids, stilbenes, lignans and flavonoids [273].

Flavonoids are a large class of polyphenols that are usually found in fruits, vegetables, medicinal plants and drinks, such as red wine, tea and beer [274,275], and thus are widely consumed. Their roles as antioxidants [276], anti-inflammatories [277], anticarcinogens [278] and protective agents against coronary disease and metabolic syndromes [279] are widely accepted. These beneficial effects make them good candidates for the development of new functional foods with potential protective/preventive properties against several diseases.

1.1 Structure and classification of flavonoids

The basic structure of flavonoids consists of 3 phenolic rings referred to as the A, B and C rings [280]. The A ring of benzene is condensed with a six-member ring (C), which carries a phenyl benzene ring (B) as a substituent at the 2-position [281]. Depending on the structure and oxidation level of the C ring, flavonoids are further divided into several subclasses. The main groups of flavonoids are anthocyanidins, flavonols, flavones, flavanones, isoflavones and flavanols or flavan-3-ols [281] (Table 4). In addition, the basic flavonoid structures can be modified and have several kinds and different patterns of substitutions (including glycosylation, hydrogenation, hydroxylation, malonylation, methylation, glucuronidation and sulfatation), which confer them different physical properties. Furthermore, the degree of polymerisation adds more variability to the flavonoids. Thus, the term flavonoid includes thousands of structures with different chemical, physical and biological properties [282].

Table 4. Classification, representative structure and food source of main flavonoids [282].

Flavonoids	Structure	Food source
Flavonols (Quercetin)		Onion, kale, broccoli, lettuce, tomato, apple, grape, berries, tea and red wine
Flavanones (Naringenin)		Citrus fruit (orange, grapefruit and lemon juice)
Flavones (Apigenin)		Celery and parsley
Anthocyanidins (Pelargonidin)		Aubergine, black grape, red cabbage and strawberry
Isoflavones (Genistein)		Soy bean and soy products
Flavanol monomer, dimers, trimers, etc. (EGCG)		Tea, chocolate, red wine, apple and berries

1.2 Chemical structure and classification of proanthocyanidins

The group of flavan-3-ols is the largest and most ubiquitous class of monomeric flavonoids and consists of not only simple monomers, such as (+)-catechin and its isomer (-)-epicatechin, but also oligomeric and polymeric proanthocyanidins [275] (Figure 15). The units of flavan-3-ols that form oligomers and polymers are mainly linked not only through the C4-C8 bond but also through the C4-C6 linkage [283]. These linkages are both called B-type linkages. However, A-type proanthocyanidins contain a linkage between C2 and C7. The designed procyanidins are proanthocyanidins that exclusively consist of (epi)catechin units and are the most common type of proanthocyanidins found in nature. The flavan-3-ols subunits may carry acyl or glycosyl substituents, with gallic acid bound as an ester (3-*O*-gallate) being the most common.

Procyanidins have a high structural diversity based on the four possible monomer units because of the different configurations of catechin and epicatechin (cis or trans, depending on the stereochemistry of their C2-C3 bond) [283].

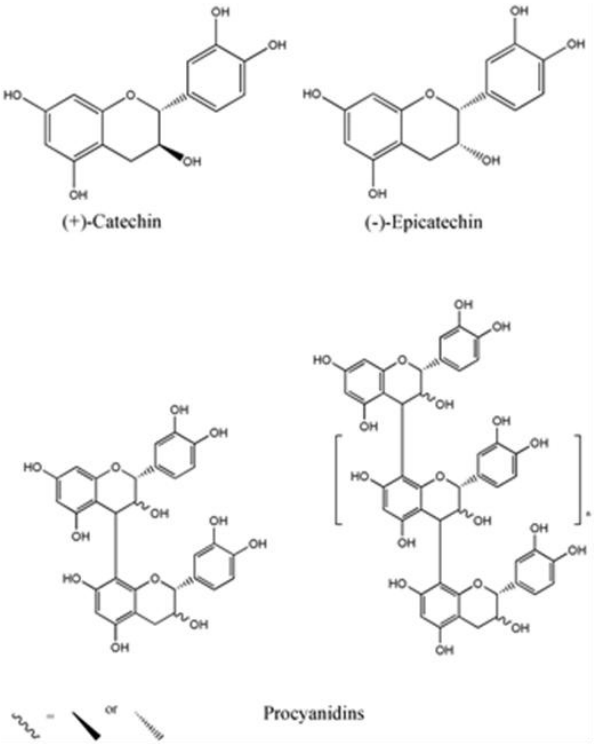


Figure 15. Structure of the flavanol monomers (+)-catechin and (-)-epicatechin and their polymers procyanidins.

1.3 Intake, bioavailability and metabolism

Various studies confirmed that proanthocyanidins are the most abundant phenolic compounds in fruits, such as grapes [284], apples [285] and strawberries [286], but these compounds are also present in beans, grains, nuts, spices, vegetables and beverages [287]. Although proanthocyanidins are widely present in the human diet, it is important to better understand their bioavailablity to humans.

Gu et al. estimated the daily average dietary intake of proanthocyanidins in the USA and found that the daily intake of proanthocyanidins is about 53.6 mg/day excluding the monomers and 57.7 mg/day including the monomers. These authors observed that about 74 % of ingested proanthocyanidins have a degree of polymerisation > 3 [288]. In another study, the dietary proanthocyanidin intake was found to vary from 10 mg to 0.5 g/day, with the B-1 and B-2 dimers most likely to be consumed [275]. Good evidence suggests that the dietary intake may be underestimated because of problems associated with extraction from food matrices prior to quantification [286].

The bioavailability of flavonoids, such as proanthocyanidins, involves liberation and digestion in the stomach and gastrointestinal tract, transport across the intestinal membrane into the blood, tissue distribution, metabolism and efficacy (biological effects) and finally elimination [289].

The mechanisms involved in flavonoid absorption have not been clearly elucidated. Following ingestion, the digestion and absorption of a few flavonoids begin in the stomach, where the aglycones may be absorbed [289]. However, the majority of dietary flavonoids, which exist predominantly as glycoside conjugates, are absorbed from the small intestine and pass through the gut wall into the circulatory system [290]. Typically, the absorption is associated with hydrolysis, releasing the aglycone, as a result of the action of lactase phloridizin hydrolase (LPH) in the brush-border of the small intestine epithelial cells. LPH exhibits broad substrate specificity for flavonoid-O-β-D-glycosides, and the released

aglycone may then enter the epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane [291]. Alternatively, hydrolysis involving cytosolic β-glucosidase (CBG) can occur within the epithelial cells. The polar glucosides must be transported into the epithelial cells in order for CBG-mediated hydrolysis to occur and possibly with the involvement of active SGLT1 (SLC5A1, sodium glucose cotransporter 1) [292]. Thus, there are two possible routes by which glucoside conjugates are hydrolysed and the resultant aglycones appear in the epithelial cells, namely, 'LPH/diffusion' and 'transport/CBG'. However, a recent investigation, in which SGLT1 was expressed in *Xenopus laevis* oocytes, indicated that SGLT1 does not transport flavonoids and that glycosylated flavonoids and some aglycones can inhibit the glucose transporter [293].

Prior to passage into the blood stream, the aglycones undergo metabolism, forming sulphate, glucuronide and/or methylated metabolites through the actions of sulfotransferases, uridine-5′-diphosphate glucuronosyltransferases and catechol-O-methyltransferases, respectively. In addition, efflux of at least some of the metabolites back into the lumen of the small intestine occurs, which is thought to involve members of the ATP-binding cassette family of transporters including multidrug resistance protein and P-glycoprotein [294].

Bacteria that normally colonise the colon also play an important role in flavonoid metabolism and absorption. Flavonoids or flavonoid metabolites that reach the colon may be further metabolised by bacterial enzymes and absorbed. Extensive colonic degradation of procyanidins, the flavanol quercetin and flavan-3-ols occurs [289]. Flavonoids known to be particularly well absorbed in humans are isoflavones, followed by quercetin-glucosides. Proanthocyanidins and the flavan-3-ol epigallocatechin gallate (EGCG) appear to be among the least well-absorbed polyphenols, which may be a result of instability once absorbed and not poor absorption [295]. Following the ingestion of a procyanidin-rich food, such as chocolate [296], tea [297] or grape seed extract [298], the oligomers of procyanidins are fragmented during digestion into monomeric units of catechin and epicatechin, which are then absorbed.

Once in the portal bloodstream, metabolites rapidly reach the liver, where they can be subjected to another metabolic phase. Further conversions and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion [290]. Metabolites of polyphenols are lost from the body via urinary and biliary excretion. Urinary excretion is an important pathway for flavanones, isoflavones and flavan-3-ols (10 % or more of the dose is excreted via urine), but biliary excretion is important for all polyphenols [289].

The diversity of flavonoid structures complicates their identification and measurement in plasma. However, despite the difficulties, several forms of modified flavonoids have been found in plasma [295,299]. Furthermore, some flavonoids have also been found within tissues such as the stomach, small intestine, colon, liver, spleen, kidney, muscle, heart, endothelium, lung, brain, thyroid, bone, skin, bladder, prostate, testes, vagina, uterus, ovary, mammary gland, fat, adrenal gland, oesophagus, eyes, lymph nodes and pituitary gland [282].

EGCG was detected in pancreas tissue as soon as 1 h after oral administration and reached about 3 times higher levels after 24 h. A second oral administration 6 h later resulted in a 4-fold increase of EGCG levels in the pancreas [300]. The isoflavone puerarin was found in pancreas 2 h after its acute administration [301]. Silibinin is the major active constituent of silymarin, the mixture of flavonoids extracted from milk thistle. Zaho et al. administered silibinin to mice starved for 24 h. They observed peak levels of free silibinin in pancreas 30 min after its administration, and peak levels of sulphate and β-glucuronidate conjugates of this compound were measured at 1 h after its administration [302]. Zhang et al. recently found quercetin and its metabolites in mice pancreas after being administered diets with 0.2 and 1 % of quercetin. Pancreas from mice fed with a 1 % quercetin diet accumulated near 4-times higher concentrations of quercetin and methylated quercetin compared with those fed with a 0.2 % quercetin diet [303].

2. Flavonoid action on insulin metabolism

Flavonoids are defined as bioactive compounds because they influence physiological or cellular activities and have beneficial health effects [276,304-307]. Several monomeric flavonoids and natural extracts rich in monomeric forms have been shown to improve hyperglycemia in streptozotocin-induced diabetic rats, in genetically altered diabetic mice and in animal models with diet-induced insulin resistance or diabetes [308-311].

The potential beneficial effects of procyanidins have also been widely studied because of their considerable intake through the diet. Procyanidins have been reported to act against coronary heart diseases and atherosclerosis as well as several metabolic processes associated with the development of those disorders [312]. Moreover, procyanidins are involved in the modulation of cholesterol and lipid metabolism [312], induce changes in vascular events [313], have antigenotoxic [314] and cardiovascular effects [315] and improve oxidative or inflammatory states. Procyanidins also have antiproliferative effects and have been studied as cancer preventive agents [316,317]. Indeed, several studies have investigated their effects on glucose homeostasis-disrupted situations, but a clear consensus has not been reached (reviewed in [318]).

2.1. Effects of procyanidins on glucose homeostasis

Published studies on the effects of procyanidins on glucose homeostasis suggest that these flavonoids may act as hypoglycemic agents. However, their effects can depend on the specific condition of glucose homeostasis disruption in which they are analysed. On the one hand, hyperglycemia may be the consequence of T1DM, in which there is a loss of β-cells due to an immune assault [319]. On the other hand, high glucose levels can occur because of the ineffectiveness of insulin. Insulin resistance is a condition in which insulin levels initially increase to compensate for the lack of insulin effect before reaching a state in which the pancreas is no longer functional (T2DM) [320].

Hyperglycemia in T1DM is the result of the body's inability to synthesise and/or secrete functional insulin. Several authors have assayed the ability of procyanidin-enriched extracts to ameliorate the physiological state caused by this situation using animal models with a destructed pancreas (mainly through the action of streptozotocin). Collectively, the published studies suggest that procyanidins have a short-lived insulin-mimetic effect on internal targets of the organism [321-323] and that they are useful in improving the general situation of the entire organism [324], most likely due to oligomeric forms [325] and a forced acute dose (summarised in [318]). On the other hand, the improved physiological state in these animal models could also result from the antioxidant effects that these flavonoids exert [326-328], because the drugs used may involve production of reactive oxygen species [329]. The action of the procyanidins avoid β-cell damage resulting in slight β-cell activity; consequently, the animals would be able to produce low levels of insulin, thus improving the physiological situation.

In normoinsulinemic animals, the effects of procyanidins on glucose homeostasis are not clear. If the inhibition of glucose absorption was the main mechanism explaining the glucose-lowering effects observed in diabetic animals, similar effects would be expected in non-diabetic animals. However, most of the experiments published do not support this hypothesis. Therefore, with a lack of insulin, procyanidins may act as insulin-mimetic agents affecting some insulin targets. Under normal insulinemia, the published studies suggest no clear effect of procyanidins on whole glucose homeostasis, probably because insulin is more effective in terms of its physiological effects [318,322,325].

Several studies have also analysed the effects of procyanidins on glucose homeostasis in an insulin-resistant state using animals fed special diets or animals with genetically induced obesity. Although preventive studies using fructose-fed models indicate that procyanidins may be useful in preventing the

induction of damage and thus in limiting hyperglycemia [330,331], the results of other studies using models such as high-fat diet-treated rats or genetically obese animals are controversial [332,333].
Pinent et al. reviewed that procyanidins may improve a slightly disrupted homeostatic situation, but such effects are highly dependent on the quantity of procyanidins that the animals receive, including the daily dose, which in turn depends on the method and period of administration [318].

2.2 The effects of flavonoids on insulin homeostasis

As previously described, procyanidins are able to modify glucose homeostasis, which could be through a peripheral and/or a central action [334]. Because of its physiological position, it is feasible that chronic procyanidin treatment targets the pancreas, the organ responsible for insulin secretion after glucose intake. However, very little information concerning the effects of procyanidins in pancreatic insulin synthesis and secretion is available. Most studies have focused on the effects of entire flavonoids in the pancreas.

Several *in vitro* and *in vivo* studies have described the effects of flavonoids in insulin secretion processes in β-cells. The author of this thesis participated in a review of this area, which serves as a complementary material of this thesis [282].

Genistein is the most studied isoflavone *in vitro*. Early studies showed that this compound increases GSIS in the β-pancreatic cell line MIN6 [335] as well as in cultured islets from mice [336] and rats [337] at concentrations up to 100 µmol/L. However, higher concentrations of genistein inhibited insulin secretion in rat islets [338]. Moreover, acute genistein treatment at physiological concentrations was shown to potentiate GSIS both in cell lines and isolated mouse islets. The insulin-secreting activity of genistein is mediated at least in part by cAMP accumulation and PKA activation [339] and is independent of oestrogen receptor mechanisms, protein tyrosine kinases and nitric oxide signalling pathways. Another study from the same research group showed that the GSIS-enhancing effect of genistein in INS-1E cells is not correlated with a modification, suggesting that genistein-enhanced GSIS is not due to the modulation of insulin synthesis. Indeed, genistein acts in a protein tyrosine kinase- and K_{ATP} channel-independent manner, and treatment with this flavonoid do not affect GLUT2 or cellular ATP production. Genistein- enhanced insulin secretion is associated with elevated intracellular Ca^{2+} concentrations and depends on PKA [340].

Quercetin at 20 µmol/L was recently reported to potentiate glucose-induced insulin secretion in a β-cell line but only had a minor effect in the absence of stimulated insulin secretion. Quercetin was also able to potentiate insulin secretion stimulated by glibenclamide, a K_{ATP} channel blocker that triggers membrane depolarisation independently of any change in glucose metabolism [341]. This work also revealed that quercetin could sensitise β-cells to primary stimulants of insulin secretion through an increase in basal $[Ca^{2+}]_i$ and subsequent ERK1/2 activation, and thereby amplify the insulin response. Indeed, quercetin was shown to increase the intracellular Ca^{2+} levels in rat islets of Langerhans [342] or inhibit ATP2A2 (responsible for cytosolic Ca^{2+} removal) [343]. Accordingly, Youl et al. observed that quercetin amplifies the depolarisation-induced intracellular Ca^{2+} increase in INS-1 cells [341].

Several molecules from the ***anthocyanin*** and ***anthocyanidin*** classes are also effective insulin secretagogues when tested in pancreatic cell lines but with different efficiencies depending on the structure. Delphinidin-3-glucoside was the most potent among the tested compounds and significantly induced insulin secretion at low and high glucose concentrations as compared with untreated cells. Although cyanidin-3-glucoside was less active than delphinidin-3-glucoside at a lower glucose concentration, it was more active at a higher glucose concentration. These results indicate that the number of hydroxyl groups in ring B of anthocyanins plays an important role in their ability to secrete

insulin. Among the anthocyanidins tested, pelargonidin was the most active at low glucose concentrations [344].

Some flavonoids interfere with glucose-induced depolarisation of the cell membrane, which initiates firing of action potentials that result in insulin secretion. Of the green tea ***catechins***, (-)-epigallocatechin-3-gallate (***EGCG***) and (-)-epicatechin-3-gallate **(*ECG***), but not (-)-epicatechin or (-)-epigallocatechin, inhibit the activity of K_{ATP} channels at tens of micromolar concentrations; indeed, ECG was 3 times more effective than EGCG. Using cloned β-cell-type K_{ATP} channels, these researchers showed that only EGCG at 1 μmol/L, a readily achievable plasma concentration by oral intake in humans, but not other epicatechins significantly blocked channel reactivation after ATP wash-out, suggesting that the interaction of phosphatidylinositol polyphosphates with the channel was impaired by EGCG [345]. Previously, Baek et al. also reported that the gallate-ester moiety of epicatechins may be critical for inhibiting the K_{ATP} channel activity via the pore-forming subunit Kir6.2, which may be a mechanism by which green tea extracts or EGCG cause unexpected side effects at the micromolar plasma level [346]. More recently, Cai and Lin published that these flavonoids could preserve the insulin secretory machinery and stimulate IRS2 signalling in rat pancreatic β-cells (RIN-m5F) under glucolipotoxic conditions [347].

Concerning the *in vivo* effects, long-term studies of the effects of soy protein containing ***genistein*** and ***daidzein*** were performed in models fed a normal and high-fat diet [348]. Chronic consumption of saturated fat increased insulin secretion associated with an increase in pancreatic islets, and soy protein ameliorated this situation. Thus, soy protein (because of its amino acid pattern as well as its isoflavones) reduces blood insulin. Using hyperglycemic clamps, rats fed soy protein were found to secrete less insulin to maintain glucose at normal levels. Interestingly, this effect was also observed in normal-fed rats. Furthermore, hyperglycemic clamps of rats infused with phytoestrogens (genistein, daidzein and equol) showed that these compounds rapidly inhibit the release of insulin, suggesting a short-term mechanism regulating this process and involving down-regulation of PPARG and GLUT2 mRNA expression [348]. More recently, another study was conducted with male CD1 mice fed with a high soy-containing diet from conception to adulthood. These animals had lower basal insulin levels and pancreatic insulin content than low phytoestrogen-fed mice [349]. Genistein and daidzein have been shown to elevate plasma insulin levels in non-obese diabetic (NOD) mice, an animal model that spontaneously develops autoimmune diabetes [350]. A 9-week treatment with genistein or daidzein (0.2 g/kg diet, animals fed *ad libitum*) suppressed an increase in blood glucose in NOD mice by elevating plasma insulin levels. Such effects were accompanied by an increase in insulin-positive β-cells, although it remained unresolved whether there was more insulin secretion from the remaining β-cells or increased β-cell mass in isoflavone-treated mice [350].

Another flavonoid, ***catechin*** from green tea, administered for 10 months with a high-fat diet in SAMP10 mice, which have characteristics of brain atrophy and cognitive dysfunction with aging, protected the pancreas from damage produced by the diet. Although the levels in serum insulin of fasted mice for 12 h were not significantly altered, the release of insulin from islet cells was significantly increased in mice fed a high-fat diet and was suppressed in mice fed a high-fat diet with green tea catechin [351].

Insulin degradation also plays an important role in insulin homeostasis. However, there have been no studies to our knowledge to determine the effects of flavonoids in insulin degradation or in relationship to IDE, the main enzyme responsible for insulin clearance.

C. References

[1] Gerozissis, K. Brain insulin, energy and glucose homeostasis; genes, environment and metabolic pathologies. *Eur.J.Pharmacol.* . 2008*,585*,38-49.
[2] Defronzo, R.A., Davidson, J.A., Del Prato, S. The Role of the Kidneys in Glucose Homeostasis:A New Path Toward Normalizing Glycaemia. *Diabetes Obes.Metab.* . 2011.
[3] Nolan, C.J., Damm, P., Prentki, M. Type 2 diabetes across generations: from pathophysiology to prevention and management. *Lancet* . 2011*,378*,169-181.
[4] Bajaj, M., Defronzo, R.A. Metabolic and molecular basis of insulin resistance. *J.Nucl.Cardiol.* . 2003*,10*,311-323.
[5] Slack, J.M. Developmental biology of the pancreas. *Development* . 1995*,121*,1569-1580.
[6] Encyclopædia Britannica Online. *Langerhans, islets of*, Art*,2011*.
[7] Weiss, F.U., Halangk, W., Lerch, M.M. New advances in pancreatic cell physiology and pathophysiology. *Best Pract.Res.Clin.Gastroenterol.* . 2008*,22*,3-15.
[8] Kulkarni, R.N. The islet beta-cell. *Int.J.Biochem.Cell Biol.* . 2004*,36*,365-371.
[9] Eberhard, D., Lammert, E. The pancreatic beta-cell in the islet and organ community. *Curr.Opin.Genet.Dev.* . 2009*,19*,469-475.
[10] Steiner, D.J., Kim, A., Miller, K., Hara, M. Pancreatic islet plasticity: interspecies comparison of islet architecture and composition. *Islets* . 2010*,2*,135-145.
[11] Kawamori, D., Kurpad, A.J., Hu, J., Liew, C.W., et al. Insulin signaling in alpha cells modulates glucagon secretion in vivo. *Cell.Metab.* . 2009*,9*,350-361.
[12] Kim, A., Miller, K., Jo, J., Kilimnik, G., et al. Islet architecture: A comparative study. *Islets* . 2009*,1*,129-136.
[13] Brissova, M., Fowler, M.J., Nicholson, W.E., Chu, A., et al. Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J.Histochem.Cytochem.* . 2005*,53*,1087-1097.
[14] Kharouta, M., Miller, K., Kim, A., Wojcik, P., et al. No mantle formation in rodent islets -- the prototype of islet revisited. *Diabetes Res.Clin.Pract.* . 2009*,85*,252-257.
[15] Soares, M.B., Schon, E., Henderson, A., Karathanasis, S.K., et al. RNA-mediated gene duplication: the rat preproinsulin I gene is a functional retroposon. *Mol.Cell.Biol.* . 1985*,5*,2090-2103.
[16] Wentworth, B.M., Schaefer, I.M., Villa-Komaroff, L., Chirgwin, J.M. Characterization of the two nonallelic genes encoding mouse preproinsulin. *J.Mol.Evol.* . 1986*,23*,305-312.
[17] Kuroda, A., Rauch, T.A., Todorov, I., Ku, H.T., et al. Insulin gene expression is regulated by DNA methylation. *PLoS One* . 2009*,4*,e6953.
[18] Kojima, H., Fujimiya, M., Matsumura, K., Nakahara, T., et al. Extrapancreatic insulin-producing cells in multiple organs in diabetes. *Proc.Natl.Acad.Sci.U.S.A.* . 2004*,101*,2458-2463.
[19] De Meyts, P. Insulin and its receptor: structure, function and evolution. *Bioessays* . 2004*,26*,1351-1362.
[20] Davidson, H.W. (Pro)Insulin processing: a historical perspective. *Cell Biochem.Biophys.* . 2004*,40*,143-158.
[21] Weiss, M.A. Proinsulin and the genetics of diabetes mellitus. *J.Biol.Chem.* . 2009*,284*,19159-19163.
[22] Eliasson, L., Abdulkader, F., Braun, M., Galvanovskis, J., et al. Novel aspects of the molecular mechanisms controlling insulin secretion. *J.Physiol.* . 2008*,586*,3313-3324.
[23] Hou, J.C., Min, L., Pessin, J.E. Insulin granule biogenesis, trafficking and exocytosis. *Vitam.Horm.* . 2009*,80*,473-506.
[24] Nenquin, M., Szollosi, A., Aguilar-Bryan, L., Bryan, J., Henquin, J.C. Both triggering and amplifying pathways contribute to fuel-induced insulin secretion in the absence of sulfonylurea receptor-1 in pancreatic beta-cells. *J.Biol.Chem.* . 2004*,279*,32316-32324.
[25] Jitrapakdee, S., Wutthisathapornchai, A., Wallace, J.C., MacDonald, M.J. Regulation of insulin secretion: role of mitochondrial signalling. *Diabetologia* . 2010*,53*,1019-1032.
[26] Farfari, S., Schulz, V., Corkey, B., Prentki, M. Glucose-regulated anaplerosis and cataplerosis in pancreatic beta-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion. *Diabetes* . 2000*,49*,718-726.
[27] Brownlee, M. A radical explanation for glucose-induced beta cell dysfunction. *J.Clin.Invest.* . 2003*,112*,1788-1790.

[28] Henquin, J.C., Nenquin, M., Ravier, M.A., Szollosi, A. Shortcomings of current models of glucose-induced insulin secretion. *Diabetes Obes.Metab.* . 2009*,11 Suppl 4*,168-179.
[29] Ravier, M.A., Nenquin, M., Miki, T., Seino, S., Henquin, J.C. Glucose controls cytosolic Ca2+ and insulin secretion in mouse islets lacking adenosine triphosphate-sensitive K+ channels owing to a knockout of the pore-forming subunit Kir6.2. *Endocrinology* . 2009*,150*,33-45.
[30] Henquin, J.C. The dual control of insulin secretion by glucose involves triggering and amplifying pathways in beta-cells. *Diabetes Res.Clin.Pract.* . 2011*,93 Suppl 1*,S27-31.
[31] Henquin, J.C. Regulation of insulin secretion: a matter of phase control and amplitude modulation. *Diabetologia* . 2009*,52*,739-751.
[32] Henquin, J.C., Nenquin, M., Stiernet, P., Ahren, B. In vivo and in vitro glucose-induced biphasic insulin secretion in the mouse: pattern and role of cytoplasmic Ca2+ and amplification signals in beta-cells. *Diabetes* . 2006*,55*,441-451.
[33] Torrejon-Escribano, B., Escoriza, J., Montanya, E., Blasi, J. Glucose-dependent changes in SNARE protein levels in pancreatic beta-cells. *Endocrinology* . 2011*,152*,1290-1299.
[34] Rorsman, P., Eliasson, L., Renstrom, E., Gromada, J., et al. The Cell Physiology of Biphasic Insulin Secretion. *News Physiol.Sci.* . 2000*,15*,72-77.
[35] Jewell, J.L., Oh, E., Thurmond, D.C. Exocytosis mechanisms underlying insulin release and glucose uptake: conserved roles for Munc18c and syntaxin 4. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* . 2010*,298*,R517-31.
[36] Rorsman, P., Renstrom, E. Insulin granule dynamics in pancreatic beta cells. *Diabetologia* . 2003*,46*,1029-1045.
[37] Seino, S., Shibasaki, T., Minami, K. Dynamics of insulin secretion and the clinical implications for obesity and diabetes. *J.Clin.Invest.* . 2011*,121*,2118-2125.
[38] Leibiger, I.B., Leibiger, B., Berggren, P.O. Insulin signaling in the pancreatic beta-cell. *Annu.Rev.Nutr.* . 2008*,28*,233-251.
[39] Leibiger, I.B., Leibiger, B., Berggren, P.O. Insulin feedback action on pancreatic beta-cell function. *FEBS Lett.* . 2002*,532*,1-6.
[40] Virkamaki, A., Ueki, K., Kahn, C.R. Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J.Clin.Invest.* . 1999*,103*,931-943.
[41] Bevan, P. Insulin signalling. *J.Cell.Sci.* . 2001*,114*,1429-1430.
[42] Saltiel, A.R., Kahn, C.R. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* . 2001*,414*,799-806.
[43] Andrali, S.S., Sampley, M.L., Vanderford, N.L., Ozcan, S. Glucose regulation of insulin gene expression in pancreatic beta-cells. *Biochem.J.* . 2008*,415*,1-10.
[44] Hay, C.W., Docherty, K. Comparative analysis of insulin gene promoters: implications for diabetes research. *Diabetes* . 2006*,55*,3201-3213.
[45] Rudnick, A., Ling, T.Y., Odagiri, H., Rutter, W.J., German, M.S. Pancreatic beta cells express a diverse set of homeobox genes. *Proc.Natl.Acad.Sci.U.S.A.* . 1994*,91*,12203-12207.
[46] Leonard, J., Peers, B., Johnson, T., Ferreri, K., et al. Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells. *Mol.Endocrinol.* . 1993*,7*,1275-1283.
[47] Boam, D.S., Docherty, K. A tissue-specific nuclear factor binds to multiple sites in the human insulin-gene enhancer. *Biochem.J.* . 1989*,264*,233-239.
[48] Marshak, S., Totary, H., Cerasi, E., Melloul, D. Purification of the beta-cell glucose-sensitive factor that transactivates the insulin gene differentially in normal and transformed islet cells. *Proc.Natl.Acad.Sci.U.S.A.* . 1996*,93*,15057-15062.
[49] German, M.S., Wang, J., Chadwick, R.B., Rutter, W.J. Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. *Genes Dev.* . 1992*,6*,2165-2176.
[50] Le Lay, J., Matsuoka, T.A., Henderson, E., Stein, R. Identification of a novel PDX-1 binding site in the human insulin gene enhancer. *J.Biol.Chem.* . 2004*,279*,22228-22235.
[51] Foulkes, N.S., Sassone-Corsi, P. Transcription factors coupled to the cAMP-signalling pathway. *Biochim.Biophys.Acta* . 1996*,1288*,F101-21.
[52] Masquilier, D., Sassone-Corsi, P. Transcriptional cross-talk: nuclear factors CREM and CREB bind to AP-1 sites and inhibit activation by Jun. *J.Biol.Chem.* . 1992*,267*,22460-22466.

[53] Kataoka, K., Shioda, S., Ando, K., Sakagami, K., et al. Differentially expressed Maf family transcription factors, c-Maf and MafA, activate glucagon and insulin gene expression in pancreatic islet alpha- and beta-cells. *J.Mol.Endocrinol.* . 2004,*32*,9-20.
[54] Campbell, S.C., Cragg, H., Elrick, L.J., Macfarlane, W.M., et al. Inhibitory effect of pax4 on the human insulin and islet amyloid polypeptide (IAPP) promoters. *FEBS Lett.* . 1999,*463*,53-57.
[55] Sander, M., Neubuser, A., Kalamaras, J., Ee, H.C., et al. Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. *Genes Dev.* . 1997,*11*,1662-1673.
[56] Karlsson, O., Edlund, T., Moss, J.B., Rutter, W.J., Walker, M.D. A mutational analysis of the insulin gene transcription control region: expression in beta cells is dependent on two related sequences within the enhancer. *Proc.Natl.Acad.Sci.U.S.A.* . 1987,*84*,8819-8823.
[57] Docherty, H.M., Hay, C.W., Ferguson, L.A., Barrow, J., et al. Relative contribution of PDX-1, MafA and E47/beta2 to the regulation of the human insulin promoter. *Biochem.J.* . 2005,*389*,813-820.
[58] Clark, A.R., Wilson, M.E., Leibiger, I., Scott, V., Docherty, K. A silencer and an adjacent positive element interact to modulate the activity of the human insulin promoter. *Eur.J.Biochem.* . 1995,*232*,627-632.
[59] Lew, A., Rutter, W.J., Kennedy, G.C. Unusual DNA structure of the diabetes susceptibility locus IDDM2 and its effect on transcription by the insulin promoter factor Pur-1/MAZ. *Proc.Natl.Acad.Sci.U.S.A.* . 2000,*97*,12508-12512.
[60] Pino, M.F., Ye, D.Z., Linning, K.D., Green, C.D., et al. Elevated glucose attenuates human insulin gene promoter activity in INS-1 pancreatic beta-cells via reduced nuclear factor binding to the A5/core and Z element. *Mol.Endocrinol.* . 2005,*19*,1343-1360.
[61] Clark, A.R., Wilson, M.E., London, N.J., James, R.F., Docherty, K. Identification and characterization of a functional retinoic acid/thyroid hormone-response element upstream of the human insulin gene enhancer. *Biochem.J.* . 1995,*309 (Pt 3)*,863-870.
[62] Cerf, M.E., Muller, C.J., Du Toit, D.F., Louw, J., Wolfe-Coote, S.A. Transcription factors, pancreatic development, and beta-cell maintenance. *Biochem.Biophys.Res.Commun.* . 2005,*326*,699-702.
[63] Qiu, Y., Guo, M., Huang, S., Stein, R. Insulin gene transcription is mediated by interactions between the p300 coactivator and PDX-1, BETA2, and E47. *Mol.Cell.Biol.* . 2002,*22*,412-420.
[64] Bernardo, A.S., Hay, C.W., Docherty, K. Pancreatic transcription factors and their role in the birth, life and survival of the pancreatic beta cell. *Mol.Cell.Endocrinol.* . 2008,*294*,1-9.
[65] Han, S.I., Yasuda, K., Kataoka, K. ATF2 interacts with beta-cell-enriched transcription factors, MafA, Pdx1, and beta2, and activates insulin gene transcription. *J.Biol.Chem.* . 2011,*286*,10449-10456.
[66] Donelan, W., Koya, V., Li, S.W., Yang, L.J. Distinct regulation of hepatic nuclear factor 1alpha by NKX6.1 in pancreatic beta cells. *J.Biol.Chem.* . 2010,*285*,12181-12189.
[67] Bartoov-Shifman, R., Hertz, R., Wang, H., Wollheim, C.B., et al. Activation of the insulin gene promoter through a direct effect of hepatocyte nuclear factor 4 alpha. *J.Biol.Chem.* . 2002,*277*,25914-25919.
[68] Okita, K., Yang, Q., Yamagata, K., Hangenfeldt, K.A., et al. Human insulin gene is a target gene of hepatocyte nuclear factor-1alpha (HNF-1alpha) and HNF-1beta. *Biochem.Biophys.Res.Commun.* . 1999,*263*,566-569.
[69] Hansen, S.K., Parrizas, M., Jensen, M.L., Pruhova, S., et al. Genetic evidence that HNF-1alpha-dependent transcriptional control of HNF-4alpha is essential for human pancreatic beta cell function. *J.Clin.Invest.* . 2002,*110*,827-833.
[70] Ashery-Padan, R., Zhou, X., Marquardt, T., Herrera, P., et al. Conditional inactivation of Pax6 in the pancreas causes early onset of diabetes. *Dev.Biol.* . 2004,*269*,479-488.
[71] Wolf, G., Hessabi, B., Karkour, A., Henrion, U., et al. The activation of the rat insulin gene II by BETA2 and PDX-1 in rat insulinoma cells is repressed by Pax6. *Mol.Endocrinol.* . 2010,*24*,2331-2342.
[72] Doyle, M.J., Loomis, Z.L., Sussel, L. Nkx2.2-repressor activity is sufficient to specify alpha-cells and a small number of beta-cells in the pancreatic islet. *Development* . 2007,*134*,515-523.
[73] Cissell, M.A., Zhao, L., Sussel, L., Henderson, E., Stein, R. Transcription factor occupancy of the insulin gene in vivo. Evidence for direct regulation by Nkx2.2. *J.Biol.Chem.* . 2003,*278*,751-756.
[74] Raum, J.C., Gerrish, K., Artner, I., Henderson, E., et al. FoxA2, Nkx2.2, and PDX-1 regulate islet beta-cell-specific mafA expression through conserved sequences located between base pairs -8118 and -7750 upstream from the transcription start site. *Mol.Cell.Biol.* . 2006,*26*,5735-5743.

[75] Meng, Z., Lv, J., Luo, Y., Lin, Y., et al. Forkhead box O1/pancreatic and duodenal homeobox 1 intracellular translocation is regulated by c-Jun N-terminal kinase and involved in prostaglandin E2-induced pancreatic beta-cell dysfunction. *Endocrinology* . 2009*,150*,5284-5293.
[76] Shu, T., Zhu, Y., Wang, H., Lin, Y., et al. AGEs decrease insulin synthesis in pancreatic beta-cell by repressing Pdx-1 protein expression at the post-translational level. *PLoS One* . 2011*,6*,e18782.
[77] Meur, G., Qian, Q., da Silva Xavier, G., Pullen, T.J., et al. Nucleo-cytosolic shuttling of FoxO1 directly regulates mouse Ins2 but not Ins1 gene expression in pancreatic beta cells (MIN6). *J.Biol.Chem.* . 2011*,286*,13647-13656.
[78] Melloul, D., Marshak, S., Cerasi, E. Regulation of pdx-1 gene expression. *Diabetes* . 2002*,51 Suppl 3*,S320-5.
[79] Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., et al. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J.Clin.Invest.* . 2002*,110*,1839-1847.
[80] Kang, H.S., Kim, Y.S., ZeRuth, G., Beak, J.Y., et al. Transcription factor Glis3, a novel critical player in the regulation of pancreatic beta-cell development and insulin gene expression. *Mol.Cell.Biol.* . 2009*,29*,6366-6379.
[81] Yang, Y., Chang, B.H., Samson, S.L., Li, M.V., Chan, L. The Kruppel-like zinc finger protein Glis3 directly and indirectly activates insulin gene transcription. *Nucleic Acids Res.* . 2009*,37*,2529-2538.
[82] Gupta, D., Kono, T., Evans-Molina, C. The role of peroxisome proliferator-activated receptor gamma in pancreatic beta cell function and survival: therapeutic implications for the treatment of type 2 diabetes mellitus. *Diabetes Obes.Metab.* . 2010*,12*,1036-1047.
[83] Sarkar, A., Zhang, M., Liu, S.H., Sarkar, S., et al. Serum response factor expression is enriched in pancreatic beta cells and regulates insulin gene expression. *FASEB J.* . 2011*,25*,2592-2603.
[84] Koranyi, L., James, D.E., Kraegen, E.W., Permutt, M.A. Feedback inhibition of insulin gene expression by insulin. *J.Clin.Invest.* . 1992*,89*,432-436.
[85] Rutter, G.A. Insulin secretion: feed-forward control of insulin biosynthesis?. *Curr.Biol.* . 1999*,9*,R443-5.
[86] Withers, D.J., Gutierrez, J.S., Towery, H., Burks, D.J., et al. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* . 1998*,391*,900-904.
[87] Kulkarni, R.N., Bruning, J.C., Winnay, J.N., Postic, C., et al. Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* . 1999*,96*,329-339.
[88] Leibiger, I.B., Leibiger, B., Moede, T., Berggren, P.O. Exocytosis of insulin promotes insulin gene transcription via the insulin receptor/PI-3 kinase/p70 s6 kinase and CaM kinase pathways. *Mol.Cell* . 1998*,1*,933-938.
[89] Xu, G.G., Gao, Z.Y., Borge, P.D.,Jr, Jegier, P.A., et al. Insulin regulation of beta-cell function involves a feedback loop on SERCA gene expression, Ca(2+) homeostasis, and insulin expression and secretion. *Biochemistry* . 2000*,39*,14912-14919.
[90] Leibiger, B., Wahlander, K., Berggren, P.O., Leibiger, I.B. Glucose-stimulated insulin biosynthesis depends on insulin-stimulated insulin gene transcription. *J.Biol.Chem.* . 2000*,275*,30153-30156.
[91] Ozcan, S., Andrali, S.S., Cantrell, J.E. Modulation of transcription factor function by O-GlcNAc modification. *Biochim.Biophys.Acta* . 2010*,1799*,353-364.
[92] Humphrey, R.K., Yu, S.M., Flores, L.E., Jhala, U.S. Glucose regulates steady-state levels of PDX1 via the reciprocal actions of GSK3 and AKT kinases. *J.Biol.Chem.* . 2010*,285*,3406-3416.
[93] Akimoto, Y., Hart, G.W., Wells, L., Vosseller, K., et al. Elevation of the post-translational modification of proteins by O-linked N-acetylglucosamine leads to deterioration of the glucose-stimulated insulin secretion in the pancreas of diabetic Goto-Kakizaki rats. *Glycobiology* . 2007*,17*,127-140.
[94] Wong, W.P., Tiano, J.P., Liu, S., Hewitt, S.C., et al. Extranuclear estrogen receptor-alpha stimulates NeuroD1 binding to the insulin promoter and favors insulin synthesis. *Proc.Natl.Acad.Sci.U.S.A.* . 2010*,107*,13057-13062.
[95] Andrali, S.S., Qian, Q., Ozcan, S. Glucose mediates the translocation of NeuroD1 by O-linked glycosylation. *J.Biol.Chem.* . 2007*,282*,15589-15596.
[96] Vanderford, N.L., Andrali, S.S., Ozcan, S. Glucose induces MafA expression in pancreatic beta cell lines via the hexosamine biosynthetic pathway. *J.Biol.Chem.* . 2007*,282*,1577-1584.

[97] Han, S.I., Aramata, S., Yasuda, K., Kataoka, K. MafA stability in pancreatic beta cells is regulated by glucose and is dependent on its constitutive phosphorylation at multiple sites by glycogen synthase kinase 3. *Mol.Cell.Biol.* . 2007*,27*,6593-6605.
[98] Portha, B., Tourrel-Cuzin, C., Movassat, J. Activation of the GLP-1 receptor signalling pathway: a relevant strategy to repair a deficient beta-cell mass. *Exp.Diabetes Res.* . 2011*,2011*,376509.
[99] Morioka, T., Asilmaz, E., Hu, J., Dishinger, J.F., et al. Disruption of leptin receptor expression in the pancreas directly affects beta cell growth and function in mice. *J.Clin.Invest.* . 2007*,117*,2860-2868.
[100] Covey, S.D., Wideman, R.D., McDonald, C., Unniappan, S., et al. The pancreatic beta cell is a key site for mediating the effects of leptin on glucose homeostasis. *Cell.Metab.* . 2006*,4*,291-302.
[101] Nielsen, J.H., Galsgaard, E.D., Moldrup, A., Friedrichsen, B.N., et al. Regulation of beta-cell mass by hormones and growth factors. *Diabetes* . 2001*,50 Suppl 1*,S25-9.
[102] Wijesekara, N., Krishnamurthy, M., Bhattacharjee, A., Suhail, A., et al. Adiponectin-induced ERK and Akt phosphorylation protects against pancreatic beta cell apoptosis and increases insulin gene expression and secretion. *J.Biol.Chem.* . 2010*,285*,33623-33631.
[103] Soriano, S., Ripoll, C., Fuentes, E., Gonzalez, A., et al. Regulation of K(ATP) channel by 17beta-estradiol in pancreatic beta-cells. *Steroids* . 2011*,76*,856-860.
[104] Nadal, A., Alonso-Magdalena, P., Soriano, S., Quesada, I., Ropero, A.B. The pancreatic beta-cell as a target of estrogens and xenoestrogens: Implications for blood glucose homeostasis and diabetes. *Mol.Cell.Endocrinol.* . 2009*,304*,63-68.
[105] Jirtle, R.L., Skinner, M.K. Environmental epigenomics and disease susceptibility. *Nat.Rev.Genet.* . 2007*,8*,253-262.
[106] Yang, B.T., Dayeh, T.A., Kirkpatrick, C.L., Taneera, J., et al. Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA(1c) levels in human pancreatic islets. *Diabetologia* . 2011*,54*,360-367.
[107] Hinke, S.A., Hellemans, K., Schuit, F.C. Plasticity of the beta cell insulin secretory competence: preparing the pancreatic beta cell for the next meal. *J.Physiol.* . 2004*,558*,369-380.
[108] Knoch, K.P., Bergert, H., Borgonovo, B., Saeger, H.D., et al. Polypyrimidine tract-binding protein promotes insulin secretory granule biogenesis. *Nat.Cell Biol.* . 2004*,6*,207-214.
[109] Fred, R.G., Welsh, N. The importance of RNA binding proteins in preproinsulin mRNA stability. *Mol.Cell.Endocrinol.* . 2009*,297*,28-33.
[110] Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* . 2004*,116*,281-297.
[111] Carthew, R.W., Sontheimer, E.J. Origins and Mechanisms of miRNAs and siRNAs. *Cell* . 2009*,136*,642-655.
[112] Kloosterman, W.P., Lagendijk, A.K., Ketting, R.F., Moulton, J.D., Plasterk, R.H. Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. *PLoS Biol.* . 2007*,5*,e203.
[113] Poy, M.N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., et al. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* . 2004*,432*,226-230.
[114] Guay, C., Roggli, E., Nesca, V., Jacovetti, C., Regazzi, R. Diabetes mellitus, a microRNA-related disease?. *Transl.Res.* . 2011*,157*,253-264.
[115] Lovis, P., Roggli, E., Laybutt, D.R., Gattesco, S., et al. Alterations in microRNA expression contribute to fatty acid-induced pancreatic beta-cell dysfunction. *Diabetes* . 2008*,57*,2728-2736.
[116] Lynn, F.C. Meta-regulation: microRNA regulation of glucose and lipid metabolism. *Trends Endocrinol.Metab.* . 2009*,20*,452-459.
[117] Huang, B., Qin, W., Zhao, B., Shi, Y., et al. MicroRNA expression profiling in diabetic GK rat model. *Acta Biochim.Biophys.Sin.(Shanghai)* . 2009*,41*,472-477.
[118] Lovis, P., Gattesco, S., Regazzi, R. Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. *Biol.Chem.* . 2008*,389*,305-312.
[119] Hennessy, E., Clynes, M., Jeppesen, P.B., O'Driscoll, L. Identification of microRNAs with a role in glucose stimulated insulin secretion by expression profiling of MIN6 cells. *Biochem.Biophys.Res.Commun.* . 2010*,396*,457-462.
[120] Tang, X., Muniappan, L., Tang, G., Ozcan, S. Identification of glucose-regulated miRNAs from pancreatic {beta} cells reveals a role for miR-30d in insulin transcription. *RNA* . 2009*,15*,287-293.
[121] Sun, L.L., Jiang, B.G., Li, W.T., Zou, J.J., et al. MicroRNA-15a positively regulates insulin synthesis by inhibiting uncoupling protein-2 expression. *Diabetes Res.Clin.Pract.* . 2011*,91*,94-100.

[122] Melkman-Zehavi, T., Oren, R., Kredo-Russo, S., Shapira, T., et al. miRNAs control insulin content in pancreatic beta-cells via downregulation of transcriptional repressors. *EMBO J.* . 2011*,30*,835-845.
[123] Zhang, Z.W., Zhang, L.Q., Ding, L., Wang, F., et al. MicroRNA-19b downregulates insulin 1 through targeting transcription factor NeuroD1. *FEBS Lett.* . 2011*,585*,2592-2598.
[124] He, A., Zhu, L., Gupta, N., Chang, Y., Fang, F. Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. *Mol.Endocrinol.* . 2007*,21*,2785-2794.
[125] Herrera, B.M., Lockstone, H.E., Taylor, J.M., Ria, M., et al. Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes. *Diabetologia* . 2010*,53*,1099-1109.
[126] Prentki, M., Tornheim, K., Corkey, B.E. Signal transduction mechanisms in nutrient-induced insulin secretion. *Diabetologia* . 1997*,40 Suppl 2*,S32-41.
[127] Johannsen, D.L., Ravussin, E. The role of mitochondria in health and disease. *Curr.Opin.Pharmacol.* . 2009*,9*,780-786.
[128] Maechler, P., Carobbio, S., Rubi, B. In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion. *Int.J.Biochem.Cell Biol.* . 2006*,38*,696-709.
[129] Schuit, F., De Vos, A., Farfari, S., Moens, K., et al. Metabolic fate of glucose in purified islet cells. Glucose-regulated anaplerosis in beta cells. *J.Biol.Chem.* . 1997*,272*,18572-18579.
[130] Newsholme, P., Bender, K., Kiely, A., Brennan, L. Amino acid metabolism, insulin secretion and diabetes. *Biochem.Soc.Trans.* . 2007*,35*,1180-1186.
[131] Nolan, C.J., Prentki, M. The islet beta-cell: fuel responsive and vulnerable. *Trends Endocrinol.Metab.* . 2008*,19*,285-291.
[132] Sener, A., Best, L.C., Yates, A.P., Kadiata, M.M., et al. Stimulus-secretion coupling of arginine-induced insulin release: comparison between the cationic amino acid and its methyl ester. *Endocrine* . 2000*,13*,329-340.
[133] Dunne, M.J., Yule, D.I., Gallacher, D.V., Petersen, O.H. Effects of alanine on insulin-secreting cells: patch-clamp and single cell intracellular Ca2+ measurements. *Biochim.Biophys.Acta* . 1990*,1055*,157-164.
[134] Sener, A., Malaisse, W.J. L-leucine and a nonmetabolized analogue activate pancreatic islet glutamate dehydrogenase. *Nature* . 1980*,288*,187-189.
[135] Gao, Z., Young, R.A., Li, G., Najafi, H., et al. Distinguishing features of leucine and alpha-ketoisocaproate sensing in pancreatic beta-cells. *Endocrinology* . 2003*,144*,1949-1957.
[136] Maechler, P., Wollheim, C.B. Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* . 1999*,402*,685-689.
[137] Hoy, M., Maechler, P., Efanov, A.M., Wollheim, C.B., et al. Increase in cellular glutamate levels stimulates exocytosis in pancreatic beta-cells. *FEBS Lett.* . 2002*,531*,199-203.
[138] Bai, L., Zhang, X., Ghishan, F.K. Characterization of vesicular glutamate transporter in pancreatic alpha - and beta -cells and its regulation by glucose. *Am.J.Physiol.Gastrointest.Liver Physiol.* . 2003*,284*,G808-14.
[139] Casimir, M., Lasorsa, F.M., Rubi, B., Caille, D., et al. Mitochondrial glutamate carrier GC1 as a newly identified player in the control of glucose-stimulated insulin secretion. *J.Biol.Chem.* . 2009*,284*,25004-25014.
[140] Rubi, B., Ishihara, H., Hegardt, F.G., Wollheim, C.B., Maechler, P. GAD65-mediated glutamate decarboxylation reduces glucose-stimulated insulin secretion in pancreatic beta cells. *J.Biol.Chem.* . 2001*,276*,36391-36396.
[141] Nolan, C.J., Leahy, J.L., Delghingaro-Augusto, V., Moibi, J., et al. Beta cell compensation for insulin resistance in Zucker fatty rats: increased lipolysis and fatty acid signalling. *Diabetologia* . 2006*,49*,2120-2130.
[142] Torres, N., Noriega, L., Tovar, A.R. Nutrient modulation of insulin secretion. *Vitam.Horm.* . 2009*,80*,217-244.
[143] Ahren, B. Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. *Nat.Rev.Drug Discov.* . 2009*,8*,369-385.
[144] Zawalich, W.S., Zawalich, K.C. Effects of glucose, exogenous insulin, and carbachol on C-peptide and insulin secretion from isolated perifused rat islets. *J.Biol.Chem.* . 2002*,277*,26233-26237.
[145] Araujo, E.P., Amaral, M.E., Souza, C.T., Bordin, S., et al. Blockade of IRS1 in isolated rat pancreatic islets improves glucose-induced insulin secretion. *FEBS Lett.* . 2002*,531*,437-442.

[146] Persaud, S.J., Asare-Anane, H., Jones, P.M. Insulin receptor activation inhibits insulin secretion from human islets of Langerhans. *FEBS Lett.* . 2002*,510*,225-228.
[147] Johnson, J.D., Misler, S. Nicotinic acid-adenine dinucleotide phosphate-sensitive calcium stores initiate insulin signaling in human beta cells. *Proc.Natl.Acad.Sci.U.S.A.* . 2002*,99*,14566-14571.
[148] Kulkarni, R.N., Roper, M.G., Dahlgren, G., Shih, D.Q., et al. Islet secretory defect in insulin receptor substrate 1 null mice is linked with reduced calcium signaling and expression of sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA)-2b and -3. *Diabetes* . 2004*,53*,1517-1525.
[149] Assmann, A., Hinault, C., Kulkarni, R.N. Growth factor control of pancreatic islet regeneration and function. *Pediatr.Diabetes* . 2009*,10*,14-32.
[150] Aspinwall, C.A., Qian, W.J., Roper, M.G., Kulkarni, R.N., et al. Roles of insulin receptor substrate-1, phosphatidylinositol 3-kinase, and release of intracellular Ca2+ stores in insulin-stimulated insulin secretion in beta -cells. *J.Biol.Chem.* . 2000*,275*,22331-22338.
[151] Park, S.H., Kim, S.Y., Baek, W.K., Lim, B., et al. Regulation of glucose-dependent insulin secretion by insulin: possible role of AMP-activated protein kinase. *Life Sci.* . 2009*,85*,178-183.
[152] Bouche, C., Lopez, X., Fleischman, A., Cypess, A.M., et al. Insulin enhances glucose-stimulated insulin secretion in healthy humans. *Proc.Natl.Acad.Sci.U.S.A.* . 2010*,107*,4770-4775.
[153] Leibiger, B., Leibiger, I.B., Moede, T., Kemper, S., et al. Selective insulin signaling through A and B insulin receptors regulates transcription of insulin and glucokinase genes in pancreatic beta cells. *Mol.Cell* . 2001*,7*,559-570.
[154] Dong, H., Kumar, M., Zhang, Y., Gyulkhandanyan, A., et al. Gamma-aminobutyric acid up- and downregulates insulin secretion from beta cells in concert with changes in glucose concentration. *Diabetologia* . 2006*,49*,697-705.
[155] Salas, M., Silvestre, R.A., Garcia-Hermida, O., Fontela, T., et al. Inhibitory effect of amylin (islet amyloid polypeptide) on insulin response to non-glucose stimuli. Study in perfused rat pancreas. *Diabete Metab.* . 1995*,21*,269-273.
[156] Ahren, B., Oosterwijk, C., Lips, C.J., Hoppener, J.W. Transgenic overexpression of human islet amyloid polypeptide inhibits insulin secretion and glucose elimination after gastric glucose gavage in mice. *Diabetologia* . 1998*,41*,1374-1380.
[157] Soty, M., Visa, M., Soriano, S., Carmona, M.D., et al. Involvement of katp channels in the loss of beta-cell function induced by human islet amyloid polypeptide. *J.Biol.Chem.* . 2011.
[158] Ahren, B., Bertrand, G., Roye, M., Ribes, G. Pancreastatin modulates glucose-stimulated insulin secretion from the perfused rat pancreas. *Acta Physiol.Scand.* . 1996*,158*,63-70.
[159] Paulmann, N., Grohmann, M., Voigt, J.P., Bert, B., et al. Intracellular serotonin modulates insulin secretion from pancreatic beta-cells by protein serotonylation. *PLoS Biol.* . 2009*,7*,e1000229.
[160] Sanchez-Margalet, V., Lucas, M., Goberna, R. Pancreastatin increases cytosolic Ca2+ in insulin secreting RINm5F cells. *Mol.Cell.Endocrinol.* . 1992*,88*,129-133.
[161] Gelling, R.W., Vuguin, P.M., Du, X.Q., Cui, L., et al. Pancreatic beta-cell overexpression of the glucagon receptor gene results in enhanced beta-cell function and mass. *Am.J.Physiol.Endocrinol.Metab.* . 2009*,297*,E695-707.
[162] Huypens, P., Ling, Z., Pipeleers, D., Schuit, F. Glucagon receptors on human islet cells contribute to glucose competence of insulin release. *Diabetologia* . 2000*,43*,1012-1019.
[163] Smith, P.A. N-type Ca(2+) -channels in murine pancreatic beta-cells are inhibited by an exclusive coupling with somatostatin receptor subtype 1. *Endocrinology* . 2009*,150*,741-748.
[164] Dezaki, K., Sone, H., Yada, T. Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis. *Pharmacol.Ther.* . 2008*,118*,239-249.
[165] Dezaki, K., Damdindorj, B., Sone, H., Dyachok, O., et al. Ghrelin attenuates cAMP-PKA signaling to evoke insulinostatic cascade in islet beta-cells. *Diabetes* . 2011*,60*,2315-2324.
[166] Wang, Y., Nishi, M., Doi, A., Shono, T., et al. Ghrelin inhibits insulin secretion through the AMPK-UCP2 pathway in beta cells. *FEBS Lett.* . 2010*,584*,1503-1508.
[167] Sekine, N., Takano, K., Kimata-Hayashi, N., Kadowaki, T., Fujita, T. Adrenomedullin inhibits insulin exocytosis via pertussis toxin-sensitive G protein-coupled mechanism. *Am.J.Physiol.Endocrinol.Metab.* . 2006*,291*,E9-E14.
[168] Elliott, R.M., Morgan, L.M., Tredger, J.A., Deacon, S., et al. Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. *J.Endocrinol.* . 1993*,138*,159-166.

[169] Thorens, B. Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1. *Proc.Natl.Acad.Sci.U.S.A.* . 1992,*89*,8641-8645.
[170] Leech, C.A., Dzhura, I., Chepurny, O.G., Kang, G., et al. Molecular physiology of glucagon-like peptide-1 insulin secretagogue action in pancreatic beta cells. *Prog.Biophys.Mol.Biol.* . 2011,*107*,236-247.
[171] Vincent, J.P., Mazella, J., Kitabgi, P. Neurotensin and neurotensin receptors. *Trends Pharmacol.Sci.* . 1999,*20*,302-309.
[172] Beraud-Dufour, S., Abderrahmani, A., Noel, J., Brau, F., et al. Neurotensin is a regulator of insulin secretion in pancreatic beta-cells. *Int.J.Biochem.Cell Biol.* . 2010,*42*,1681-1688.
[173] Taylor, A.I., Irwin, N., McKillop, A.M., Patterson, S., et al. Evaluation of the degradation and metabolic effects of the gut peptide xenin on insulin secretion, glycaemic control and satiety. *J.Endocrinol.* . 2010,*207*,87-93.
[174] Karlsson, S., Ahren, B. CCK-8-stimulated insulin secretion in vivo is mediated by CCKA receptors. *Eur.J.Pharmacol.* . 1992,*213*,145-146.
[175] Ahren, B., Pettersson, M., Uvnas-Moberg, K., Gutniak, M., Efendic, S. Effects of cholecystokinin (CCK)-8, CCK-33, and gastric inhibitory polypeptide (GIP) on basal and meal-stimulated pancreatic hormone secretion in man. *Diabetes Res.Clin.Pract.* . 1991,*13*,153-161.
[176] Lo, C.M., Obici, S., Dong, H.H., Haas, M., et al. Impaired insulin secretion and enhanced insulin sensitivity in cholecystokinin-deficient mice. *Diabetes* . 2011,*60*,2000-2007.
[177] Ahren, B., Holst, J.J., Efendic, S. Antidiabetogenic action of cholecystokinin-8 in type 2 diabetes. *J.Clin.Endocrinol.Metab.* . 2000,*85*,1043-1048.
[178] Kieffer, T.J., Heller, R.S., Leech, C.A., Holz, G.G., Habener, J.F. Leptin suppression of insulin secretion by the activation of ATP-sensitive K+ channels in pancreatic beta-cells. *Diabetes* . 1997,*46*,1087-1093.
[179] Pallett, A.L., Morton, N.M., Cawthorne, M.A., Emilsson, V. Leptin inhibits insulin secretion and reduces insulin mRNA levels in rat isolated pancreatic islets. *Biochem.Biophys.Res.Commun.* . 1997,*238*,267-270.
[180] Ahren, B., Havel, P.J. Leptin inhibits insulin secretion induced by cellular cAMP in a pancreatic B cell line (INS-1 cells). *Am.J.Physiol.* . 1999,*277*,R959-66.
[181] Picinato, M.C., Haber, E.P., Cipolla-Neto, J., Curi, R., et al. Melatonin inhibits insulin secretion and decreases PKA levels without interfering with glucose metabolism in rat pancreatic islets. *J.Pineal Res.* . 2002,*33*,156-160.
[182] Ahren, B. Autonomic regulation of islet hormone secretion--implications for health and disease. *Diabetologia* . 2000,*43*,393-410.
[183] Duttaroy, A., Zimliki, C.L., Gautam, D., Cui, Y., et al. Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in m3 muscarinic acetylcholine receptor-deficient mice. *Diabetes* . 2004,*53*,1714-1720.
[184] Gilon, P., Henquin, J.C. Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. *Endocr.Rev.* . 2001,*22*,565-604.
[185] Ahren, B. Role of pituitary adenylate cyclase-activating polypeptide in the pancreatic endocrine system. *Ann.N.Y.Acad.Sci.* . 2008,*1144*,28-35.
[186] Pan, M., Yang, G., Cui, X., Yang, S.N. Subthreshold alpha-adrenergic activation counteracts glucagon-like peptide-1 potentiation of glucose-stimulated insulin secretion. *Exp.Diabetes Res.* . 2011,*2011*,604989.
[187] Ahren, B., Lindskog, S. Galanin and the regulation of islet hormone secretion. *Int.J.Pancreatol.* . 1992,*11*,147-160.
[188] Morgan, D.G., Kulkarni, R.N., Hurley, J.D., Wang, Z.L., et al. Inhibition of glucose stimulated insulin secretion by neuropeptide Y is mediated via the Y1 receptor and inhibition of adenylyl cyclase in RIN 5AH rat insulinoma cells. *Diabetologia* . 1998,*41*,1482-1491.
[189] Skoglund, G., Gross, R., Ahren, B., Loubatieres-Mariani, M.M. Different mechanisms are involved in neuropeptide Y-induced pancreatic vasoconstriction and inhibition of insulin secretion. *Eur.J.Pharmacol.* . 1993,*236*,69-74.
[190] Maechler, P., Wollheim, C.B. Mitochondrial function in normal and diabetic beta-cells. *Nature* . 2001,*414*,807-812.

[191] Watkins, D.T. Pyridine nucleotide stimulation of insulin release from isolated toadfish insulin secretion granules. *Endocrinology* . 1972,*90*,272-276.
[192] Ivarsson, R., Quintens, R., Dejonghe, S., Tsukamoto, K., et al. Redox control of exocytosis: regulatory role of NADPH, thioredoxin, and glutaredoxin. *Diabetes* . 2005,*54*,2132-2142.
[193] Reinbothe, T.M., Ivarsson, R., Li, D.Q., Niazi, O., et al. Glutaredoxin-1 mediates NADPH-dependent stimulation of calcium-dependent insulin secretion. *Mol.Endocrinol.* . 2009,*23*,893-900.
[194] Wiederkehr, A., Wollheim, C.B. Mitochondrial signals drive insulin secretion in the pancreatic beta-cell. *Mol.Cell.Endocrinol.* . 2011.
[195] Kibbey, R.G., Pongratz, R.L., Romanelli, A.J., Wollheim, C.B., et al. Mitochondrial GTP regulates glucose-stimulated insulin secretion. *Cell.Metab.* . 2007,*5*,253-264.
[196] Wiederkehr, A., Szanda, G., Akhmedov, D., Mataki, C., et al. Mitochondrial matrix calcium is an activating signal for hormone secretion. *Cell.Metab.* . 2011,*13*,601-611.
[197] Seino, S., Takahashi, H., Fujimoto, W., Shibasaki, T. Roles of cAMP signalling in insulin granule exocytosis. *Diabetes Obes.Metab.* . 2009,*11 Suppl 4*,180-188.
[198] Leech, C.A., Chepurny, O.G., Holz, G.G. Epac2-dependent rap1 activation and the control of islet insulin secretion by glucagon-like peptide-1. *Vitam.Horm.* . 2010,*84*,279-302.
[199] Fridlyand, L.E., Philipson, L.H. Coupling of metabolic, second messenger pathways and insulin granule dynamics in pancreatic beta-cells: A computational analysis. *Prog.Biophys.Mol.Biol.* . 2011,*107*,293-303.
[200] Ammala, C., Ashcroft, F.M., Rorsman, P. Calcium-independent potentiation of insulin release by cyclic AMP in single beta-cells. *Nature* . 1993,*363*,356-358.
[201] Seino, S., Shibasaki, T. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol.Rev.* . 2005,*85*,1303-1342.
[202] Li, C., Buettger, C., Kwagh, J., Matter, A., et al. A signaling role of glutamine in insulin secretion. *J.Biol.Chem.* . 2004,*279*,13393-13401.
[203] Rabaglia, M.E., Gray-Keller, M.P., Frey, B.L., Shortreed, M.R., et al. Alpha-Ketoisocaproate-induced hypersecretion of insulin by islets from diabetes-susceptible mice. *Am.J.Physiol.Endocrinol.Metab.* . 2005,*289*,E218-24.
[204] Pi, J., Bai, Y., Zhang, Q., Wong, V., et al. Reactive oxygen species as a signal in glucose-stimulated insulin secretion. *Diabetes* . 2007,*56*,1783-1791.
[205] Rutter, G.A., Leclerc, I. The AMP-regulated kinase family: enigmatic targets for diabetes therapy. *Mol.Cell.Endocrinol.* . 2009,*297*,41-49.
[206] Xue, B., Kahn, B.B. AMPK integrates nutrient and hormonal signals to regulate food intake and energy balance through effects in the hypothalamus and peripheral tissues. *J.Physiol.* . 2006,*574*,73-83.
[207] Dufer, M., Noack, K., Krippeit-Drews, P., Drews, G. Activation of the AMP-activated protein kinase enhances glucose-stimulated insulin secretion in mouse beta-cells. *Islets* . 2010,*2*,156-163.
[208] Beall, C., Piipari, K., Al-Qassab, H., Smith, M.A., et al. Loss of AMP-activated protein kinase alpha2 subunit in mouse beta-cells impairs glucose-stimulated insulin secretion and inhibits their sensitivity to hypoglycaemia. *Biochem.J.* . 2010,*429*,323-333.
[209] Uchida, K., Tominaga, M. The role of thermosensitive TRP (transient receptor potential) channels in insulin secretion. *Endocr.J.* . 2011.
[210] Valera Mora, M.E., Scarfone, A., Calvani, M., Greco, A.V., Mingrone, G. Insulin clearance in obesity. *J.Am.Coll.Nutr.* . 2003,*22*,487-493.
[211] Duckworth, W.C., Bennett, R.G., Hamel, F.G. Insulin degradation: progress and potential. *Endocr.Rev.* . 1998,*19*,608-624.
[212] Poy, M.N., Yang, Y., Rezaei, K., Fernstrom, M.A., et al. CEACAM1 regulates insulin clearance in liver. *Nat.Genet.* . 2002,*30*,270-276.
[213] Najjar, S.M. Regulation of insulin action by CEACAM1. *Trends Endocrinol.Metab.* . 2002,*13*,240-245.
[214] DeAngelis, A.M., Heinrich, G., Dai, T., Bowman, T.A., et al. Carcinoembryonic antigen-related cell adhesion molecule 1: a link between insulin and lipid metabolism. *Diabetes* . 2008,*57*,2296-2303.
[215] BROH-KAHN, R.H., MIRSKY, I.A. The inactivation of insulin by tissue extracts; the effect of fasting on the insulinase content of rat liver. *Arch.Biochem.* . 1949,*20*,10-14.
[216] Seta, K.A., Roth, R.A. Overexpression of insulin degrading enzyme: cellular localization and effects on insulin signaling. *Biochem.Biophys.Res.Commun.* . 1997,*231*,167-171.

[217] Qiu, W.Q., Walsh, D.M., Ye, Z., Vekrellis, K., et al. Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. *J.Biol.Chem.* . 1998,*273*,32730-32738.
[218] Vekrellis, K., Ye, Z., Qiu, W.Q., Walsh, D., et al. Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. *J.Neurosci.* . 2000,*20*,1657-1665.
[219] Kuo, W.L., Montag, A.G., Rosner, M.R. Insulin-degrading enzyme is differentially expressed and developmentally regulated in various rat tissues. *Endocrinology* . 1993,*132*,604-611.
[220] Yfanti, C., Mengele, K., Gkazepis, A., Weirich, G., et al. Expression of metalloprotease insulin-degrading enzyme insulysin in normal and malignant human tissues. *Int.J.Mol.Med.* . 2008,*22*,421-431.
[221] Bennett, R.G., Duckworth, W.C., Hamel, F.G. Degradation of amylin by insulin-degrading enzyme. *J.Biol.Chem.* . 2000,*275*,36621-36625.
[222] Bennett, R.G., Hamel, F.G., Duckworth, W.C. Characterization of the insulin inhibition of the peptidolytic activities of the insulin-degrading enzyme-proteasome complex. *Diabetes* . 1997,*46*,197-203.
[223] Hamel, F.G., Gehm, B.D., Rosner, M.R., Duckworth, W.C. Identification of the cleavage sites of transforming growth factor alpha by insulin-degrading enzymes. *Biochim.Biophys.Acta* . 1997,*1338*,207-214.
[224] Song, E.S., Juliano, M.A., Juliano, L., Fried, M.G., et al. ATP effects on insulin-degrading enzyme are mediated primarily through its triphosphate moiety. *J.Biol.Chem.* . 2004,*279*,54216-54220.
[225] Noinaj, N., Bhasin, S.K., Song, E.S., Scoggin, K.E., et al. Identification of the allosteric regulatory site of insulysin. *PLoS One* . 2011,*6*,e20864.
[226] Hulse, R.E., Ralat, L.A., Wei-Jen, T. Structure, function, and regulation of insulin-degrading enzyme. *Vitam.Horm.* . 2009,*80*,635-648.
[227] Camberos, M.C., Perez, A.A., Udrisar, D.P., Wanderley, M.I., Cresto, J.C. ATP inhibits insulin-degrading enzyme activity. *Exp.Biol.Med.(Maywood)* . 2001,*226*,334-341.
[228] Del Carmen Camberos, M., Cresto, J.C. Insulin-degrading enzyme hydrolyzes ATP. *Exp.Biol.Med.(Maywood)* . 2007,*232*,281-292.
[229] Cordes, C.M., Bennett, R.G., Siford, G.L., Hamel, F.G. Redox regulation of insulin degradation by insulin-degrading enzyme. *PLoS One* . 2011,*6*,e18138.
[230] Farris, W., Mansourian, S., Leissring, M.A., Eckman, E.A., et al. Partial loss-of-function mutations in insulin-degrading enzyme that induce diabetes also impair degradation of amyloid beta-protein. *Am.J.Pathol.* . 2004,*164*,1425-1434.
[231] Nordman, S., Ostenson, C.G., Efendic, S., Gu, H.F. Loci of TCF7L2, HHEX and IDE on chromosome 10q and the susceptibility of their genetic polymorphisms to type 2 diabetes. *Exp.Clin.Endocrinol.Diabetes* . 2009,*117*,186-190.
[232] Furukawa, Y., Shimada, T., Furuta, H., Matsuno, S., et al. Polymorphisms in the IDE-KIF11-HHEX gene locus are reproducibly associated with type 2 diabetes in a Japanese population. *J.Clin.Endocrinol.Metab.* . 2008,*93*,310-314.
[233] Ragvin, A., Moro, E., Fredman, D., Navratilova, P., et al. Long-range gene regulation links genomic type 2 diabetes and obesity risk regions to HHEX, SOX4, and IRX3. *Proc.Natl.Acad.Sci.U.S.A.* . 2010,*107*,775-780.
[234] Abdul-Hay, S.O., Kang, D., McBride, M., Li, L., et al. Deletion of insulin-degrading enzyme elicits antipodal, age-dependent effects on glucose and insulin tolerance. *PLoS One* . 2011,*6*,e20818.
[235] Leissring, M.A. The AbetaCs of Abeta-cleaving proteases. *J.Biol.Chem.* . 2008,*283*,29645-29649.
[236] Qiu, W.Q., Folstein, M.F. Insulin, insulin-degrading enzyme and amyloid-beta peptide in Alzheimer's disease: review and hypothesis. *Neurobiol.Aging* . 2006,*27*,190-198.
[237] Farris, W., Mansourian, S., Chang, Y., Lindsley, L., et al. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. *Proc.Natl.Acad.Sci.U.S.A.* . 2003,*100*,4162-4167.
[238] American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* . 2005,*28 Suppl 1*,S37-42.
[239] Prentki, M., Nolan, C.J. Islet beta cell failure in type 2 diabetes. *J.Clin.Invest.* . 2006,*116*,1802-1812.
[240] Defronzo, R.A. Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* . 2009,*58*,773-795.
[241] Unger, R.H., Scherer, P.E. Gluttony, sloth and the metabolic syndrome: a roadmap to lipotoxicity. *Trends Endocrinol.Metab.* . 2010,*21*,345-352.

[242] DeFronzo, R.A., Abdul-Ghani, M.A. Preservation of beta-cell function: the key to diabetes prevention. *J.Clin.Endocrinol.Metab.* . 2011,*96*,2354-2366.
[243] Abdul-Ghani, M.A., Tripathy, D., DeFronzo, R.A. Contributions of beta-cell dysfunction and insulin resistance to the pathogenesis of impaired glucose tolerance and impaired fasting glucose. *Diabetes Care* . 2006,*29*,1130-1139.
[244] Saad, M.F., Knowler, W.C., Pettitt, D.J., Nelson, R.G., et al. Sequential changes in serum insulin concentration during development of non-insulin-dependent diabetes. *Lancet* . 1989,*1*,1356-1359.
[245] Polonsky, K.S., Sturis, J., Bell, G.I. Seminars in Medicine of the Beth Israel Hospital, Boston. Non-insulin-dependent diabetes mellitus - a genetically programmed failure of the beta cell to compensate for insulin resistance. *N.Engl.J.Med.* . 1996,*334*,777-783.
[246] Faber, O.K., Hagen, C., Binder, C., Markussen, J., et al. Kinetics of human connecting peptide in normal and diabetic subjects. *J.Clin.Invest.* . 1978,*62*,197-203.
[247] Kahn, S.E., Hull, R.L., Utzschneider, K.M. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* . 2006,*444*,840-846.
[248] de Koning, E.J., Bonner-Weir, S., Rabelink, T.J. Preservation of beta-cell function by targeting beta-cell mass. *Trends Pharmacol.Sci.* . 2008,*29*,218-227.
[249] Bonner-Weir, S., O'Brien, T.D. Islets in type 2 diabetes: in honor of Dr. Robert C. Turner. *Diabetes* . 2008,*57*,2899-2904.
[250] Salehi, M., Aulinger, B.A., D'Alessio, D.A. Targeting beta-cell mass in type 2 diabetes: promise and limitations of new drugs based on incretins. *Endocr.Rev.* . 2008,*29*,367-379.
[251] Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., et al. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* . 2003,*52*,102-110.
[252] Ritzel, R.A., Butler, A.E., Rizza, R.A., Veldhuis, J.D., Butler, P.C. Relationship between beta-cell mass and fasting blood glucose concentration in humans. *Diabetes Care* . 2006,*29*,717-718.
[253] van Raalte, D.H., Diamant, M. Glucolipotoxicity and beta cells in type 2 diabetes mellitus: target for durable therapy?. *Diabetes Res.Clin.Pract.* . 2011,*93 Suppl 1*,S37-46.
[254] Eizirik, D.L., Cardozo, A.K., Cnop, M. The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr.Rev.* . 2008,*29*,42-61.
[255] Marchetti, P., Bugliani, M., Lupi, R., Marselli, L., et al. The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. *Diabetologia* . 2007,*50*,2486-2494.
[256] Poitout, V., Robertson, R.P. Glucolipotoxicity: fuel excess and beta-cell dysfunction. *Endocr.Rev.* . 2008,*29*,351-366.
[257] Li, N., Frigerio, F., Maechler, P. The sensitivity of pancreatic beta-cells to mitochondrial injuries triggered by lipotoxicity and oxidative stress. *Biochem.Soc.Trans.* . 2008,*36*,930-934.
[258] Li, N., Brun, T., Cnop, M., Cunha, D.A., et al. Transient oxidative stress damages mitochondrial machinery inducing persistent beta-cell dysfunction. *J.Biol.Chem.* . 2009,*284*,23602-23612.
[259] Wajchenberg, B.L. Beta-Cell Failure in Diabetes and Preservation by Clinical Treatment. *Endocr.Rev.* . 2007,*28*,187-218.
[260] Cnop, M., Welsh, N., Jonas, J.C., Jorns, A., et al. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* . 2005,*54 Suppl 2*,S97-107.
[261] Paolisso, G., Tataranni, P.A., Foley, J.E., Bogardus, C., et al. A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. *Diabetologia* . 1995,*38*,1213-1217.
[262] Teusink, B., Voshol, P.J., Dahlmans, V.E., Rensen, P.C., et al. Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake. *Diabetes* . 2003,*52*,614-620.
[263] Poitout, V., Robertson, R.P. Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. *Endocrinology* . 2002,*143*,339-342.
[264] Lupi, R., Dotta, F., Marselli, L., Del Guerra, S., et al. Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* . 2002,*51*,1437-1442.
[265] Oprescu, A.I., Bikopoulos, G., Naassan, A., Allister, E.M., et al. Free fatty acid-induced reduction in glucose-stimulated insulin secretion: evidence for a role of oxidative stress in vitro and in vivo. *Diabetes* . 2007,*56*,2927-2937.

[266] Cnop, M., Igoillo-Esteve, M., Cunha, D.A., Ladriere, L., Eizirik, D.L. An update on lipotoxic endoplasmic reticulum stress in pancreatic beta-cells. *Biochem.Soc.Trans.* . 2008,*36*,909-915.
[267] Cnop, M. Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes. *Biochem.Soc.Trans.* . 2008,*36*,348-352.
[268] Ryffel, G.U. Mutations in the human genes encoding the transcription factors of the hepatocyte nuclear factor (HNF)1 and HNF4 families: functional and pathological consequences. *J.Mol.Endocrinol.* . 2001,*27*,11-29.
[269] Fajans, S.S., Bell, G.I., Polonsky, K.S. Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. *N.Engl.J.Med.* . 2001,*345*,971-980.
[270] Maassen, J.A., Kadowaki, T. Maternally inherited diabetes and deafness: a new diabetes subtype. *Diabetologia* . 1996,*39*,375-382.
[271] Maassen, J.A., 'T Hart, L.M., Van Essen, E., Heine, R.J., et al. Mitochondrial diabetes: molecular mechanisms and clinical presentation. *Diabetes* . 2004,*53 Suppl 1*,S103-9.
[272] Russell, W., Duthie, G. Plant secondary metabolites and gut health: the case for phenolic acids. *Proc.Nutr.Soc.* . 2011,*70*,389-396.
[273] Manach, C., Scalbert, A., Morand, C., Remesy, C., Jimenez, L. Polyphenols: food sources and bioavailability. *Am.J.Clin.Nutr.* . 2004,*79*,727-747.
[274] Bhagwat, S., Holden, J., Haytowitz, D., Gebhardt, S., et al. USDA database for the flavonoid content of selected foods. *Available from: http://www.ars.usda.gov/nutrientdata* .
[275] Aron, P.M., Kennedy, J.A. Flavan-3-ols: nature, occurrence and biological activity. *Mol.Nutr.Food Res.* . 2008,*52*,79-104.
[276] Williams, R.J., Spencer, J.P., Rice-Evans, C. Flavonoids: antioxidants or signalling molecules?. *Free Radic.Biol.Med.* . 2004,*36*,838-849.
[277] Li, W.G., Zhang, X.Y., Wu, Y.J., Tian, X. Anti-inflammatory effect and mechanism of proanthocyanidins from grape seeds. *Acta Pharmacol.Sin.* . 2001,*22*,1117-1120.
[278] Lu, Y., Zhao, W.Z., Chang, Z., Chen, W.X., Li, L. Procyanidins from grape seeds protect against phorbol ester-induced oxidative cellular and genotoxic damage. *Acta Pharmacol.Sin.* . 2004,*25*,1083-1089.
[279] Bagchi, D., Sen, C.K., Ray, S.D., Das, D.K., et al. Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutat.Res.* . 2003,*523-524*,87-97.
[280] Kuhnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev.Nutr.Diet.* . 1976,*24*,117-191.
[281] Aherne, S.A., O'Brien, N.M. Dietary flavonols: chemistry, food content, and metabolism. *Nutrition* . 2002,*18*,75-81.
[282] Pinent, M., Castell, A., Baiges, I., Montagut, G., et al. Bioactivity of Flavonoids on Insulin-Secreting Cells. *Comprehensive Reviews in Food Science and Food Safety* . 2008,*7*,299-308.
[283] Hummer, W., Schreier, P. Analysis of proanthocyanidins. *Mol.Nutr.Food Res.* . 2008,*52*,1381-1398.
[284] Mane, C., Souquet, J.M., Olle, D., Verries, C., et al. Optimization of simultaneous flavanol, phenolic acid, and anthocyanin extraction from grapes using an experimental design: application to the characterization of champagne grape varieties. *J.Agric.Food Chem.* . 2007,*55*,7224-7233.
[285] Wojdylo, A., Oszmianski, J., Laskowski, P. Polyphenolic compounds and antioxidant activity of new and old apple varieties. *J.Agric.Food Chem.* . 2008,*56*,6520-6530.
[286] Tarascou, I., Souquet, J.M., Mazauric, J.P., Carrillo, S., et al. The hidden face of food phenolic composition. *Arch.Biochem.Biophys.* . 2010,*501*,16-22.
[287] Gu, L., Kelm, M.A., Hammerstone, J.F., Beecher, G., et al. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J.Agric.Food Chem.* . 2003,*51*,7513-7521.
[288] Gu, L., Kelm, M.A., Hammerstone, J.F., Beecher, G., et al. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J.Nutr.* . 2004,*134*,613-617.
[289] Erdman, J.W.,Jr, Balentine, D., Arab, L., Beecher, G., et al. Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31-June 1, 2005, Washington, DC. *J.Nutr.* . 2007,*137*,718S-737S.
[290] JL Donovan, C Manach, RM Faulks, PA Kroon, *Absorption and Metabolism of Dietary Plant Secondary Metabolites - Plant Secondary Metabolites*, Blackwell Publishing Ltd 2006; 2007,pp. 303-351.

[291] Day, A.J., Canada, F.J., Diaz, J.C., Kroon, P.A., et al. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* . 2000,*468*,166-170.
[292] Gee, J.M., DuPont, M.S., Day, A.J., Plumb, G.W., et al. Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J.Nutr.* . 2000,*130*,2765-2771.
[293] Kottra, G., Daniel, H. Flavonoid glycosides are not transported by the human Na+/glucose transporter when expressed in Xenopus laevis oocytes, but effectively inhibit electrogenic glucose uptake. *J.Pharmacol.Exp.Ther.* . 2007,*322*,829-835.
[294] Crozier, A., Jaganath, I.B., Clifford, M.N. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat.Prod.Rep.* . 2009,*26*,1001-1043.
[295] Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am.J.Clin.Nutr.* . 2005,*81*,230S-242S.
[296] Rein, D., Lotito, S., Holt, R.R., Keen, C.L., et al. Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. *J.Nutr.* . 2000,*130*,2109S-14S.
[297] Warden, B.A., Smith, L.S., Beecher, G.R., Balentine, D.A., Clevidence, B.A. Catechins are bioavailable in men and women drinking black tea throughout the day. *J.Nutr.* . 2001,*131*,1731-1737.
[298] Tsang, C., Auger, C., Mullen, W., Bornet, A., et al. The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br.J.Nutr.* . 2005,*94*,170-181.
[299] Lotito, S.B., Frei, B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon?. *Free Radic.Biol.Med.* . 2006,*41*,1727-1746.
[300] Suganuma, M., Okabe, S., Oniyama, M., Tada, Y., et al. Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* . 1998,*19*,1771-1776.
[301] Prasain, J.K., Peng, N., Moore, R., Arabshahi, A., et al. Tissue distribution of puerarin and its conjugated metabolites in rats assessed by liquid chromatography-tandem mass spectrometry. *Phytomedicine* . 2009,*16*,65-71.
[302] Zhao, J., Agarwal, R. Tissue distribution of silibinin, the major active constituent of silymarin, in mice and its association with enhancement of phase II enzymes: implications in cancer chemoprevention. *Carcinogenesis* . 1999,*20*,2101-2108.
[303] Zhang, L., Angst, E., Park, J.L., Moro, A., et al. Quercetin aglycone is bioavailable in murine pancreas and pancreatic xenografts. *J.Agric.Food Chem.* . 2010,*58*,7252-7257.
[304] Zern, T.L., Fernandez, M.L. Cardioprotective effects of dietary polyphenols. *J.Nutr.* . 2005,*135*,2291-2294.
[305] Rivera, L., Moron, R., Sanchez, M., Zarzuelo, A., Galisteo, M. Quercetin ameliorates metabolic syndrome and improves the inflammatory status in obese Zucker rats. *Obesity (Silver Spring)* . 2008,*16*,2081-2087.
[306] Kim, H.P., Park, H., Son, K.H., Chang, H.W., Kang, S.S. Biochemical pharmacology of biflavonoids: implications for anti-inflammatory action. *Arch.Pharm.Res.* . 2008,*31*,265-273.
[307] Amin, A., Buratovich, M. The anti-cancer charm of flavonoids: a cup-of-tea will do!. *Recent.Pat.Anticancer Drug Discov.* . 2007,*2*,109-117.
[308] Cao, H., Hininger-Favier, I., Kelly, M.A., Benaraba, R., et al. Green tea polyphenol extract regulates the expression of genes involved in glucose uptake and insulin signaling in rats fed a high fructose diet. *J.Agric.Food Chem.* . 2007,*55*,6372-6378.
[309] Bose, M., Lambert, J.D., Ju, J., Reuhl, K.R., et al. The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. *J.Nutr.* . 2008,*138*,1677-1683.
[310] Park, S.Y., Choi, Y.H., Lee, W. Dangnyohwan improves glucose utilization and reduces insulin resistance by increasing the adipocyte-specific GLUT4 expression in Otsuka Long-Evans Tokushima Fatty rats. *J.Ethnopharmacol.* . 2008,*115*,473-482.
[311] Potenza, M.A., Marasciulo, F.L., Tarquinio, M., Tiravanti, E., et al. EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. *Am.J.Physiol.Endocrinol.Metab.* . 2007,*292*,E1378-87.
[312] Blade, C., Arola, L., Salvado, M.J. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Mol.Nutr.Food Res.* . 2010,*54*,37-59.

[313] Corder, R., Mullen, W., Khan, N.Q., Marks, S.C., et al. Oenology: red wine procyanidins and vascular health. *Nature* . 2006,*444*,566.
[314] Llopiz, N., Puiggros, F., Cespedes, E., Arola, L., et al. Antigenotoxic effect of grape seed procyanidin extract in Fao cells submitted to oxidative stress. *J.Agric.Food Chem.* . 2004,*52*,1083-1087.
[315] Karthikeyan, K., Bai, B.R., Devaraj, S.N. Cardioprotective effect of grape seed proanthocyanidins on isoproterenol-induced myocardial injury in rats. *Int.J.Cardiol.* . 2007,*115*,326-333.
[316] Faria, A., Calhau, C., de Freitas, V., Mateus, N. Procyanidins as antioxidants and tumor cell growth modulators. *J.Agric.Food Chem.* . 2006,*54*,2392-2397.
[317] Mantena, S.K., Baliga, M.S., Katiyar, S.K. Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. *Carcinogenesis* . 2006,*27*,1682-1691.
[318] Pinent, M., Cedó, L., Montagut, G., Blay, M., Ardévol, A. Procyanidins improve some disrupted glucose homoeostatic situations: an analysis of doses and treatments according to different animal models. *Crit.Rev.Food Sci.Nutr.* . 2011.
[319] Ichinose, K., Kawasaki, E., Eguchi, K. Recent advancement of understanding pathogenesis of type 1 diabetes and potential relevance to diabetic nephropathy. *Am.J.Nephrol.* . 2007,*27*,554-564.
[320] Kashyap, S.R., Defronzo, R.A. The insulin resistance syndrome: physiological considerations. *Diab Vasc.Dis.Res.* . 2007,*4*,13-19.
[321] Al-Awwadi, N., Azay, J., Poucheret, P., Cassanas, G., et al. Antidiabetic activity of red wine polyphenolic extract, ethanol, or both in streptozotocin-treated rats. *J.Agric.Food Chem.* . 2004,*52*,1008-1016.
[322] Pinent, M., Blay, M., Blade, M.C., Salvado, M.J., et al. Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. *Endocrinology* . 2004,*145*,4985-4990.
[323] Maritim, A., Dene, B.A., Sanders, R.A., Watkins, J.B.,3rd. Effects of pycnogenol treatment on oxidative stress in streptozotocin-induced diabetic rats. *J.Biochem.Mol.Toxicol.* . 2003,*17*,193-199.
[324] Li, B.Y., Cheng, M., Gao, H.Q., Ma, Y.B., et al. Back-regulation of six oxidative stress proteins with grape seed proanthocyanidin extracts in rat diabetic nephropathy. *J.Cell.Biochem.* . 2008,*104*,668-679.
[325] Lee, Y.A., Kim, Y.J., Cho, E.J., Yokozawa, T. Ameliorative effects of proanthocyanidin on oxidative stress and inflammation in streptozotocin-induced diabetic rats. *J.Agric.Food Chem.* . 2007,*55*,9395-9400.
[326] Coskun, O., Kanter, M., Korkmaz, A., Oter, S. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. *Pharmacol.Res.* . 2005,*51*,117-123.
[327] Kim, M.J., Ryu, G.R., Chung, J.S., Sim, S.S., et al. Protective effects of epicatechin against the toxic effects of streptozotocin on rat pancreatic islets: in vivo and in vitro. *Pancreas* . 2003,*26*,292-299.
[328] Song, E.K., Hur, H., Han, M.K. Epigallocatechin gallate prevents autoimmune diabetes induced by multiple low doses of streptozotocin in mice. *Arch.Pharm.Res.* . 2003,*26*,559-563.
[329] Lenzen, S. The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia* . 2008,*51*,216-226.
[330] Yokozawa, T., Kim, H.J., Cho, E.J. Gravinol ameliorates high-fructose-induced metabolic syndrome through regulation of lipid metabolism and proinflammatory state in rats. *J.Agric.Food Chem.* . 2008,*56*,5026-5032.
[331] Tsai, H.Y., Wu, L.Y., Hwang, L.S. Effect of a proanthocyanidin-rich extract from longan flower on markers of metabolic syndrome in fructose-fed rats. *J.Agric.Food Chem.* . 2008,*56*,11018-11024.
[332] Montagut, G., Blade, C., Blay, M., Fernandez-Larrea, J., et al. Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. *J.Nutr.Biochem.* . 2010,*21*,961-967.
[333] Agouni, A., Lagrue-Lak-Hal, A.H., Mostefai, H.A., Tesse, A., et al. Red wine polyphenols prevent metabolic and cardiovascular alterations associated with obesity in Zucker fatty rats (Fa/Fa). *PLoS One* . 2009,*4*,e5557.
[334] Montagut Pino, G. Procyanidin effects on an impaired glucose metabolism: a further insight into procyanidin signalling in adipose cells.
[335] Ohno, T., Kato, N., Ishii, C., Shimizu, M., et al. Genistein augments cyclic adenosine 3'5'-monophosphate(cAMP) accumulation and insulin release in MIN6 cells. *Endocr.Res.* . 1993,*19*,273-285.

[336] Jonas, J.C., Plant, T.D., Gilon, P., Detimary, P., et al. Multiple effects and stimulation of insulin secretion by the tyrosine kinase inhibitor genistein in normal mouse islets. *Br.J.Pharmacol.* . 1995*,114*,872-880.
[337] Sorenson, R.L., Brelje, T.C., Roth, C. Effect of tyrosine kinase inhibitors on islets of Langerhans: evidence for tyrosine kinases in the regulation of insulin secretion. *Endocrinology* . 1994*,134*,1975-1978.
[338] Persaud, S.J., Harris, T.E., Burns, C.J., Jones, P.M. Tyrosine kinases play a permissive role in glucose-induced insulin secretion from adult rat islets. *J.Mol.Endocrinol.* . 1999*,22*,19-28.
[339] Liu, D., Zhen, W., Yang, Z., Carter, J.D., et al. Genistein acutely stimulates insulin secretion in pancreatic beta-cells through a cAMP-dependent protein kinase pathway. *Diabetes* . 2006*,55*,1043-1050.
[340] Fu, Z., Liu, D. Long-term exposure to genistein improves insulin secretory function of pancreatic beta-cells. *Eur.J.Pharmacol.* . 2009*,616*,321-327.
[341] Youl, E., Bardy, G., Magous, R., Cros, G., et al. Quercetin potentiates insulin secretion and protects INS-1 pancreatic beta-cells against oxidative damage via the ERK1/2 pathway. *Br.J.Pharmacol.* . 2010*,161*,799-814.
[342] Hii, C.S., Howell, S.L. Effects of flavonoids on insulin secretion and 45Ca2+ handling in rat islets of Langerhans. *J.Endocrinol.* . 1985*,107*,1-8.
[343] Ogunbayo, O.A., Harris, R.M., Waring, R.H., Kirk, C.J., Michelangeli, F. Inhibition of the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase by flavonoids: a quantitative structure-activity relationship study. *IUBMB Life* . 2008*,60*,853-858.
[344] Jayaprakasam, B., Vareed, S.K., Olson, L.K., Nair, M.G. Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. *J.Agric.Food Chem.* . 2005*,53*,28-31.
[345] Jin, J.Y., Park, S.H., Bae, J.H., Cho, H.C., et al. Uncoupling by (--)-epigallocatechin-3-gallate of ATP-sensitive potassium channels from phosphatidylinositol polyphosphates and ATP. *Pharmacol.Res.* . 2007*,56*,237-247.
[346] Baek, W.K., Jang, B.C., Lim, J.H., Kwon, T.K., et al. Inhibitory modulation of ATP-sensitive potassium channels by gallate-ester moiety of (-)-epigallocatechin-3-gallate. *Biochem.Pharmacol.* . 2005*,70*,1560-1567.
[347] Cai, E.P., Lin, J.K. Epigallocatechin gallate (EGCG) and rutin suppress the glucotoxicity through activating IRS2 and AMPK signaling in rat pancreatic beta cells. *J.Agric.Food Chem.* . 2009*,57*,9817-9827.
[348] Noriega-Lopez, L., Tovar, A.R., Gonzalez-Granillo, M., Hernandez-Pando, R., et al. Pancreatic insulin secretion in rats fed a soy protein high fat diet depends on the interaction between the amino acid pattern and isoflavones. *J.Biol.Chem.* . 2007*,282*,20657-20666.
[349] Cederroth, C.R., Vinciguerra, M., Gjinovci, A., Kuhne, F., et al. Dietary phytoestrogens activate AMP-activated protein kinase with improvement in lipid and glucose metabolism. *Diabetes* . 2008*,57*,1176-1185.
[350] Choi, M.S., Jung, U.J., Yeo, J., Kim, M.J., Lee, M.K. Genistein and daidzein prevent diabetes onset by elevating insulin level and altering hepatic gluconeogenic and lipogenic enzyme activities in non-obese diabetic (NOD) mice. *Diabetes Metab.Res.Rev.* . 2008*,24*,74-81.
[351] Unno, K., Yamamoto, H., Maeda, K., Takabayashi, F., et al. Protection of brain and pancreas from high-fat diet: effects of catechin and caffeine. *Physiol.Behav.* . 2009*,96*,262-269.

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II. HYPOTHESIS AND OBJECTIVES

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Several studies have reported that **procyanidins** (one of the most abundant group of flavonoids) can protect against coronary heart diseases and atherosclerosis as well as act on several metabolic processes that are associated with the development of these disorders. Evidence supports procyanidin bioactivity in lipid metabolism and as antioxidant agents, but the data are less clear regarding the effectiveness of procyanidins in other physiological processes.

Previous studies published by our research group (Nutrigenomics group) have shown that procyanidins have **positive effects on glucose metabolism** in situations of slightly disrupted glucose homeostasis. Part of this effect was explained by the activity of procyanidins on adipose cells. However, this work was based on a rat cafeteria-diet model treated with GSPE for 30 days, exhibiting **decreased fasting plasma insulin** levels concomitantly with **unchanged glucose**. The same effect was observed in non-fasting insulin levels. These results seem to indicate that procyanidins **can modulate insulin secretion and/or insulin synthesis** through their action on β-cells.

The importance of the endocrine pancreas in whole body nutrient equilibrium is highlighted by the emergence of several pathologies of nutrient metabolism, such as type 1 and 2 diabetes mellitus, that involve pancreatic cell deregulation. However, **little data are available that address whether procyanidins have central effects on the endocrine pancreas**, a key organ involved in metabolic control. This hypothesis could be supported by the fact that the **pancreas is located immediately after the location of enteric absorption**.

Therefore, taking into account the data presented above, our hypothesis was as follows:

Procyanidins can modulate β-cell functionality

The aim of this thesis was to assess whether procyanidins affect insulinemia through their action on pancreatic insulin synthesis and secretion.

The main objectives proposed to achieve this aim were as follows:

1. To develop a **tool to evaluate the bioactivity of plant extracts** on pancreatic β-cells.

2. To evaluate whether **GSPE modulates β-cell functionality**.

3. To assess whether the effects of **GSPE** on β-cells **prevent or improve β-cell dysfunction**.

4. To identify the **mechanisms used by GSPE** to modulate β-cell functionality.

The work presented in this thesis was performed in the Nutrigenomics group at the Universitat Rovira i Virgili, together with a 3 month stay at the Faculty of Medicine at the University of Geneva. Funding came from three different institutions. The first six months of funding came from Universitat Rovira i Virgili. After that, I received the FI fellowship from the Generalitat de Catalunya. Finally, I won a FPU fellowship from the Ministerio de Educación of the Spanish government that covered the last 3 years of this thesis.

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III. RESULTS

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The results of this thesis are presented as accepted or submitted manuscripts for peer-reviewed journals. A brief description of the relationship between the objectives and chapters follows.

OBJECTIVE 1: *To develop a tool to evaluate the bioactivity of plant extracts on pancreatic β-cells*.

To overcome the limitation of working with natural extracts, we designed a coculture system to reproduce *in vivo* conditions, where compounds from natural extracts cross the epithelium barrier before reaching the pancreas. We seeded Caco-2 cells onto culture inserts. After 21 days, these cells were cocultured with pancreatic β-cells, INS-1E, on the base of the well. To prove the reliability of this method, we assayed essential functions of each cell type both alone or under co-culture conditions. The results from this study are described in the **Chapter 1** and were published in **Planta Medica (Planta Med 2010; 76: 1576-1581)**.

OBJECTIVE 2: *To evaluate whether GSPE modulates β-cell functionality*.

To determine the effects of GSPE on β-cell functionality, we assayed various doses of GSPE in different animal models. We used an acute high dose and chronic moderate treatments in healthy animals. To better understand how GSPE acts on β-cells, we also used the INS-1E β-cell line. The results of these experiments are reported in **Chapter 2** and have been accepted in the **Journal of Nutritional Biochemistry (JNB 2012; *in press*)**.

OBJECTIVE 3: *To assess whether the effects of GSPE on β-cells prevent or improve β-cell dysfunction.*

To prove whether the GSPE effects could be beneficial to prevent or improve disrupted β-cell functionality, we used animal models that presented β-cell dysfunction caused by diet (animals fed with a cafeteria diet) or genetics (Zucker fatty rats). As a complementary approach, we used an *in vitro* model of β-cell dysfunction induced by treatment with the fatty acid oleate. The results of these experiments are described in **Chapter 4** (submitted to the **Journal of Nutritional Biochemistry**) and **Chapter 5** (submitted to the **Journal of Proteome Research**).

OBJECTIVE 4: *To identify the mechanisms used by GSPE to modulate β-cell functionality.*

All of the presented studies were designed to determine the mechanisms that the procyanidin extract use to modulate β-cell functionality. Moreover, taking into account the role of microRNA (miRNA) in the regulation of metabolic processes in diabetes and that some published data reported the modulation of miRNA expression by flavonoids in other tissues than pancreas, we analysed whether GSPE could modify β-cell functionality through its effect on the miRNA expression pattern. The results from this study are reported in **Chapter 3** and have been submitted to the **Molecular Nutrition & Food Research** as a Food & Function manuscript.

Most of this work has been accomplished together with my colleague Lidia Cedó. Only the results related to the GSPE effects to insulin secretion and synthesis belong to this thesis. All the other data belong to the future theses of my colleagues.

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Development of a Coculture System to Evaluate the Bioactivity of Plant Extracts on Pancreatic β-cells

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CHAPTER 1

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Development of a Coculture System to Evaluate the Bioactivity of Plant Extracts on Pancreatic β-Cells

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Key words
- coculture
- pancreatic β-cells
- Caco-2 cells
- insulin secretion
- plant extracts
- bioavailability

Abstract

Natural plant extracts are candidates for the development of new functional foods. Most of them are usually complex mixtures of molecules of uncertain bioavailability that are often partially metabolized before they finally reach the target cells *in vivo*. *In vitro* studies of the bioactivity of these extracts suggest that their direct application to some cell cultures might be a long way from becoming a reality. To overcome this limitation, we seeded Caco-2 cells onto culture inserts and after 21 days, cocultured these with INS-1E on the base of the well. After 24 hours of coculture, TEER (transepithelium electrical resistance) measurements indicated no changes in the permeability of the Caco-2 barrier. We also found no changes in either the ability of Caco-2 cells to metabolize the flavan-3-ol component of a grape-seed procyanidin-rich extract, or in the flavanols' ability to pass through the barrier. However, the expression of the Caco-2 SGLT-1 gene increased due to the coculture. GSIS (glucose stimulated insulin secretion) was maintained in the INS-1E cells with higher levels of insulin secretion despite the fact that the insulin gene expression was unmodified by the cocultivation. Furthermore, we found that in some of the assays requiring several medium changes there was a tendency to lose β-cells. Neutral red assay showed that seeded cells should only be cocultured for a short time to obtain a higher consistency. In conclusion, four hours coculture with Caco-2 cells and INS-1E is a suitable method for checking the bioactivity of natural plant extracts of unknown bioavailability on β-cells.

Introduction

Designing functional food requires a lot of time and many expensive studies to demonstrate food's bioactivity. The first steps usually involve screening a large number of molecules before moving on to human intervention studies to provide the human data required to demonstrate that the food is functional [1]. In an attempt to restrict animal experimentation as much as possible, the European Union recommends that a previous screening of a food's bioactivity on cell lines should be carried out when testing the biological effects of compounds [2]. Extracts from natural plant sources are candidates for formulating and developing new functional foods. However, natural extracts are complex mixtures of molecules which have to be uptaken and metabolized if they are to finally reach the cells *in vivo* [3–5]. Therefore, directly applying the extracts to the cell cultures might be a long way from becoming a reality in *in vivo* situations [6,7]. To overcome this problem many studies have analyzed the activity of pure molecules [7–13]. Yet, obtaining pure components might be very difficult given the technical limitations, the huge diversity of the structures contained in the extracts, and the very small quantity in which they are found. Moreover, only a limited amount of original molecules present in the extracts have been detected in plasma after their intake, and a huge proportion of the metabolites detected and quantified in plasma corresponded to modified forms of the original ingested molecules [3,14,15]. Working with pure molecules directly on the cell line in order to analyze their bioactivity therefore does not completely overcome the problem of whether a potential beneficial compound will be absorbed. Finally, and very importantly, the *in vivo* effects of natural extracts may be due not only to the pure molecules but also to synergies between them [16]. If the molecules present in extracts are to be absorbed they have to cross the intestinal epithelium barrier. Human intestinal epithe-

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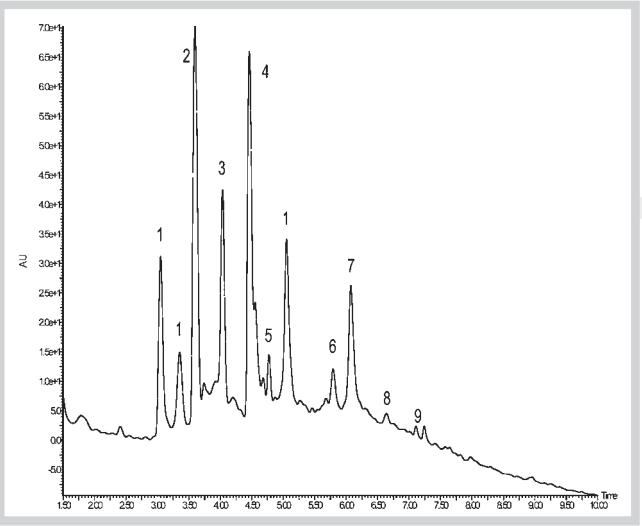

Fig. 1 Chromatographic analysis of GSPE. HPLC chromatogram (at 280 nm) of procyanidin extract from grape seed. (**1**) Dimer, (**2**) catechin, (**3**) epicatechin, (**4**) epigallocatechin, (**5**) trimer, (**6**) epigallocatechin, (**7**) epicatechin gallate, (**8**) tetramer, (**9**) pentamer.

lial Caco-2 cells grown on permeable inserts have been shown to possess many of the morphological and functional characteristics of intestinal enterocytes and to enable intestinal permeability to be evaluated [17]. Furthermore, they have already been used as an *in vitro* model in studies of the absorption of natural bioactive compounds [13, 17–20]. They have also been assayed in a double-layered system that reproduces absorption in the intestine. Co-culturing Caco-2 with hepatocytes has been proved a feasible system for analyzing drug toxicity in hepatocytes, even when the chemicals remain unidentified [21]. Also it has proved a suitable model for analyzing the bioactivity of a grape-seed derived procyanidin extract on HepG2 cells (submitted results). The bioactivity of plant extracts on β-pancreatic cells has received little attention [5]. In fact, there is much controversy, at least regarding the flavonoid group, about the effects of plant extracts on β-cell. In this study, we show the advantages and limitations of a coculture system based on physically separated human intestinal epithelial Caco-2 cells and β-cell lines when used to evaluate how the bioactivity of plant extracts of unknown bioavailability affects β-cells.

Materials and Methods

Chemical

Cell culture reagents were obtained from BioWhittaker. A grape seed procyanidin extract (GSPE) was used as source of procyanidins (Dérivés Résiniques et Terpéniques; batch no. 031751). The extract contained monomers (catechin and epicatechin), oligomers (dimer to pentamer), epigallocatechin galate, epigallocatechin and epicatechin gallate (**Fig. 1**). The procyanidins content of the extract was determined by liquid chromatography tandem mass spectrometry according with the method described by Serra et al. [14]. Results of the quantification are showed in **Table 1**.

Table 1 Analysis of procyanidins of grape seed extract.

Procyanidin	mg/g extract
Catechin	31.16
Epicatechin	30.77
Epigallocatechin gallate	58.29
Epigallocatechin	0.92
Epicatechin gallate	1.40
Dimer	123.32
Trimer	17.39
Tetramer	0.97
Pentamer	0.29

Cell culture

Caco-2 cells were obtained from ATCC (American Tissue Culture Collection). MIN6 cells (mouse derived pancreatic β-cells) were kindly provided by Dr. Anders Tengholm, Uppsala University [22]. Rat insulinoma INS-1E cells were kindly provided by Prof. Pierre Maechler, University of Geneva [23]. Caco-2 cells were kept in Dulbecco's modified minimum essential medium (DMEM) supplemented with 20% foetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin. MIN6 were cultured in DMEM (4.5 g/L glucose) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol, 15% FBS (heat inactivated at 56° C for 30 min), and 2 mM L-glutamine. INS-1E cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Single- and double-layered culture

Caco-2 were seeded onto a culture insert (6- or 12-well Millicell Hanging Cell Culture Inserts; Millipore) at a cell density of 5.3×10^4 cells/cm^2. The cells were then used for the experiments after 21 days once the confluent monolayer had formed, and the cells expressed a constant transepithelium electrical resistant (TEER) measured with the Millicell-ERS system (Millipore). The volume of the culture medium was 0.4 mL on the apical side and 1 mL on the basolateral side. For the coculture with MIN6, β-cells were seeded into the 6-well plates at a cell density of 2.5×10^5 cells/cm^2. The volume of the culture medium was 2.5 mL. For the coculture with INS-1E, β-cells were seeded onto the 12-well plates at a cell density of 1×10^5 cells/cm^2. The volume of the culture medium was 1 mL. In both cases, once the cells reached confluence, we prepared the following different culture systems: culture of Caco-2 cells, culture of MIN6 cells, culture of INS-1E cells, double-layered coculture of Caco-2 cells and MIN6 cells, and double-layered coculture of Caco-2 cells and INS-1E cells. Caco-2 cells and MIN6 cells were cocultured for 2 and 5 days. Caco-2 cells in the inserts and INS-1E cells on the base of the well were cocultured for 4 and 24 hours (**Fig. 2**).

Viability and glucose measures

Following 4 and 24 h of coculture, INS-1E cells were incubated with neutral red dye to assess toxicity as previously described [24]. Essentially, 1 mL of freshly prepared neutral red solution (50 µg/mL) was prewarmed to 37 °C and added to each well (12-well plate). The cells were then incubated for 2 h at 37 °C. After the cells had been washed twice with PBS, 750 µL of glacial acetic acid solution (1% (v/v) glacial acetic acid, 50% (v/v) absolute ethanol in MQ water) were added to each well and plates were shaken

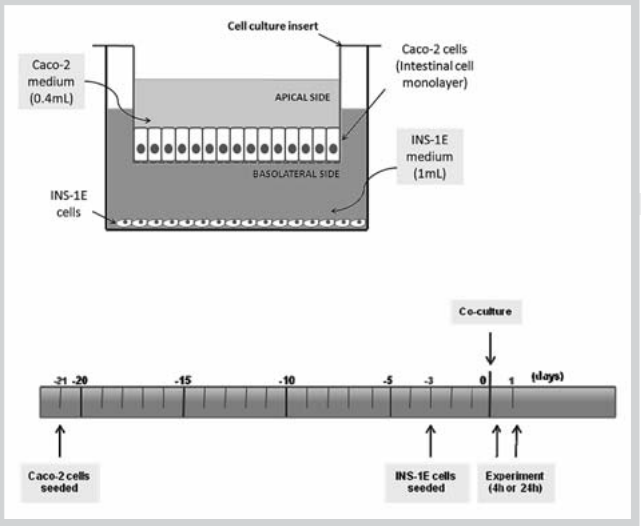

Fig. 2 Coculture system constructed with Caco-2 cells and INS-1E cells. Caco-2 were seeded alone at a cell density of 5.3×10^4 cells/cm^2. The volume of culture medium was 0.4 mL on the apical side and 1 mL on the basolateral side. INS-1E cells were seeded onto the 12-well plates at a cell density of 1×10^5 cells/cm^2. The volume of the culture medium was 1 mL. After the cells reached confluence, cells were cocultured for 4 and 24 hours.

for 20 min to release all of the dye from the cells. The absorbance was read at 540 nm. After 24 h of double coculturing Caco-2 cells and INS-1E cells, the basolateral medium was removed and the glucose concentration was analyzed using an enzymatic colorimetric kit (QCA) following the manufacturer's instructions

Insulin secretion

The secretory responses to glucose were tested in INS-1E cells, after the coculture treatment, as previously described [23]. The cells (Caco-2 and INS-1E) were maintained for 2 h in glucose-free culture medium. The cells were then washed twice and preincubated for 30 min at 37 °C in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 135 mM NaCl, 3.6 mM KCL, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 10 mM HEPES, pH 7.4. BSA (0.1%) was added as an insulin carrier. Next, the cells were washed once with glucose-free KRBH and then incubated for 30 min in KRBH 2.5 mM (basal) or 20 mM glucose (stimulated). Glucose stimulated insulin secretion (GSIS) was measured by Insulin ELISA (Mercodia).

Quantitative RT-PCR

The total RNA was extracted using the TRIzol reactive following the manufacturer's instructions. cDNA was generated using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Quantitative PCR amplification and detection were done using TaqMan assay-on-demand probes (Applied Biosystems): Hs00165793_m1 for SGLT1 (SLC5A1) and Rn01774648_g1 for insulin. The results were referenced to cyclophiline Hs99999 904_m1 in the Caco-2 cells and β-actin Rn00667869_m1 in the INS-1E cells.

Analyses of procyanidins and their metabolites in the culture cell mediums

The culture cell mediums were pretreated with off-line microelution SPE plates (2 mg of OASIS HLB; Waters) before chromatographic analysis. The plates were conditioned sequentially with 250 µL of methanol and 0.2% acetic acid. After that, 350 µL of cellular medium with 200 µL of phosphoric acid 4% containing the internal standard catechol (50 µL at a concentration of 10 mg/L) were loaded into the plate. The internal standard (IS) was prepared in 4% phosphoric acid. Then, 200 µL of MilliQ water and 0.2% acetic acid were passed through the plate in order to eliminate any possible interference in the sample. Finally, 2 × 50 µL of acetone/Milli-Q/acetic acid (70/29.5/0.5, v/v/v) solution was used to elute the procyanidins. The eluted solution was directly injected into the UPLC-MS/MS and the sample volume was 2.5 µL. The chromatographic analyses of the procyanidins and their metabolites were done using UPLC along with tandem MS in accordance with a previous study [14].

Calculations and statistical analysis

Results are expressed as the mean ± SEM. Effects were assessed by ANOVA or Student's t-test. All calculations were made with SPSS software.

Results and Discussion

Working with cell lines is a useful way to easily prove and understand the bioactivity of natural extracts; however, the complex composition of the extract and its real bioavailability can be a very big limitation to this method. Kroon et al. strongly recommended using only physiological relevant flavonoids and their conjugates to study the biological responses of dietary polyphenols in *in vitro* models [6]. In the present paper, we describe an approach that fully meets this objective when used to analyze the bioactivity of plant extracts with unknown bioavailability on pancreatic β-cells. Our approach is based on the coculture of intestinal Caco-2 cells and pancreatic β-cells. Several studies have shown that coculturing other cell types is an appropriate method for: adipocyte-macrophage cross-talk [25], improving gastrointestinal permeability modelling [26], or coculturing Caco-2 and HepG2 [21]. We have also previously characterized a coculture of Caco-2 and HepG2 cells to study the bioactivity of plant extracts (submitted results). In our first attempt, we cocultured MIN6 cells on the base of the wells and grew Caco-2 cells on the inserts suspended on the same well. The TEER measurements in **Fig. 3a** show that coculture does not affect the permeability barrier established by Caco-2 cells. On the periphery of the base of the well, the cells maintained a similar morphology to cells growing without any other well coculture (**Fig. 3b**, upper panel). In contrast, **Fig. 3b** shows that after 2 days of coculture, MIN6 cells that were situated directly below the insert of Caco-2 changed their morphology towards a phenotype more typical of an undifferentiated cell, that is, it was individualized and with a rounded appearance (**Fig. 3b**, lower panel). The challenge for β-cell lines is to properly reproduce glucose stimulated insulin secretion (GSIS) [27]. We had difficulties in our lab in reproducing GSIS in MIN6 cells (results not shown) and this, together with the change in morphology due to coculture, meant that we decided to use another β-cell line (the INS-1E cell line) to develop our approach which shows very good GSIS [23].

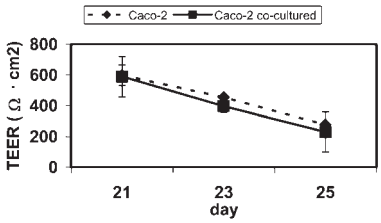

Fig. 3a Coculture Caco-2/MIN6. Caco-2 cells grown on inserts for 20 days were cocultured with MIN6 cells (squares) or grown alone (rhombs) during 5 more days. Each day, TEER measurements were taken from 2 wells of each treatment during 3 different passages.

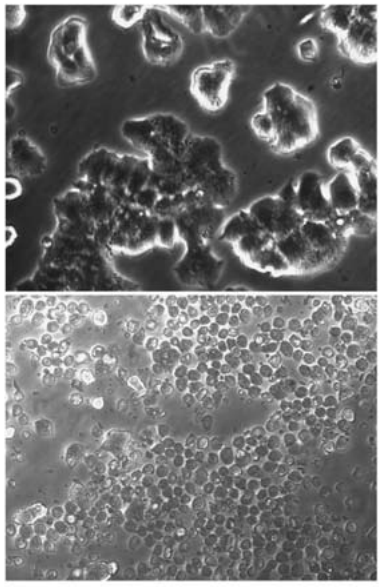

Fig. 3b The same MIN6 cells that were cocultured in ▶ **Fig. 3a** were used to show phenotype changes. Periphery of the cocultured MIN6 (upper panel). Center of the cocultured MIN6 (lower panel). These pictures show the cells in each situation after 2 days of coculture.

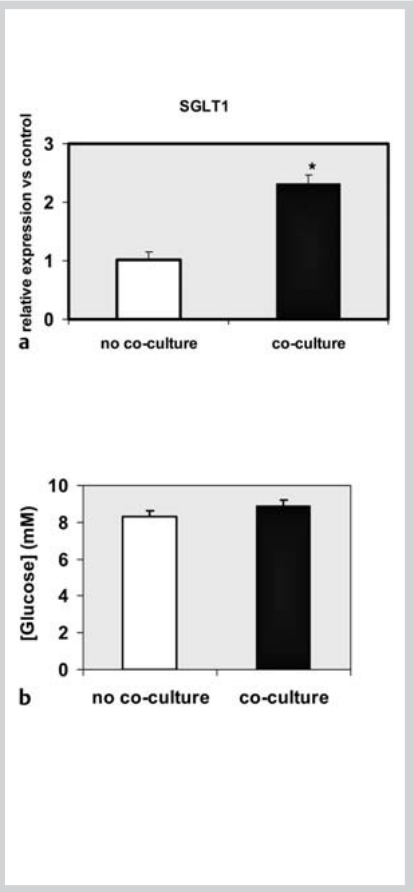

Fig. 4 Effect of coculture on glucose uptake ability of Caco-2 cells. **a** SGLT-1 expression of Caco-2 cells (n = 3): Cocultured Caco-2 cells (according to ▶ **Fig. 2**) and parallel non-cocultured Caco-2 cells, 24 hours after beginning the coculture, were rinsed with PBS. RNA was extracted using TRIZOL solution on 2 wells of each treatment during 3 different passages. cDNA was obtained from this RNA and SGLT-1 analyzed and normalized using cyclophiline by RT-PCR. **b** Glucose amount in the basolateral medium (n = 6): basolateral media were collected from the same cells for glucose measurement according to the procedure detailed on Materials and Methods. * Indicates $p < 0.05$ versus its respective non-cocultured Caco-2.

After 24 hours of coculturing the Caco-2 and INS-1E cells, we found that they had slight differences compared to when they grew alone. The TEER measurements showed no change in the Caco-2 barrier permeability ($197 \pm 4\ \Omega \cdot cm^2$) and that it was similar to that found by some other authors [28]. TEER measurements were used to assess the integrity of membrane barriers, and the results showed that the barrier was maintained, and that there was no reduction in Caco-2 cell viability due to the coculture. No change in the TEER value suggested no changes on leakiness or tightness [29] in the paracellular pathway which is used by some compounds to cross intestinal barrier. But for some other bioactive compounds, the transepithelial pathway is the preferred way of crossing the intestinal barrier [30]. For example, certain flavonoid glycosides used some habitual transporters of the enterocyte, i.e., SGLT1 [31], whereas other structures such as epigallocatechin (EGC) did not [9]. This is important since we found slight differences between cells when they were cocultured and when they grew alone. Specifically, our coculture system increased SGLT-1 gene expression (▶ **Fig. 4a**). This effect contrasts with the effect that we previously observed in Caco-2 cocultured with HepG2 cells, where we found that the coculture downregulated SGLT-1 expression (submitted results). The upregulation of SGLT1 suggested that Caco-2 cells had a possible effect on the amount of glucose in the basolateral side which could affect β-cell function. We therefore measured it and found no significant changes in the concentration of glucose that reached the β-cells (see ▶ **Fig. 4b**).

The intestinal barrier is also active at modifying the natural components of natural extracts. Galijatovie et al. [32,33] described the presence of enzymes responsible for glucuronidation and sulfatation in Caco-2 cells. Likewise, we found that Caco-2 cells were able to sulphate and glucuronidate the catechin components of a grape-seed procyanidin-rich extract (GSPE). Our results also show that coculturing caused no changes in the ability of Caco-2 cells to metabolize the flavan-3-ols components of GSPE or in the flavanols' ability to pass through the barrier (▶ **Fig. 5**). Therefore, applying the natural extracts to the coculture, instead of incubating the extracts directly over the β-cells, is better because the media in the basolateral side of the Caco-2 cells more closely resemble the plasma flavonoid composition after the ingestion of natural extracts [34]. Moreover, the number of oligomeric forms present in both situations is another very strong difference because the coculture system mimicked both the physiological concentrations reached by these oligomeric forms and the size of the compounds that truly reach internal body cell types [14]. Then Caco-2 cells act as a filter for natural extracts and also modify some of their structures, thus providing a basolateral media for β-cells that resembles *in vivo* natural plasma after oral intake of this extract.

The proper functionality of INS-1E cells should show a reproducible GSIS. ▶ **Fig. 6** shows that GSIS was maintained, although with higher levels of basal insulin secretion. The insulin gene expression was, however, unmodified by cocultivation (no coculture 1.06 ± 0.32, coculture 1.22 ± 0.35). As previously stated, we

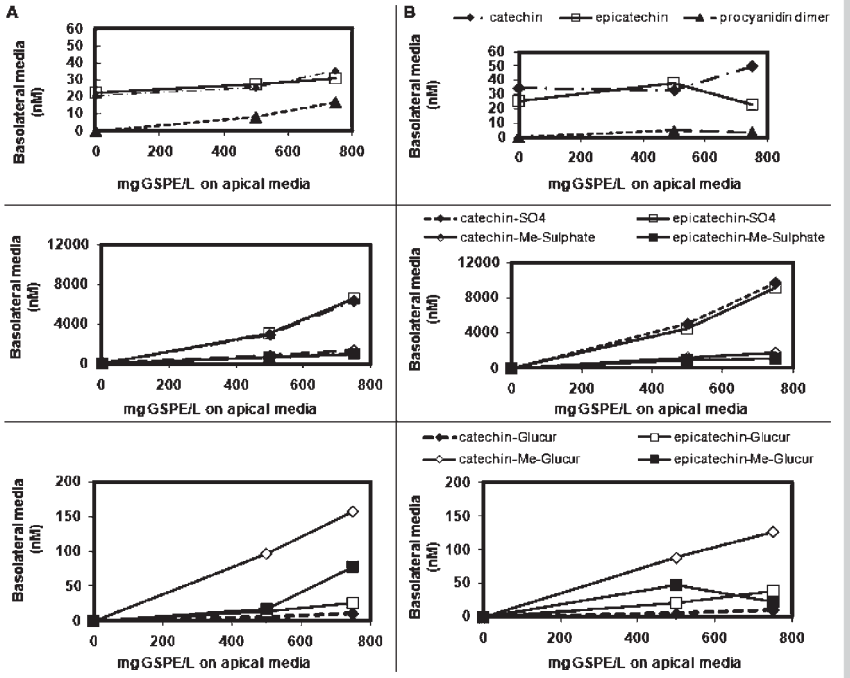

Fig. 5 Effect of coculture on the number of components of grape-seed procyanidin extract (GSPE) in basolateral media of Caco-2 cells. Cocultured Caco-2 cells (left pictures indicated as **A**) and non-cocultured Caco-2 cells (right pictures indicated as **B**) were seeded as indicated in **Fig. 2**. After twenty-four hours of coculture, Caco-2 cells were treated on apical media with two doses (500 mg/L and 750 mg/L) of GSPE. After 4 hours of treatment, basolateral media were collected and treated to analyze the presence of flavan-3-ols and derivatives, as stated in the Material and Methods. Pictures show the nM concentration of each compound in the control situation and in each apical GSPE concentration. First row of pictures refers to non-modified compounds, second row includes sulphate conjugates, and third row includes glucuronide conjugates.

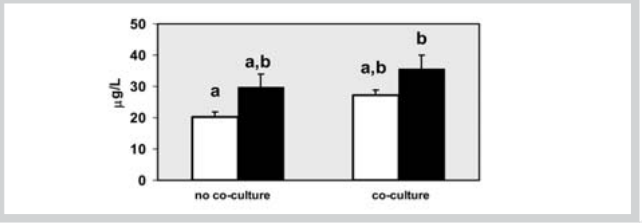

Fig. 6 INS-1E cells' glucose response. INS-1 cells were cultured or cocultured 24 hours according to **Fig. 2**, after which the coculture cells were kept for 2 h in glucose-free culture medium. The cells were then washed twice and preincubated for 30 min at 37 °C in glucose-free KRBH. Next, the cells were washed once with glucose-free KRBH and then incubated for 30 min in KRBH 2.5 mM (white column) or 20 mM glucose (black column). Glucose stimulated insulin secretion (GSIS) was measured by Insulin ELISA (n = 6). Different letters indicate statistically significant differences between treatments ($p < 0.05$) by ANOVA (Tukey method as post hoc test).

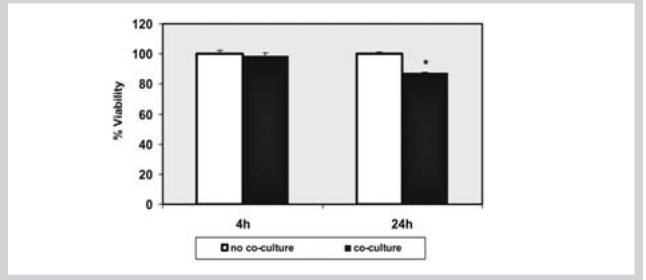

Fig. 7 INS-1E cell viability after 4 and 24 h of coculture. INS-1 cells were cultured or cocultured for 4 and 24 hours according to **Fig. 2**. The coculture cells were then cultured with a neutral red enriched medium and further processed according to the Materials and Methods procedure (n = 6). 100 % Viability corresponds to non-cocultured cells in each treatment. * Indicates $p < 0.05$ versus its respective non-cocultured well.

ruled out the idea that the coculture affects the cell culture media by increasing the amount of glucose. Instead, these results could reflect a phenotypic adaptation of the cells to the new culture situation.

Finally, we found that some of the assays that required several medium changes tended to lose β-cells. Therefore we carried out a neutral red assay after 4 and 24 hours of coculture and compared this to a non-coculture situation (**Fig. 7**). Our results suggest that it is better to coculture cells only for a short time to obtain a higher constancy of seeded cells and better results reproducibility.

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Conflict of interest statement

There were no financial or commercial conflicts of interest.

References

1 *Aggett PJ, Antoine JM, Asp NG, Bellisle F, Contor L, Cummings JH, Howlett J, Müller DJ, Persin C, Pijls LT, Rechkemmer G, Tuijtelaars S, Verhagen H.* PASSCLAIM: Consensus on criteria. Eur J Nutr 2005; 44: 5–30

2 *European Union.* Council directive 86/609/EEC of 24 November 1986 on the approximation of lows, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes. OJL 1986; 358: 1–28

3 *Manach C, Williamson G, Morand C, Scalbert A, Remesy C.* Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005; 81: 230S–242S

4 *Hackman RM, Polagruto JA, Zhu QY, Sun B, Fuji H, Keen CL.* Flavanols: digestion, absorption and bioactivity. Phytochem Rev 2008; 7: 195–208

5 *Pinent M, Castell A, Baiges I, Montagut G, Arola L, Ardévol A.* Bioactivity of flavonoids on insulin-secreting cell. Compr Rev Food Sci Food Saf 2008; 7: 299–308

6 *Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, Williamson G.* How should we assess the effects of exposure to dietary polyphenols *in vitro*? Am J Clin Nutr 2004; 80: 15–21

7 *Kim M, Ryu G, Kang J, Sim S, Min D, Rhie D, Yoon S, Hahn S, Jeong I, Hong K, Kim M, Jo Y.* Inhibitory effects of epicatechin on interleukin-1β-induced inducible nitric oxide synthase expression in RINm5F cells and rat pancreatic islets by down-regulation of NF-κB activation. Biochem Pharmacol 2004; 68: 1775–1785

8 *Mackenzie GG, Carrasquedo F, Delfino JM, Keen CL, Fraga CG, Oteiza PI.* Epicatechin, catechin, and dimeric procyanidins inhibit PMA-induced NF-{kappa}B activation at multiple steps in jurkat T cells. FASEB J 2004; 18: 167–169

9 *Vaidyanathan JB, Walle T.* Cellular uptake and efflux of the tea flavonoid (−)epicatechin-3-gallate in the human intestinal cell line Caco-2. J Pharmacol Exp Ther 2003; 307: 745–752

10 *Baba S, Osakabe N, Natsume M, Terao J.* Absorption and urinary excretion of procyanidin B2 [epicatechin-(4[beta]-8)-epicatechin] in rats. Free Radic Biol Med 2002; 33: 142–148

11 *Waltner-Law ME, Wang XL, Law BK, Hall RK, Nawano M, Granner DK.* Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. J Biol Chem 2002; 277: 34933–34940

12 *Bose M, Lambert JD, Ju J, Reuhl KR, Shapses SA, Yang CS.* The major green tea polyphenol, (−)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. J Nutr 2008; 138: 1677–1683

13 *Chan KY, Zhang L, Zuo Z.* Intestinal efflux transport kinetics of green tea catechins in Caco-2 monolayer model. J Pharm Pharmacol 2007; 59: 395–400

14 *Serra A, Macià A, Romero M, Salvadó M, Bustos M, Fernández-Larrea J, Motilva M.* Determination of procyanidins and their metabolites in plasma samples by improved liquid chromatography–tandem mass spectrometry. J Chromatogr B 2009; 877: 1169–1176

15 *Garcia-Ramirez B, Fernandez-Larrea J, Salvado MJ, Ardevol A, Arola L, Blade C.* Tetramethylated dimeric procyanidins are detected in rat plasma and liver early after oral administration of synthetic oligomeric procyanidins. J Agric Food Chem 2006; 54: 2543–2551

16 *Kris-Etherton PM, Hu FB, Ros E, Sabate J.* The role of tree nuts and peanuts in the prevention of coronary heart disease: multiple potential mechanisms. J Nutr 2008; 138: 1746S–1751S

17 *Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F.* The caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol 2005; 21: 1–26

18 *Tsukura Y.* Effects of capsaicin on cellular damage and monolayer permeability in human intestinal Caco-2 cells. Biol Pharm Bull 2007; 30: 1982–1986

19 *Yang X.* Establishment of Caco-2 cell monolayer model and standard operation procedure for assessing intestinal absorption of chemical components of traditional chinese medicine. Zhong Xi Yi Jie He Xue Bao 2007; 5: 634–641

20 *Yamashita S, Furubayashi T, Kataoka M, Sakane T, Sezaki H, Tokuda H.* Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. Eur J Pharm Sci 2000; 10: 195–204

21 *Hyung Choi S, Nishikawa M, Sakoda A, Sakai Y.* Feasibility of a simple double-layered coculture system incorporating metabolic processes of the intestine and liver tissue: application to the analysis of benzo [a]pyrene toxicity. Toxicol In Vitro 2004; 18: 393–402

22 *Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K.* Establishment of a pancreatic b cell line that retains glucose inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology 1990; 127: 126–132

23 *Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P.* Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. Endocrinology 2004; 145: 667–678

24 *Borenfreund E, Puerner JA.* Toxicity determined *in vitro* by morphological alternations and neutral red absorption. Toxicol Lett 1985; 24: 119–123

25 *Suganami T, Nishida J, Ogawa Y.* A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor {alpha}. Arterioscler Thromb Vasc Biol 2005; 25: 2062–2068

26 *Nollevaux G, Devillé C, El Moualij B, Zorzi W, Deloyer P, Schneider Y, Peulen O, Dandrifosse G.* Development of a serum-free co-culture of human intestinal epithelium cell-lines (Caco-2/HT29-5M21). BMC Cell Biol 2006; 7: 20

27 *Neville H, McClenaghan P, Flatt R.* Engineering cultured insulin-secreting pancreatic B-cell lines. J Mol Med 1999; 77: 235–243

28 *Markowska M, Oberle R, Juzwin S, Hsu C, Gryszkiewicz M, Streeter AJ.* Optimizing Caco-2 cell monolayers to increase throughput in drug intestinal absorption analysis. J Pharmacol Toxicol Methods 2001; 46: 51–55

29 *Konsoula R, Barile FA.* Correlation of *in vitro* cytotoxicity with paracellular permeability in mortal rat intestinal cells. J Pharmacol Toxicol Methods 2007; 55: 176–183

30 *Deprez S, Mila I, Huneau JF, Tome D, Scalbert A.* Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. Antioxid Redox Signal 2001; 3: 957–967

31 *Walgren RA, Lin J, Kinne RK, Walle T.* Cellular uptake of dietary flavonoid quercetin 4′-beta-glucoside by sodium-dependent glucose transporter SGLT1. J Pharmacol Exp Ther 2000; 294: 837–843

32 *Galijatovic A, Otake Y, Walle UK, Walle T.* Extensive metabolism of the flavonoid chrysin by human Caco-2 and hep G2 cells. Xenobiotica 1999; 29: 1241–1256

33 *Galijatovic A, Otake Y, Walle UK, Walle T.* Induction of UDP-glucuronosyltransferase UGT1A1 by the flavonoid chrysin in caco-2 cells – potential role in carcinogen bioinactivation. Pharm Res 2001; 18: 374–379

34 *Tsang C, Auger C, Mullen W, Bornet A, Rouanet J, Crozier A, Teissedre P.* The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. Br J Nutr 2005; 94: 170

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CHAPTER 2

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Procyanidins modify insulinemia by affecting insulin production and degradation☆

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Abstract

Previous studies from our research group have suggested that procyanidins modify glycemia and insulinemia. The aim of this work was to evaluate the effects of procyanidins on β-cell functionality in a nonpathological system. Four groups of healthy rats were studied. The animals were given daily acute doses of grape seed procyanidins extract (GSPE) for different time periods and at different daily amounts. A β-cell line (INS-1E) was treated with 25 mg GSPE/L for 24 h to identify possible mechanisms of action for the procyanidins. *In vivo* experiments showed that different doses of GSPE affected insulinemia in different ways by modifying β-cell functionality and/or insulin degradation. The islets isolated from rats that were treated with 25 mg GSPE/kg of body weight for 45 days exhibited a limited response to glucose stimulation. In addition, insulin gene expression, insulin synthesis and expression of genes related to insulin secretion were all down-regulated. *In vitro* studies revealed that GSPE decreased the ability of β-cells to secrete insulin in response to glucose. GSPE increased glucose uptake in β-cells under high-glucose conditions but impaired glucose-induced mitochondrial hyperpolarization, decreased adenosine triphosphate synthesis and altered cellular membrane potentials. GSPE also modified Glut2, glucokinase and Ucp2 gene expression as well as altered the expression of hepatic insulin-degrading enzyme (Ide), thereby altering insulin degradation. At some doses, procyanidins changed β-cell functionality by modifying insulin synthesis, secretion and degradation under nonpathological conditions. Membrane potentials and Ide provide putative targets for procyanidins to induce these effects.
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1. Introduction

It is now generally accepted that food can have health-promoting properties beyond its traditional nutritional value [1]. Procyanidins are a class of bioactive compounds that are usually found in fruits and other plant organs and are widely consumed. Procyanidins can protect against coronary heart diseases and atherosclerosis as well as act on several metabolic processes that are associated with the development of these disorders [2].

Most of the studies describing the beneficial effects of procyanidins have shown the peripheral activity of these molecules [3–7]. However, there are little data addressing whether procyanidins have central effects on the endocrine pancreas, a key organ of metabolic control [8]. Hanhineva et al. demonstrated that water extract from *Eriobotrya japonica* increased the insulin secretion of INS-1E cells, but treatment with procyanidin B-2 isolated from the extract decreased insulin secretion [9]. Previously, pretreatment and a daily administration of proanthocyanidins for 72 h were shown to protect β-cell function in alloxan-diabetic rats, suggesting a protective effect against the generation of reactive oxygen species [10].

The importance of the endocrine pancreas in whole-body nutrient equilibrium is highlighted by the emergence of several pathologies of nutrient metabolism, such as type 1 and 2 diabetes, that involve pancreatic cell deregulation. In addition, the pancreas is exposed to bioactive compounds immediately after their enteric absorption, suggesting that bioactive absorbed flavonoids can achieve high concentrations in this organ [11]. Therefore, the pancreas may be a target for procyanidins and their effects on metabolic processes.

Procyanidins act positively on glucose metabolism [7] by modifying both glycemia and insulinemia. We have previously described the peripheral targets of procyanidins that partially account for these effects [12,13]. However, the question remains whether procyanidins affect β-cell functionality. To address this question, healthy rats were treated with different grape seed procyanidins extracts (GSPEs). In addition, a β-cell line (INS-1E) was treated with GSPE to gain a better understanding of potential mechanisms of action, and work done in this cell line focused on the central pathways that regulate glucose-driven insulin secretion.

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t1.1 Table 1
t1.2 Summary of animal experimental procedures

t1.3 Animal group	Group A	Group B	Group C	Group D
t1.4 Sex	Female	Male	Female	Female
t1.5 Weight (g)	150–175	130–150	225–250	175–200
t1.6 Doses GSPE (mg/kg of bw)	0, 2.5, 5, 10, 25, 50	0,5, 15, 25, 50	0, 25	0, 1000
t1.7 Treatment period	36 days	21 days	45 days	1 h
t1.8 Doses GSPE (mg/kg of bw*days of treatment)	0, 90, 180, 360, 900, 1800	0, 105, 315, 525, 1050	0, 1125	0, 1000
t1.9 Vehicle	Sweetened condensed milk	Sweetened condensed milk	Sweetened condensed milk diluted 1:6 with water	Tap water
t1.10 Fasting period before sacrifice	5 h	Overnight	Overnight	Overnight
t1.11 Last dose time	9 a.m.	9 a.m.	8 p.m. day before	9 a.m.
t1.12 Sacrifice time	2 p.m.	12 p.m.	9 a.m.	10 a.m.
t1.13 Anesthetic (mg/kg of bw)	Ketamine (70) and xylazine (5)	Ketamine (70) and xylazine (5)	Pentobarbital sodium (75)	Pentobarbital sodium (75)

2. Materials and methods

2.1. Chemicals

According to the manufacturer, GSPE (*Les Dérives Résiniques et Terpéniques*, Dax, France) contained monomeric (16.6%), dimeric (18.8%), trimeric (16.0%), tetrameric (9.3%) and oligomeric procyanidins (5–13 units, 35.7%) as well as phenolic acids (4.2%).

2.2. Cell culture

INS-1E cells were kindly provided by Prof. Pierre Maechler, University of Geneva [14]. The cell line was cultured as previously described [15]. Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium).

2.3. Animal procedures

Four groups of Wistar rats were studied. All animals were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22°C with a 12-h light, 12-h dark cycle. Treatment began after 1 week in quarantine, as detailed in Table 1. Pancreatic islets were isolated from groups that underwent treatments C and D (Table 1). Blood was collected from all of the animal groups using heparin. Tissues from all of the animal groups were excised, frozen immediately in liquid nitrogen and stored at −80°C until analysis. All procedures were approved by the Experimental Animals Ethics Committee of the Universitat Rovira i Virgili. Insulin and C-peptide plasma levels were measured by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden) following the manufacturer's instructions. Glucose plasma levels were determined using an enzymatic colorimetric kit (GOD-PAP method from QCA, Amposta, Spain).

2.4. Islet isolation

Islets from animals in groups C and D were prepared by collagenase digestion as described previously [16]. Briefly, the rats were anesthetized, and the pancreas was infused with 7 ml of ice-cold collagenase P (Roche, Barcelona, Spain) solution (1 mg/ml) before removal. After the pancreas was removed, it was incubated at 37°C for 15 min. Islets were purified on a Histopaque gradient (Sigma, Madrid, Spain) and handpicked until a population of pure islets was obtained.

2.5. Glucose-stimulated insulin secretion (GSIS)

Secretory responses to glucose were tested in INS-1E cells as previously described [15]. GSIS and cellular insulin contents were measured by radioimmunoassay (RIA) using rat insulin as a standard [17]. GSIS was tested in islets from rats in groups C and D. Islets were maintained for 24 h in RPMI-supplemented medium. The islets were then washed twice and incubated for 1 h at 37°C in Krebs–Ringer bicarbonate HEPES buffer (KRBH) with 2.8 mM glucose (basal) or 16.8 mM glucose (stimulated). Insulin secretion was measured using the Insulin ELISA Kit (Mercodia, Uppsala, Sweden). The islet protein content for each sample was measured using the Bradford method [18].

2.6. Glucose uptake

Glucose transport was determined by measuring the uptake of 2-deoxy-D-[^{3}H] glucose in INS-1E cells cultured in 24-well plates using a methodology adapted from Ref. [19]. Briefly, pancreatic cells were maintained for 30 min at 37°C in glucose-free KRBH. The cells were then incubated for 10 min in a KRBH transport solution containing 2.5 mM or 15 mM glucose and 0.75 μCi 2-deoxy-D-[^{3}H]glucose. Uptake was halted by adding 100 mM glucose, and cells were disrupted by adding 0.1 M NaOH/0.1% phosphate-buffered saline. Glucose uptake was assessed by scintillation counting, and protein content was used to normalize the glucose transport values. The protein content was determined by the Bradford method [18].

2.7. Mitochondrial membrane potential ($\Delta\Psi_m$) and cellular membrane potential measurements

The $\Delta\Psi_m$ and cellular membrane potential were measured as described previously [14].

2.8. Cytosolic adenosine triphosphate (ATP) levels

The cytosolic ATP levels were monitored in cells expressing an ATP-sensitive bioluminescent luciferase probe 1 day after transduction. Pancreatic β-cells were maintained for 2 h in glucose-free culture medium and then stimulated with 15 mM glucose in the presence of 200 μM luciferin. Finally, 2 mM NaN_3 was added as a mitochondrial poison [14].

2.9. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from INS-1E cells grown in six-well plates was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA), and 2 μg of RNA was converted into cDNA [20]. Total RNA was extracted from the pancreas and the liver of animals in groups A and B using TRIzol reagent following the manufacturer's instructions, and cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RNA was extracted from the islets using the miRNeasy Mini Kit (Qiagen, Barcelona, Spain), and cDNA was generated using a kit from Applied Biosystems. The cDNA from all the experiments was subjected to quantitative RT-PCR amplification using the TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA). Specific TaqMan probes (Applied Biosystems, Foster City, CA, USA) were used for different genes: Rn01774648-g1 for insulin, Rn00755591-m1 for pancreatic duodenal homeobox 1 (Pdx1), Rn00561265-m1 for glucokinase, Rn00563565-m1 for Glut2, Rn00565839-m1 for insulin-degrading enzyme (Ide) and Rn01754856-m1 for uncoupling protein 2 (Ucp2). β-Actin was used as the reference gene (Rn00667869-m1). The reactions were run on a quantitative RT-PCR 7300 System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

2.10. Calculations and statistical analysis

The results are expressed as the mean±S.E.M. The effects were assessed by the Student's *t* test. All calculations were performed with the SPSS software.

Fig. 1. Effects of procyanidin on insulinemia after different GSPE treatments. Animals (five or six per group) were sacrificed, and their plasma and tissues were collected according to Table 1. (a) Plasma insulin levels and the HOMA index vs. control for different doses of GSPE (seeTable 1). Glucose and insulin levels were analyzed by colorimetric and ELISA methods, respectively. (b) Pancreatic insulin and Pdx1 gene expression vs. control for different doses of GSPE (seeTable 1). mRNA levels were determined by quantitative RT-PCR. (c) The ratio between plasma insulin and insulin gene expression after treatment with different doses of GSPE for 21 days. (d) Plasma C-peptide levels after a treatment with 25 mg GSPE/kg of bw for 21 days (525 mg GSPE/kg of bw*days of treatment). The plasma C-peptide levels were quantified using ELISA methodology. (e) Liver Ide gene expression after treatment with 15 mg of GSPE/kg of bw for 21 days (315 mg GSPE/kg of bw*days of treatment). The data are presented as the mean±S.E.M. *P<.05 vs. the control; #P<.1 vs. the control.

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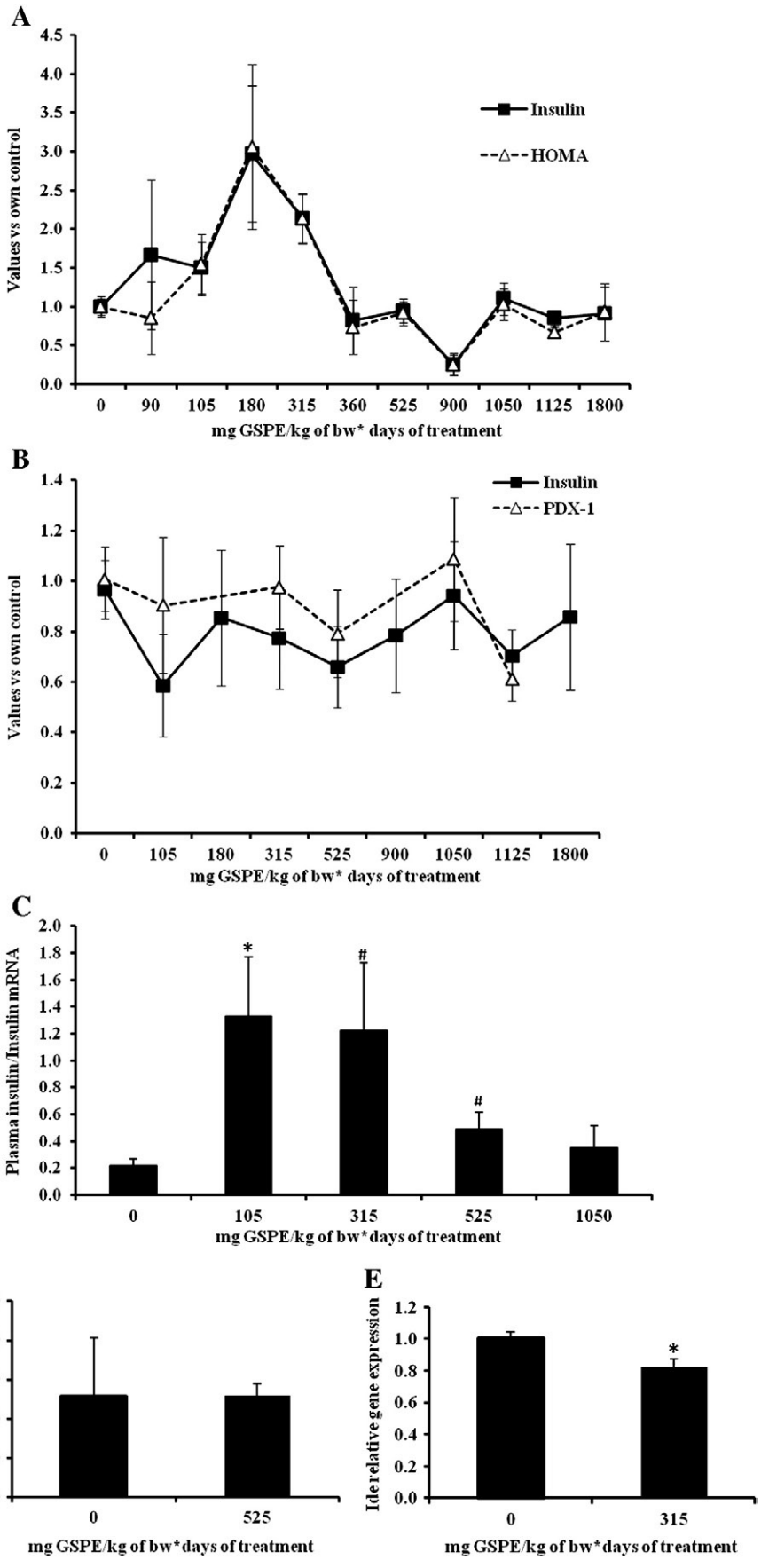

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t2.1 Table 2
t2.2 Statistical results of the effects on plasma insulin, HOMA index, insulin mRNA and Pdx1 mRNA after a treatment with different doses of GSPE

t2.3 GSPE doses (mg GSPE/kg of bw*days)	90	105	180	315	360	525	900	1050	1125	1800
t2.4 Plasma insulin vs. control	NS	NS	NS	$P<.05$	NS	NS	$P<.05$	NS	NS	NS
t2.5 HOMA vs. control	NS	NS	NS	$P<.05$	NS	NS	$P<.06$	NS	$P<.05$	NS
t2.6 Insulin mRNA vs. control	–	$P<.1$	NS	NS	–	$P<.1$	NS	NS	$P<.1$	–
t2.7 Pdx-1 mRNA vs. control	–	NS	–	NS	–	NS	–	NS	$P<.05$	–

t2.8 NS, not significant.

3. Results and discussion

3.1. Procyanidins affect insulinemia due to their effects on insulin synthesis and degradation

Procyanidins have been shown to have beneficial effects on glucose homeostasis [7], but most of these studies focused on the bioactivity of procyanidins in the liver and adipose tissues. The present study shows that a daily acute administration of ~~a~~ GSPE to healthy rats at different concentrations and for different time periods results in a peculiar effect on insulinemia. To better compare the GSPE effects between different animal studies, the effects of each treatment are shown relative to its own control group [insulin reference values for each group (μg/L): A, 0.47±0.2; B, 0.24±0.0; C, 0.90±0.1]. Fig. 1 shows that the lowest doses of GPSE did not affect insulinemia, and statistically significant results were found at moderate doses (summarized in Table 2). Treatments at 5 mg and 15 mg GSPE/kg of body weight (bw) for 21 to 36 days (180 mg and 315 mg GSPE/kg of bw*days of treatment) increased insulinemia, whereas treatments at 25 mg/kg of bw for 36 days (900 mg GSPE/kg of bw*days of treatment) decreased insulinemia. Higher doses did not show any effect. The homeostasis model assessment (HOMA) index [21] for these treatment groups exhibited a similar pattern (Fig. 1a) and showed that the changes in insulin did not provoke significant changes in glycemia (as seen in Table 2) (HOMA reference values for each group: A, 9.49±4.1; B, 1.61±0.3; C, 10.24±2.2). These results agree with our previous results that suggested that procyanidins alter insulinemia, although the relationship between the dose and the effect was unclear [12]. In a retrospective review, we highlighted that the efficacy of procyanidins or procyanidin extracts depends on the dose and the metabolic situation [7].

To better understand this procyanidin effect, insulin production was analyzed. Fig. 1b shows that the insulin gene expression profile in these animals exhibits the tendency for lower insulin mRNA levels at lower doses. The same tendency was found for Pdx1 mRNA (Fig. 1b), a key controller in insulin synthesis. Fig. 1c shows the relationship between plasma insulin and insulin mRNA at different GSPE concentrations. Insulin mRNA levels reflect the amount of insulin synthesis. Insulin plasma levels reflect the amount of insulin from pancreatic production and the clearance of this hormone in different tissues. At some GSPE doses, the plasma insulin protein levels were higher than the pancreatic mRNA levels. These ratios may reflect modifications in insulin production (synthesis and/or secretion) and/or in insulin removal. Fig. 1b showed no effect on insulin mRNA synthesis at these doses. To determine if insulin secretion was altered, the C-peptide levels were analyzed. Fig. 1d shows that GSPE treatment did not modify pancreatic insulin secretion. Therefore, insulin removal was analyzed. Because Ide is responsible for the removal of insulin and Ide activity is high in the liver [22], liver Ide gene expression was measured in the treatment group that showed significantly increased insulinemia: 15 mg GSPE/kg of bw*21 days (315 mg GSPE/kg of bw*days of treatment). At this dose, procyanidins decreased Ide mRNA levels (Fig. 1e),

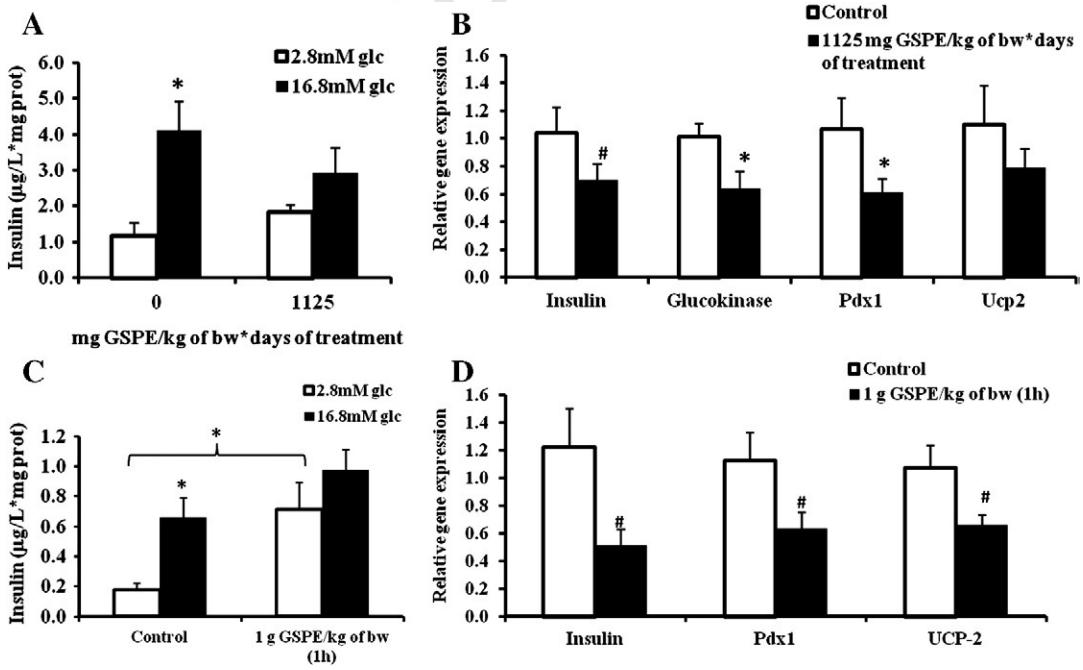

Fig. 2. Effects of GSPE treatment on rat islets. (a and b) The rats were treated with 25 mg GSPE/kg of bw for 45 days. (a) GSIS measurements and (b) insulin, glucokinase, Pdx1 and Ucp2 gene expression. (c and d) The rats were treated acutely with 1000 mg GSPE/kg of bw. (c) GSIS measurements and (d) insulin, Pdx1 and Ucp2 gene expression. To analyze the glucose response in both experiments, isolated islets were maintained for 24 h in RPMI-supplemented medium and then cultured for 1 h at low (2.8 mM) or high (16.8 mM) glucose concentrations. Insulin levels were quantified by ELISA methodology, and mRNA levels were determined by quantitative RT-PCR. The data are presented as the mean±S.E.M. *$P<.05$ vs. the control; #$P<.1$ vs. the control.

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suggesting that these animals have limited insulin degradation activity, which could explain their increased insulinemia. These data suggest that Ide is a target for procyanidins, and to our knowledge, this is the first data describing the effect of procyanidins on Ide. The transcriptional regulation of Ide and its effect on insulin homeostasis are still not well understood, and there are little data describing the factors that regulate Ide gene expression. Insulin increases Ide gene expression in HepG2 cells but only under high-glucose conditions [23]. Concerning other tissues, Du et al. showed that peroxisome proliferator-activated receptor-γ (PPARγ) plays an important role in regulating Ide gene expression in rat primary neurons through its interaction with a functional peroxisome proliferator-response element on the Ide promoter, thereby activating Ide gene transcription [24]. We do not exclude that the effect of GSPE on Ide expression could involve PPARγ regulation since chronic GSPE treatment down-regulates PPARγ expression in 3T3-L1 adipocytes [25].

Therefore, the effects of procyanidins on plasma insulin could be related to their bioactivity on Ide as well as their effects on β-cells.

3.2. Islets from GSPE-treated animals show decreased responsiveness to glucose

To directly test whether the islets of Langerhans are the targets of procyanidins, islets from rats treated with 25 mg GSPE/kg of bw for 45 days were isolated, and their response to glucose stimulation was measured after 24 h in culture. Fig. 2a shows that there were a higher basal level of insulin production and a clearly limited response to glucose stimulation in islets from GSPE-treated animals (GSIS rate for the control group, 4.94±1.32, was significantly different from the GSIS rate for GSPE-treated animals, 1.45±0.43). At the mRNA level (Fig. 2b), there was a decrease in both insulin and Pdx1 mRNA levels. Glucokinase gene expression was significantly down-regulated, and there was also a small, but not statistically significant, decrease in Ucp2 mRNA levels. Taken together, these data suggest that procyanidins modify islet functionality by decreasing their sensitivity to glucose and by modifying mRNA expression levels.

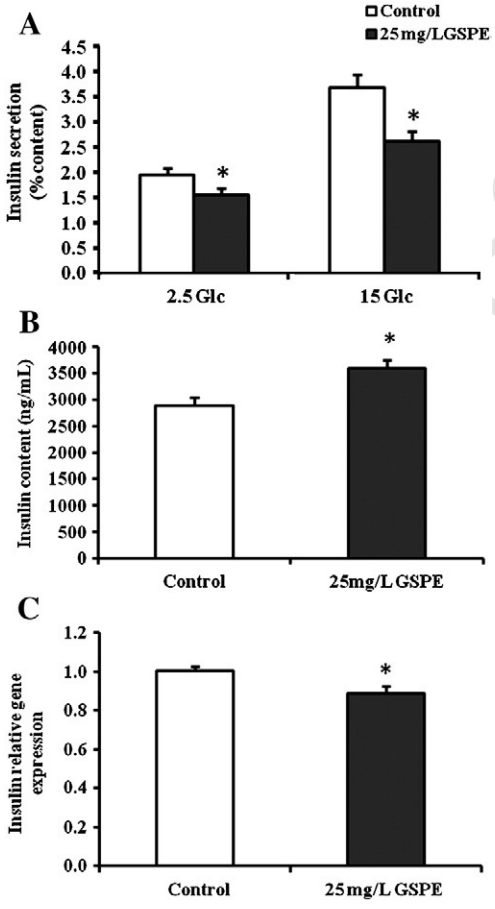

Fig. 3. Effects of procyanidin treatment on insulin synthesis and secretion in INS-1E cells. INS-1E cells were treated with 25 mg/L of GSPE for 24 h. After 2 h of starvation in RPMI without glucose medium, cells were cultured in medium with basal (2.5 mM) or stimulated (15 mM) glucose levels. (a) GSIS measurements and (b) insulin content in the cells were determined from an acid-ethanol extract. Insulin was measured by insulin RIA. (c) Insulin mRNA levels were measured by quantitative RT-PCR. The data are presented as the mean±S.E.M. *P<.05 vs. the control.

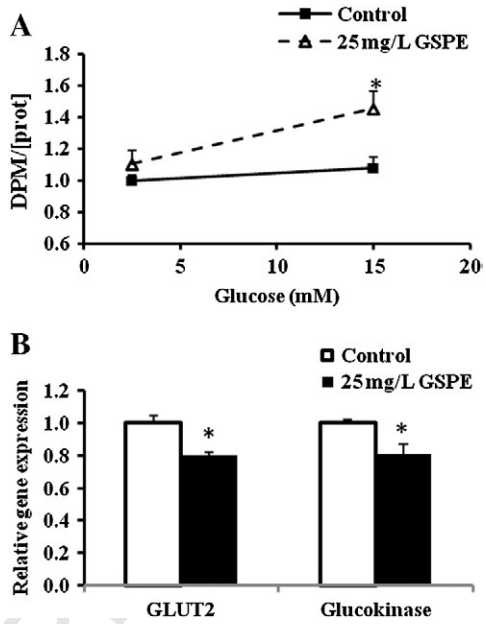

Fig. 4. Effects of a 24-h GSPE treatment (25 mg/L) on glucose entry in INS-1E cells. The cells were treated as indicated in Fig. 3. (a) Glucose uptake was determined by measuring 2-deoxy-D-[^{3}H]glucose uptake by scintillation counting. (b) Glut2 and glucokinase gene expression was analyzed by quantitative RT-PCR. The data are presented as the mean±S.E.M. *P<.05 vs. the control.

Next, we tested whether the same effects could be induced by a similar amount of procyanidins in an acute dose. Healthy female rats were treated with an acute dose of 1000 mg/kg of bw. After 1 h, which corresponded to peak procyanidin levels in the blood [26], islets from these animals were isolated and cultured as described above, and their response to glucose stimulation was measured. Fig. 2c shows that this treatment led to higher basal levels of insulin production, which limited islet sensitivity to glucose stimulation. Similar to islets from animals in the 45-day treatment group, these islets exhibited lower insulin, Pdx1 and Ucp2 mRNA levels (Fig. 2d), and there was no effect on glycemia (control: 6.22±0.31 mM, GSPE treatment: 6.11±0.28 mM).

Similar effects were produced *in vitro* after long-term fatty acid treatment [27,28] or after hyperglycemia [29]. The molecules that are used as antidiabetic drugs also produced similar effects. Chronic treatment of islets with glibenclamide, a sulfonylurea, inhibited proinsulin biosynthesis at basal and intermediate glucose concentrations and promoted insulin secretion independently of glucose concentration [30].

Surprisingly, when two different administrations of the same total amount of procyanidins were compared, we found very similar effects. Both treatments limited glucose sensitivity in the islets that were removed from GSPE-treated animals and cultured for 24 h. These effects did not correlate with plasma procyanidin levels in either treatment group. In the acute treatment group, dimeric

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procyanidins reached 0.5 nM, and trimeric procyanidins reached 2.5 nM [26]. In the chronic treatment group, dimeric procyanidins reached 11.5±1.25 nM, but there was no measurable amount of trimeric procyanidins. These results suggest that a minimum amount of dimeric procyanidins (approximately 0.5 nM) is necessary to induce the described effects. However, unpublished results from our research group, working with pure structures, do not support this conclusion. We expected that other components from the extract that we were not able to identify could play a role in eliciting these effects. However, measuring the bioavailability of different molecules in plant extracts is beyond the scope of this paper [8,15,31]. We included this information to highlight the relationship between two very different procyanidin administrations, both in their pancreatic effects and in the amount of procyanidins achieved in plasma.

3.3. GSPE limits mitochondrial function

To better understand how procyanidins modify β-cell insulin secretion, the study was carried out using the INS-1 β-cell line. The cells were treated for 24 h with 25 mg GSPE/L (Fig. 3a), and we found that insulin secretion decreased under basal glucose conditions, and this effect was even stronger under stimulated glucose conditions. In addition, the amount of insulin content was higher (Fig. 3b) and insulin gene expression was lower (Fig. 3c) in cells treated with 25 mg GPSE/L.

Because insulin secretion depends on cell energetics, key pathways in cell energetics from glucose entry to insulin secretion [32] were analyzed to identify possible targets of procyanidins that could limit insulin secretion and/or insulin synthesis.

Fig. 4a shows that GSPE administration led to an increase in glucose uptake in β-cells under high-glucose conditions. In contrast, procyanidins lowered mRNA levels for the Glut2 glucose transporter and glucokinase, which are key effectors of glucose uptake (Fig. 4b). After glucose enters the cell and is metabolized through the glycolytic pathway, it reaches the mitochondria and enters the Krebs cycle. At the mRNA level, there was no effect on citrate synthase enzyme levels (1.03±0.04 vs. control 1.00±0.03). In contrast, there was a clear effect on the $\Delta\Psi_m$.Fig. 5a shows that INS-1E cells treated with 25 mg GSPE/L for 24 h exhibited a decrease in glucose-induced mitochondrial hyperpolarization (~5%). The total $\Delta\Psi_m$ revealed by *p*-trifluoromethoxyphenylhydrazone (FCCP) was reduced by GSPE treatment compared with the control (Fig. 5b). A possible cause for this uncoupling could be the increase in Ucp2 expression (Fig. 5c). Uncoupling protein-2 is thought to catalyze a mitochondrial inner-membrane H^+ leak that bypasses ATP synthase, thereby reducing the cellular ATP content [33]. These data suggest that although there was an increased entry of glucose under high-glucose conditions, coupling with the mitochondria was altered and resulted in lower levels of ATP synthesis.Fig. 6a shows that the GSPE strongly inhibits cytosolic ATP production after glucose stimulation, which may lower the ability of the GSPE-treated cells to close ATP-sensitive potassium (K^+_{ATP}) channels as suggested inFig. 6b. GSPE treatment for 24 h did not affect the cellular membrane potential after glucose stimulation, but addition of 30 mM KCl in the presence of 25 mg GSPE/L resulted in an increased depolarization. These results suggest that absolute cellular membrane potential levels are lower in INS-1E cells treated with the highest GSPE dose than in control cells. Therefore, GPSE decreases the ability of these cells to secrete insulin in response to glucose entry by uncoupling the entire process.

Thus, our results indicate that procyanidins limit insulin secretion through modifying membrane permeability and the glucose-stimulated insulin secretion pathway, which lead to an increase in insulin content in β-cells. Accumulation of insulin in the cell could be responsible for the inhibition of insulin mRNA levels, as there is

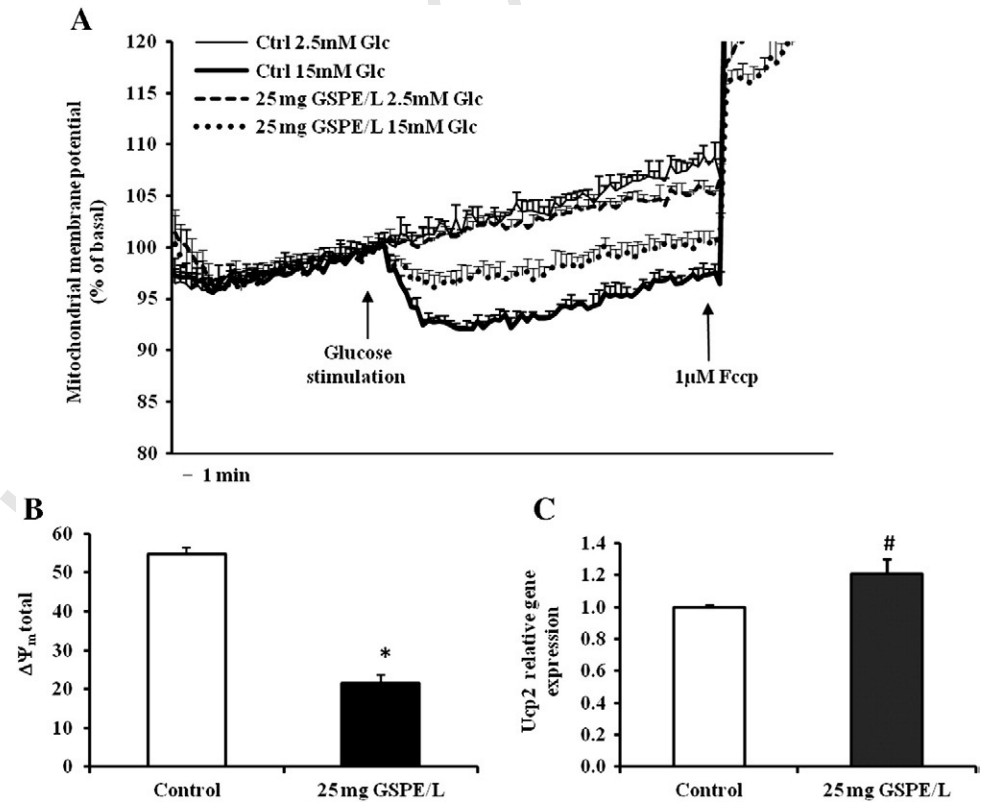

Fig. 5. Effects of a 24-h GSPE treatment (25 mg/L) on mitochondrial function in INS-1E cells. The cells were treated as indicated in Fig. 3. (a) The $\Delta\Psi_m$ was monitored by rhodamine 123 fluorescence. Hyperpolarization of $\Delta\Psi_m$ was induced with 15 mM glucose, and after 10 min of glucose stimulation, depolarization was induced by FCCP. (b) Total $\Delta\Psi_m$ revealed by FCCP. (c) Ucp2 mRNA levels were measured by quantitative RT-PCR. The data are presented as the mean±S.E.M. *P<.05 vs. the control; #P<.06 vs. the control.

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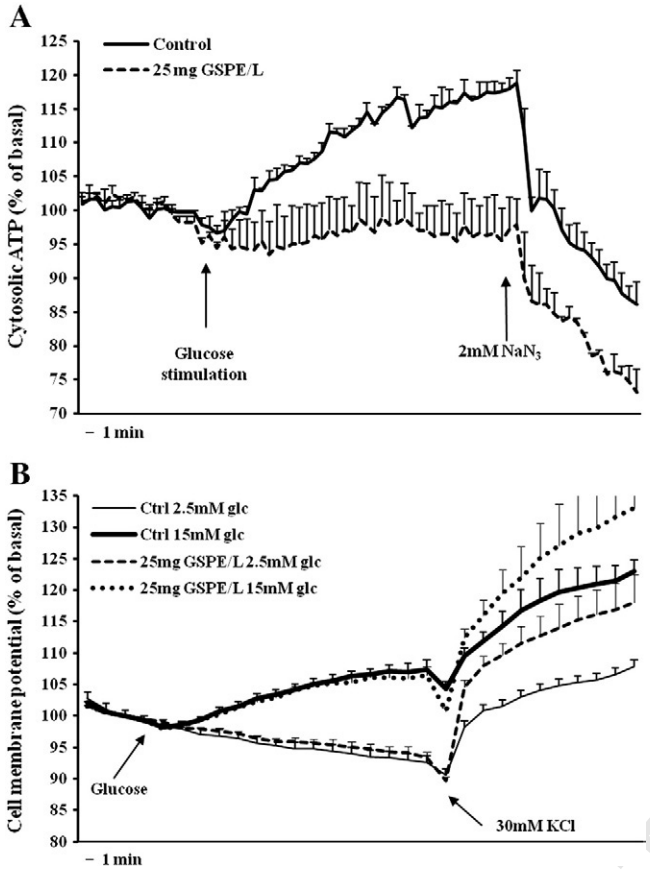

Fig. 6. Effects of a 24-h GSPE treatment (25 mg/L) on ATP generation and cellular membrane potential in INS-1E cells. (a) Cytosolic ATP levels were monitored in cells expressing luciferase. The cells were stimulated by raising glucose levels from 2.5 mM to 15 mM. After 10 min of stimulation, 2 mM NaN_3 was added as a mitochondrial poison. (b) The cellular membrane potential was monitored by bisoxonol fluorescence. Depolarization of cellular membranes was induced with 15 mM glucose for 10 min, followed by control depolarization, which was induced by 30 mM KCl. The data are presented as the mean±S.E.M.

sufficient insulin protein synthesized. However, according to published data [34], it seems not to be responsible to explain the effects on insulin biosynthesis; therefore, GSPE could also act directly at the insulin promoter to inhibit gene transcription. We do not exclude this possibility because we found it in all the models we have assayed.

A recently published study demonstrated that treating INS-1E cells with resveratrol, another phenolic compound, for 24 h promoted GSIS by increasing glucose oxidation, ATP production and mitochondrial oxygen consumption [35]. Vetterli et al. have also shown that resveratrol up-regulates key genes in β-cell function, such as Glut2, glucokinase, Pdx1, hepatocyte nuclear factor 1 homeobox A and mitochondrial transcription factor A. The differences between this result and our results could be due to the differences in chemical structure between procyanidins and resveratrol; in fact, there have been several controversial results reported for the effects of different flavonoids on β-cells [8].

Alternatively, acute treatment with pioglitazone (Pio), a thiazolidinedione (TZD), in INS 832/13 cells and in isolated rat islets produced effects similar to those for GSPE treatment. Pio reduced the GSIS in β-cells at intermediate glucose concentrations, which altered ATP content and inhibited glucose-induced mitochondrial membrane hyperpolarization. A previous study published by Kim et al. showed that chronic treatment with other TZD molecules, such as rosiglitazone, stimulated insulin release and synthesis. These molecules upregulated Glut2 and glucokinase gene expression after a 24-h treatment period through PPARγ activation [36]. The results from this study and other studies suggest that the effects of TZDs, which are PPARγ agonists, on pancreatic β-cells remain controversial, and the effect depends on the dose and treatment period of antidiabetic agents, similar to our results with procyanidins.

We speculate that during chronic treatment, GSPE can act as a PPARγ antagonist in β-cells; this is similar to its effect on adipocytes, where procyanidins limit adipogenesis during the induction of differentiation [12,25]. In β-cells, GSPE limited glucose-induced insulin secretion by uncoupling the process and down-regulated the expression of genes that act directly on insulin synthesis and secretion. Furthermore, Moibi et al. showed that PPARγ induces Pdx1 expression and, consequently, induces the expression of Glut2, glucokinase and insulin [37]. The present study showed that the expression of these genes decreased after GSPE treatment at different doses and at different treatment periods. However, the precise role of PPARγ in the molecular mechanisms by which GSPE alters β-cell functionality remains elusive.

In conclusion, we showed that procyanidins play an important role in β-cell function by limiting glucose sensitivity and insulin biosynthesis. GPSE treatment altered the $\Delta\Psi_m$, ATP production and the cellular membrane potential. Various *in vivo* experiments corroborate this procyanidin effect. Both acute and chronic treatment reduced glucose-induced insulin secretion and down-regulated insulin and Pdx1 mRNA levels, the β-cell master gene, in rat islets. Moreover, our results demonstrated that low doses of procyanidins increased the plasma insulin levels and inhibited insulin gene expression, which led to reduced Pdx1 mRNA levels in the pancreas and reduced hepatic Ide gene expression.

Acknowledgments

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References

[1] Saris WH, Asp NG, Bjorck I, Blaak E, Bornet F, Brouns F, et al. Functional food science and substrate metabolism. Br J Nutr 1998;80(Suppl 1):S47–75.
[2] Blade C, Arola L, Salvado MJ. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. Mol Nutr Food Res 2010;54:37–59.
[3] Koo SI, Noh SK. Green tea as inhibitor of the intestinal absorption of lipids: potential mechanism for its lipid-lowering effect. J Nutr Biochem 2007;18: 179–83.
[4] Meeran SM, Katiyar SK. Cell cycle control as a basis for cancer chemoprevention through dietary agents. Front Biosci 2008;13:2191–202.
[5] Pinent M, Blade C, Salvado MJ, Blay M, Pujadas G, Fernandez-Larrea J, et al. Procyanidin effects on adipocyte-related pathologies. Crit Rev Food Sci Nutr 2006; 46:543–50.
[6] Vafeiadou K, Vauzour D, Spencer JP. Neuroinflammation and its modulation by flavonoids. Endocr Metab Immune Disord Drug Targets 2007;7:211–24.
[7] Pinent M, CedÃ³L, Montagut G, Blay M, ArdÃ©vol A. Procyanidins improve some disrupted glucose homoeostatic situations: an analysis of doses and treatments according to different animal models. Crit Rev Food Sci Nutr 2011. Q2 Q3
[8] Pinent M, Castell A, Baiges I, Montagut G, Arola L, Ardévol A. Bioactivity of flavonoids on insulin-secreting cells. Compr Rev Food Sci Food Saf 2008;7: 299–308.
[9] Hanhineva K, Torronen R, Bondia-Pons I, Pekkinen J, Kolehmainen M, Mykkanen H, et al. Impact of dietary polyphenols on carbohydrate metabolism. Int J Mol Sci 2010;11:1365–402.
[10] El-Alfy AT, Ahmed AA, Fatani AJ. Protective effect of red grape seeds proanthocyanidins against induction of diabetes by alloxan in rats. Pharmacol Res 2005;52:264–70.
[11] Suganuma M, Okabe S, Oniyama M, Tada Y, Ito H, Fujiki H. Wide distribution of [3H](−)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. Carcinogenesis 1998;19:1771–6.
[12] Montagut G, Blade C, Blay M, Fernandez-Larrea J, Pujadas G, Salvado MJ, et al. Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. J Nutr Biochem 2010;21:961–7.

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[13] Montagut G, Onnockx S, Vaque M, Blade C, Blay M, Fernandez-Larrea J, et al. Oligomers of grape-seed procyanidin extract activate the insulin receptor and key targets of the insulin signaling pathway differently from insulin. J Nutr Biochem 2010;21:476–81.
[14] Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P. Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. Endocrinology 2004;145: 667–78.
[15] Castell-Auvi A, Cedo L, Pallares V, Blay MT, Pinent M, Motilva MJ, et al. Development of a coculture system to evaluate the bioactivity of plant extracts on pancreatic beta-cells. Planta Med 2010;76:1576–81.
[16] Estil les E, Tellez N, Soler J, Montanya E. High sensitivity of beta-cell replication to the inhibitory effects of interleukin-1beta: modulation by adenoviral over-expression of IGF2 in rat islets. J Endocrinol 2009;203:55–63.
[17] Maechler P, Wollheim CB. Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. Nature 1999;402:685–9.
[18] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[19] Pinent M, Blay M, Blade MC, Salvado MJ, Arola L, Ardevol A. Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. Endocrinology 2004;145:4985–90.
[20] Frigerio F, Chaffard G, Berwaer M, Maechler P. The antiepileptic drug topiramate preserves metabolism-secretion coupling in insulin secreting cells chronically exposed to the fatty acid oleate. Biochem Pharmacol 2006;72: 965–73.
[21] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28: 412–9.
[22] Valera Mora ME, Scarfone A, Calvani M, Greco AV, Mingrone G. Insulin clearance in obesity. J Am Coll Nutr 2003;22:487–93.
[23] Pivovarova O, Gogebakan O, Pfeiffer AF, Rudovich N. Glucose inhibits the insulin-induced activation of the insulin-degrading enzyme in HepG2 cells. Diabetologia 2009;52:1656–64.
[24] Du J, Zhang L, Liu S, Zhang C, Huang X, Li J, et al. PPARgamma transcriptionally regulates the expression of insulin-degrading enzyme in primary neurons. Biochem Biophys Res Commun 2009;383:485–90.
[25] Pinent M, Blade MC, Salvado MJ, Arola L, Hackl H, Quackenbush J, et al. Grape-seed derived procyanidins interfere with adipogenesis of 3T3-L1 cells at the onset of differentiation. Int J Obes (Lond) 2005;29:934–41.
[26] Serra A, Macia A, Romero MP, Valls J, Blade C, Arola L, et al. Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. Br J Nutr 2010;103:944–52.
[27] Lupi R, Del Guerra S, Marselli L, Bugliani M, Boggi U, Mosca F, et al. Rosiglitazone prevents the impairment of human islet function induced by fatty acids: evidence for a role of PPARgamma2 in the modulation of insulin secretion. Am J Physiol Endocrinol Metab 2004;286:E560–7.
[28] Olofsson CS, Collins S, Bengtsson M, Eliasson L, Salehi A, Shimomura K, et al. Long-term exposure to glucose and lipids inhibits glucose-induced insulin secretion downstream of granule fusion with plasma membrane. Diabetes 2007;56: 1888–97.
[29] Robertson RP, Harmon JS. Diabetes, glucose toxicity, and oxidative stress: a case of double jeopardy for the pancreatic islet beta cell. Free Radic Biol Med 2006;41: 177–84.
[30] Alarcon C, Wicksteed B, Rhodes CJ. Exendin 4 controls insulin production in rat islet beta cells predominantly by potentiation of glucose-stimulated proinsulin biosynthesis at the translational level. Diabetologia 2006;49:2920–9.
[31] Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81:230S–42S.
[32] Maechler P, Wollheim CB. Mitochondrial signals in glucose-stimulated insulin secretion in the beta cell. J Physiol 2000;529(Pt 1):49–56.
[33] Chan CB, De Leo D, Joseph JW, McQuaid TS, Ha XF, Xu F, et al. Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action. Diabetes 2001;50:1302–10.
[34] Leibiger IB, Leibiger B, Berggren PO. Insulin signaling in the pancreatic beta-cell. Annu Rev Nutr 2008;28:233–51.
[35] Vetterli L, Brun T, Giovannoni L, Bosco D, Maechler P. Resveratrol potentiates glucose-stimulated insulin secretion in INS-1E beta-cells and human islets through Sirt1 dependent mechanism. J Biol Chem 2010; Q4
[36] Kim HS, Noh JH, Hong SH, Hwang YC, Yang TY, Lee MS, et al. Rosiglitazone stimulates the release and synthesis of insulin by enhancing GLUT-2, glucokinase and BETA2/NeuroD expression. Biochem Biophys Res Commun 2008;367:623–9.
[37] Moibi JA, Gupta D, Jetton TL, Peshavaria M, Desai R, Leahy JL. Peroxisome proliferator-activated receptor-gamma regulates expression of PDX-1 and NKX6.1 in INS-1 cells. Diabetes 2007;56:88–95.

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THE EFFECTS OF GRAPE SEED PROCYANIDIN EXTRACT ON INSULIN SYNTHESIS AND SECRETION
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DL:T. 271-2012

Procyanidins Modulate MicroRNA Expression in Pancreatic Islets

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CHAPTER 3

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ABSTRACT

Procyanidins modulate glucose metabolism, and the results of recent experiments also suggest that the pancreas is a target for procyanidins. Given the role of microRNAs (miRNAs) in the regulation of metabolic processes in diabetes and that of flavonoids in the modulation of miRNAs in tissues other than the pancreas, we hypothesised that procyanidins might target miRNAs in the pancreas. We assessed the miRNA expression profile in pancreatic islets isolated from rats that had been chronically treated with a daily dose of grape seed procyanidin extract (GSPE). Our data indicate that GSPE treatment modulates the miRNA gene expression pattern, and *in silico* prediction studies suggest that ion transport and response to glucose might be among the pathways affected.

MANUSCRIPT FOOD&FUNCTION

MicroRNAs (miRNAs) comprise a family of small non-coding RNAs that post-transcriptionally regulate gene expression [1]. Currently, it is known that miRNAs are important not only for normal organism development and physiology but also for the pathologies of cancer, heart disease and inflammation [2]. MiRNAs are also involved in diabetes [3] and are required for both pancreas development [4,5] and the regulation of glucose-stimulated insulin secretion [6]. The most widely studied miRNA in the pancreas is miR-375 [2,7]. However, other miRNAs have recently been described as regulators of pancreas functionality, modifying insulin secretion [6,8,9] and insulin biosynthesis [10,11] ; additionally, some miRNAs have been shown to be related to β-cell apoptosis [12]. Given the role of miRNAs in regulating the metabolic processes that are important in some pathologies and in diabetes, miRNAs are likely targets for bioactive compounds that affect these pathologies. Among these bioactive compounds are the procyanidins, which are phenolic compounds found in fruits and vegetables [13]. Procyanidins modulate glucose metabolism by modifying both glycemia and insulinemia (reviewed in [14]). We recently observed that, *in vivo*, different doses of grape seed procyanidin extract (GSPE) affected insulinemia by modifying β-cell functionality and/or insulin degradation activity [15]. Considering the fact that miRNAs modify pancreas functionality and apoptosis, we hypothesised that another mechanism for procyanidins to act on glucose homeostasis might be via the modulation of miRNA expression. A few recent studies support the idea that flavonoids can alter the miRNA expression profile [16-19]. In addition there are evidences that procyanidins treatment modulate miRNA expression pattern, since our research group has recently published that GSPE modulate miRNA profile in an hepatocyte cell line (HepG2) [20]. These studies describe *in vitro* experiments in different cell lines, but, to our knowledge, there have been no studies to determine the effects of flavonoids in the pancreas. The aim of this study, therefore, was to investigate whether procyanidins modify the expression pattern of miRNAs in rat pancreatic islets after a chronic treatment.

Rats were treated with a daily dose of GSPE (25 mg/kg of body weight (bw)) for 45 days, and the expression of 680 miRNAs in freshly isolated islets was analysed. In Figure 1, the 50 miRNAs with the highest absolute values of logarithmised fold changes are presented. We found 4 miRNAs with significant differential expression due to the GSPE treatment: miR-1249, miR-483, miR-30c-1* (each of which was down-regulated), and miR-3544 (which was up-regulated). Previous studies have reported that miR-375, miR-9, miR-124a, and miR-96 play a role in the regulation of insulin secretion in rodent pancreatic β-cell lines [6,8,9]. All of these referenced miRNAs were present in our biochip, but GSPE did not alter the expression of any of them. Instead, GSPE modulated the gene expression of other miRNAs for which a role in the pancreas has not been previously described. There is very little available information describing the roles of these miRNAs. Data are available for only miR-483, which is a malignancy marker in adrenocorticol tumours in humans [21], and miR-30c-1*, which is associated with the recurrence of non-small-cell lung cancer following surgical resection in humans [22]. Our results suggest that miR-1249, miR-3544, miR-483, and miR-30c-1* may have functions in the pancreas and that GSPE might exert its effects on the pancreas via modulation of the gene expression of these miRNAs; however, more information concerning the function and targets of these miRNAs is necessary to further elucidate the roles of these miRNAs.

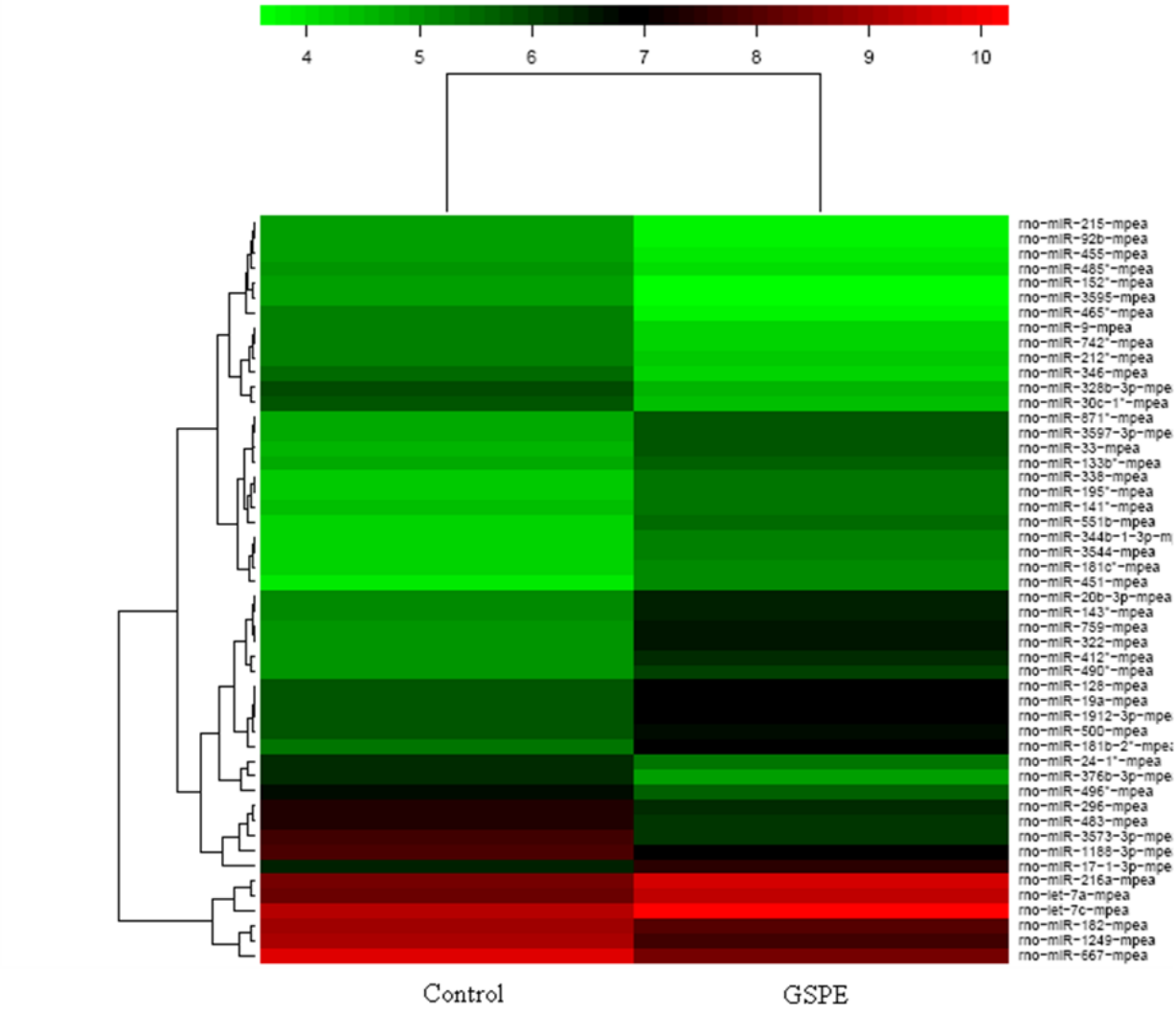

Figure 1. Differential expression of miRNAs in control rats vs GSPE-treated rats and a hierarchical clustering/heatmap of the 50 miRNA genes with higher logFC values. The 4 miRNA genes with significantly changed expression are included. Each row represents a miRNA, and each column represents an individual sample. The colorgram depicts high (red), average (black), and low (green) expression levels.

To investigate the function of these differentially expressed miRNAs, we analysed their putative target genes using the miRWalk database [23]. miR-1249 and miR-3544 do not have any validated or predicted targets. miR-483 has 4 validated and 1592 predicted targets, and miR-30c-1* has 19 validated and 2442 predicted targets. Nearly half of the validated targets of miR-483 and miR-30c-1* (Table 1) are related to apoptosis or proliferation in pancreatic islets. Moreover, most of the published studies concerning flavonoids and miRNA describe the activity of these phenolic compounds in cancer and, therefore, the involvement of these compounds in apoptosis and proliferation [24-26]. To complement these observations, we analysed whether GSPE plays a role in apoptosis and proliferation in the pancreas. Our immunohistochemistry results did not show any sign of apoptosis in the islets or the exocrine pancreas. The β-cell mass also remained unmodified by treatment with GSPE (0.72 ± 0.09 % vs. 0.85 ± 0.06 % for GSPE-treated and control cells, respectively). The gene expression of the anti-apoptotic marker Bcl2 showed a tendency ($p < 0.1$) to decrease (0.75 ± 0.09 vs. 1.02 ± 0.10) in the islets of rats treated with GSPE. Although the gene expression of the proliferation marker MKI67 remained statistically unchanged (0.71 ± 0.22 vs. 1.02 ± 0.11), that of cyclin D2 was significantly ($p < 0.05$) down-regulated by GSPE (0.77 ± 0.05 vs. 1.02 ± 0.10). In summary, nearly half of the validated targets of GSPE-modulated miR-483 and miR-30c-1* are related to apoptosis or proliferation processes. Additionally, GSPE slightly modulated the gene expression of Bcl2 and cyclin D2 in the islets, but it did not modulate the islet content or apoptosis in the pancreas.

Table 1. rno-miRNA-483 and rno-miRNA-30c-1* validated targets.

MiRNA	Official symbol	Name
rno-miR-483	Myc	Myelocytomatosis oncogene
	Mzf1	Myeloid zinc finger 1
	Nos2	Nitric oxide synthase 2, inducible
	Nos3	Nitric oxide synthase 3, endothelial cell
*rno-miR-30c-1**	Abcb1b	ATP-binding cassette, subfamily B (MDR/TAP), member 1B
	Akt1	v-akt murine thymoma viral oncogene homolog 1
	Bcl2	B-cell leukemia/lymphoma 2
	Egfr	Epidermal growth factor receptor
	F2	Coagulation factor II (thrombin)
	Gria2	Glutamate receptor, ionotropic, AMPA 2
	Id1	Inhibitor of DNA binding 1
	Inhba	Inhibin beta-A
	LOC503116	Similar to lin-28 homolog
	Met	Met proto-oncogene
	Nfyb	Nuclear transcription factor-Y beta
	Notch1	Notch homolog 1, translocation-associated (Drosophila)
	Smad2	SMAD family member 2
	Snca	Synuclein, alpha (non A4 component of amyloid precursor
	Sox2	(Sex determining region Y)-box 2
	Tgfbr1	Transforming growth factor, beta receptor 1
	Tp53	Tumor protein p53
	Xiap	X-linked inhibitor of apoptosis
	Xpo5	Exportin 5

We next performed an *in silico* prediction to investigate the functions of these predicted target genes using the ontology classification of genes based on the gene annotation and summary information that is available through DAVID. We restricted our analysis to the 599 common predicted and validated targets of miR-483 and miR-30c-1* because it has been shown that the effects of binding multiple miRNA complexes to the 3′-untranslated region (UTR) are likely to be cooperative; therefore, effects greater than those of a single miRNA in suppressing protein synthesis are expected [27]. The significantly enriched Gene Ontology (GO) terms in the common predicted miRNA target genes are listed in Table 2 and classified according to the “biological processes” in which they are involved. We observed GO enrichment for genes involved in the processes of ion transport and response to stimuli such as hormones and organic substances. Our previous studies support GSPE action through changes to membrane potentials [15]. The islets isolated from rats subject to the same treatment described above exhibited an altered glucose-stimulated insulin secretion. This alteration could partially be mediated by the modification of cell and mitochondrial membrane potentials by GSPE, which has been observed in INS-1E cells. The involvement of other miRNAs in the regulation of pancreas functionality by modulating membrane proteins has previously been shown. For example, miR-15a regulates insulin synthesis by inhibiting the expression of uncoupling protein-2 [10], a mitochondrial inner membrane uncoupler that modifies mitochondrial membrane permeability.

Also, the expression of the plasma membrane monocarboxylate transporter-1 is silenced in pancreatic β cells at least in part by miRNAs (miR-29a, miR-29b, and miR-124), thus affecting insulin release [28].

Table 2. The significantly enriched GO terms in the predicted common target genes of miR-483 and miR-30c-1*.

Biological process									***GO term***	***Genes in pathway***
Biological regulation	Regulation of biological process	Regulation of anatomical structure morphogenesis	Regulation of cell morphogenesis						0022604	14
		Regulation of cellular process								
		Regulation of cellular component organization								
Cellular process	Cellular component movement	Cell motility	Cell migration						0016477	19
	Cellular developmental process	Cell differentiation	Neuron differentiation						0030182	30
Developmental process	Anatomical structure development	Cell development	Neuron development						0048666	24
Localization	Establishment of localization	Transport	Ion transport						0006811	36
				Cation transport					0006812	28
					Metal ion transport				0030001	27
						Sodium ion transport			0006814	14
					Monovalent inorganic cation transport				0015672	22
						Sodium ion transport			0006814	14
			Neurotransmitter transport						0006836	14
Locomotion		Cell motility	Cell migration						0016477	19
Multicellular organismal process	System process	Neurological system process	Transmission of nerve impulse						0019226	20
Signalling	Multicellular organismal signalling									
Response to stimulus	Response to chemical stimulus	Response to organic substance							0010033	51
			Response to hormone stimulus						0009725	32
			Response to carbohydrate stimulus						0009743	12
				Response to monosaccharide stimulus					0034284	11
					Response to hexose stimulus				0009746	11
						Response to glucose stimulus			0009749	10
		Response to oxygen levels							0070482	16
	Response to endogenous stimulus								0009719	34
		Response to hormone stimulus							0009725	32

Therefore, the effects of GSPE on the functionality of the islets could, in part, be due to GSPE's effects on the expression profile of the miRNA, which would contribute to changes in the cell and mitochondrial membrane permeabilities by varying the expression of ion transport proteins. Finally, we also observed enrichments in genes with roles included in the category "neuron differentiation and neuron development". Some miRNAs are known to regulate the translation of genes that are involved in development of the central nervous system [29]. Observation of this result was unexpected in the present study because we focused on the pancreas. However, most of the genes classified in this GO category are also expressed in other tissues; thus, the putative modulation of these genes via miRNAs could be linked to other processes and not detected as being significantly modified in the present common set. One such process could be the differentiation of the islet cells because some of the genes found in these categories (e.g., Dlk, SDF-1) have been related to this process [30,31]. Consequently, although these results are interesting because they suggest that procyanidins may act on the nervous system, more studies in a more appropriate model must be carried out to confirm this hypothesis.

In conclusion, we show that a chronic GSPE treatment in rats modulates the miRNA gene expression pattern in pancreatic islets, down-regulating the expression of miR-1249, miR-30c-1*, and miR-483 and up-regulating that of miR-3544.The scarce knowledge about these miRNAs difficult to finely describe the consequences of their modulation by GSPE, our *in silico* prediction studies, in accordance with cell culture studies, suggest that ion transport and response to glucose might be among the pathways affected.

Acknowledgments

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References

[1] Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* . 2004*,116*,281-297.
[2] Lynn, F.C. Meta-regulation: microRNA regulation of glucose and lipid metabolism. *Trends Endocrinol.Metab.* . 2009*,20*,452-459.
[3] Herrera, B.M., Lockstone, H.E., Taylor, J.M., Ria, M., et al. Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes. *Diabetologia* . 2010*,53*,1099-1109.
[4] Kloosterman, W.P., Lagendijk, A.K., Ketting, R.F., et al. Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. *PLoS Biol.*. 2007*,5*,e203.
[5] Lynn, F.C., Skewes-Cox, P., Kosaka, Y., McManus, M.T., et al. MicroRNA expression is required for pancreatic islet cell genesis in the mouse. *Diabetes* . 2007*,56*,2938-2945.
[6] Plaisance, V., Abderrahmani, A., Perret-Menoud, V., et al. MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. *J.Biol.Chem.* . 2006*,281*,26932-26942.
[7] Guay, C., Roggli, E., Nesca, V., Jacovetti, C., Regazzi, R. Diabetes mellitus, a microRNA-related disease?. *Transl.Res.* . 2011*,157*,253-264.
[8] Lovis, P., Gattesco, S., Regazzi, R. Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. *Biol.Chem.* . 2008*,389*,305-312.
[9] Baroukh, N., Ravier, M.A., Loder, M.K., Hill, E.V., et al. MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic beta-cell lines. *J.Biol.Chem.* . 2007*,282*,19575-19588.
[10] Sun, L.L., Jiang, B.G., Li, W.T., Zou, J.J., et al. MicroRNA-15a positively regulates insulin synthesis by inhibiting uncoupling protein-2 expression. *Diabetes Res.Clin.Pract.* . 2011*,91*,94-100.

[11] Melkman-Zehavi, T., Oren, R., Kredo-Russo, S., Shapira, T., et al. miRNAs control insulin content in pancreatic beta-cells via downregulation of transcriptional repressors. *EMBO J.* . 2011,*30*,835-845.
[12] Lovis, P., Roggli, E., Laybutt, D.R., Gattesco, S., et al. Alterations in microRNA expression contribute to fatty acid-induced pancreatic beta-cell dysfunction. *Diabetes* . 2008,*57*,2728-2736.
[13] Bhagwat, S., Holden, J., Haytowitz, D., Gebhardt, S., et al. USDA database for the flavonoid content of selected foods. *Available from: http://www.ars.usda.gov/nutrientdata* .
[14] Pinent, M., Cedó, L., Montagut, G., Blay, M., Ardévol, A. Procyanidins improve some disrupted glucose homoeostatic situations: an analysis of doses and treatments according to different animal models. *Crit.Rev.Food Sci.Nutr.* . 2011. In press.
[15] Castell-Auví, A., Cedó, L., Pallarès, V., Blay, M., et al. Procyanidins modify insulinemia by affecting insulin production and degradation. *J.Nutr.Biochem.* . 2011. In press.
[16] Murase, T., Misawa, K., Minegishi, Y., Aoki, M., et al. Coffee polyphenols suppress diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice. *Am.J.Physiol.Endocrinol.Metab.* . 2011,*300*,E122-33.
[17] Boesch-Saadatmandi, C., Loboda, A., Wagner, A.E., Stachurska, A., et al. Effect of quercetin and its metabolites isorhamnetin and quercetin-3-glucuronide on inflammatory gene expression: role of miR-155. *J.Nutr.Biochem.* . 2011,*22*,293-299.
[18] Sun, Q., Cong, R., Yan, H., Gu, H., et al. Genistein inhibits growth of human uveal melanoma cells and affects microRNA-27a and target gene expression. *Oncol.Rep.* . 2009,*22*,563-567.
[19] Tsang, W.P., Kwok, T.T. Epigallocatechin gallate up-regulation of miR-16 and induction of apoptosis in human cancer cells. *J.Nutr.Biochem.* . 2010,*21*,140-146.
[20] Arola-Arnal, A., Blade, C. Proanthocyanidins Modulate MicroRNA Expression in Human HepG2 Cells. *PLoS One* . 2011,*6*,e25982.
[21] Patterson, E.E., Holloway, A.K., Weng, J., Fojo, T., Kebebew, E. MicroRNA profiling of adrenocortical tumors reveals miR-483 as a marker of malignancy. *Cancer* . 2011,*117*,1630-1639.
[22] Patnaik, S.K., Kannisto, E., Knudsen, S., Yendamuri, S. Evaluation of microRNA expression profiles that may predict recurrence of localized stage I non-small cell lung cancer after surgical resection. *Cancer Res.* . 2010,*70*,36-45.
[23] Dweep, H., Sticht, C., Pandey, P., Gretz, N. miRWalk - Database: Prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J.Biomed.Inform.* . 2011.
[24] Siddiqui, I.A., Asim, M., Hafeez, B.B., Adhami, V.M., et al. Green tea polyphenol EGCG blunts androgen receptor function in prostate cancer. *FASEB J.* . 2011,*25*,1198-1207.
[25] Tsang, W.P., Kwok, T.T. Epigallocatechin gallate up-regulation of miR-16 and induction of apoptosis in human cancer cells. *J.Nutr.Biochem.* . 2010,*21*,140-146.
[26] Sun, Q., Cong, R., Yan, H., Gu, H., et al. Genistein inhibits growth of human uveal melanoma cells and affects microRNA-27a and target gene expression. *Oncol.Rep.* . 2009,*22*,563-567.
[27] Doench, J.G., Petersen, C.P., Sharp, P.A. siRNAs can function as miRNAs. *Genes Dev.* . 2003,*17*,438-442.
[28] Pullen, T.J., da Silva Xavier, G., Kelsey, G., Rutter, G.A. miR-29a and miR-29b Contribute to Pancreatic β-Cell-Specific Silencing of Monocarboxylate Transporter 1 (Mct1). *Mol.Cell.Biol.* . 2011,*31*,3182-3194.
[29] Sempere, L.F., Freemantle, S., Pitha-Rowe, I., Moss, E., et al. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.* . 2004,*5*,R13.
[30] May, R., Sureban, S.M., Lightfoot, S.A., Hoskins, A.B., et al. Identification of a novel putative pancreatic stem/progenitor cell marker DCAMKL-1 in normal mouse pancreas. *Am.J.Physiol.Gastrointest.Liver Physiol.* . 2010,*299*,G303-10.
[31] Cheng, H., Zhang, Y.C., Wolfe, S., Valencia, V., et al. Combinatorial treatment of bone marrow stem cells and stromal cell-derived factor 1 improves glycemia and insulin production in diabetic mice. *Mol.Cell.Endocrinol.* . 2011.

SUPPORTING MATERIAL

Materials and methods

Chemicals. According to the manufacturer, GSPE (Les Dérives Résiniques et Terpéniques, Dax, France) contained monomeric (16.6 %), dimeric (18.8 %), trimeric (16.0 %), tetrameric (9.3 %), and oligomeric procyanidins (5 to 13 units, 35.7 %) as well as phenolic acids (4.2 %).

Animal procedures. Female Wistar rats weighing 225-250 g were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22ºC with 12 h light/12 h dark cycles. Treatment began after the rats had spent 1 week in quarantine. The animals were divided into two groups a control group and a group treated for 45 days with 25 mg GSPE /kg of body weight (bw) * day. The rats' food (standard chow) was withdrawn at 8 a.m. every day, and, at 8 p.m., the rats were treated with GSPE or its vehicle (sweetened condensed milk diluted 1:6 with tap water), and the food was renewed. On the sacrifice day, the animals, which had fasted overnight, were anesthetised at 9 a.m. using pentobarbital sodium (75 mg/kg of bw) and then sacrificed by exsanguination. The blood was collected and the pancreatic islets were isolated from ten animals per group, and one half of the pancreas from six rats per group were fixed overnight in 4% (w/v) formaldehyde (QCA, Amposta, Spain) and embedded in paraffin. This procedure was approved by the Experimental Animals Ethics Committee of the Universitat Rovira i Virgili (Permission number from Government of Catalonia: 4250).

Islet isolation. The islets were prepared by collagenase digestion as previously described. Briefly, the rats were anesthetised, and the pancreas was infused with 7 mL of an ice-cold collagenase P (Roche, Barcelona, Spain) solution (1 mg/mL) before its removal and incubation at 37 ºC for 15 min. The islets were purified on a Histopaque gradient (Sigma-Aldrich, St. Louis, MO) and handpicked until a population of pure islets was obtained.

miRNA profile analysis. The total RNA from freshly isolated islets was extracted using the Qiagen miRNeasy isolation kit (Qiagen, Barcelona, Spain) and stored at -80ºC. The quality of the total RNA was determined with an Agilent 2100 Bioanalyzer using the RNA 6000 Nano kit according to the manufacturer's instructions. All samples have Abs 260/280 ~2.0 and Abs 260/230 > 1.4. The samples were analysed with a Geniom Real-time Analyser (GRTA, febit GmbH, Heidelberg, Germany) using the Geniom Biochip MPEA *Rattus norvegicus*. The probes were designed as the reverse complements of all of the major mature miRNAs and the mature sequences as published in the current Sanger miRBase release (version 16.0 September 2010 for *Rattus norvegicus*) [1]. For each array, the RNA was suspended in febit's proprietary miRNA hybridisation buffer (25 µL per array). Hybridisation was performed automatically for 16 h at 42 ºC using GRTA. Next, the biochip was stringently washed. Following the labelling procedure, febit was applied to the microfluidic-based primer extension assay [2]. This assay utilises the bound miRNAs as a primer for the enzymatic elongation using labelled nucleotides. The elongation was performed with Klenow fragments and biotinylated nucleotides at 37ºC for 15 minutes. Finally, the biochip was washed automatically. For maximum sensitivity, febit used biotin and its detection with streptavidin-phycoerythrin (SAPE) in combination with febit's consecutive signal enhancement (CSE) procedure. The feature recognition (using a Cy3 filter set) and signal calculation were performed automatically within milliseconds. The Geniom technology provided accurate detection of the miRNA profiles. The microarray data were normalised by the variance stabilisation normalisation method [3], and statistics were analysed with linear models as implemented in the Limma Bioconductor package [4]. The miRNAs with adjusted p-values less than 0.2 were considered significant.

Pathway analysis and prediction. The validated and predicted target genes for rno-miRNAs that had statistically significant modifications to their expression after GSPE treatment were obtained from the online database miRWalk [5]. A miRWalk miRNA target prediction was provided by the match among

eight established miRNA prediction programs on 3' UTRs (RNA22, miRanda, miRDB, TargetScan, RNAhybrid, PITA, PICTAR, and Diana-microT) with a p-value less than 0.05.

To determine the functions of the common predicted and validated target genes, we used DAVID (Database for Annotation, Visualisation and Integrated Discovery) [6]. This database allowed us to assign predicted target genes to functional groups based on molecular function, biological process and specific pathways. The GO terms with p-values less than 0.05 after adjustment using the Benjamini method were considered significantly enriched.

Measurement of mRNA expression of apoptosis and proliferation markers. For gene expression experiments, the total RNA from freshly isolated islets was extracted as described above. A total RNA of 0.5-1 µg was reverse transcribed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), and cDNA was amplified for 40 cycles in a quantitative RT-PCR 7300 System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions using a Taqman Master Mix and the specific Taqman probes for different genes (Applied Biosystems, Foster City, CA): Rn99999125_m1 for Bcl2, Rn01492401_m1 for cyclin D2 (CCND2), Rn01451446_m1 for MKI67 and Rn00667869-m1 for β-actin as the reference gene. The relative mRNA expression levels were calculated using the ΔΔCt method.

Immunohistochemical analysis of apoptosis and pancreatic islet mass. Each block of the pancreas that had been embedded in paraffin was serially sectioned throughout its length in cuts of 6 µm and mounted on slides. One section of every interval of approximately 40 slides was deparaffinated, rehydrated and used to study apoptosis. Apoptotic cells were detected with the ApopTag Peroxidase In Situ Apoptosis Detection kit (Millipore, Billerica, MA) as previously described [7].

Pancreas sections were then immunostained for insulin. First, they were blocked for 50 min at room temperature with goat serum (Vector Laboratories, Burlingame, CA) diluted 1:10 with Tris buffer and incubated overnight at 4ºC with an anti-insulin antibody from guinea pig (MP Biomedicals, Illkirch, France) diluted 1:300 with Tris buffer. After being washed, the samples were incubated for 1 h at room temperature with an alkaline phosphatase-conjugated polyclonal anti-guinea pig antibody from rabbit (Abcam, Cambridge, UK) diluted 1:200 with Tris buffer and stained using an Alkaline phosphatase Substrate kit I (Vector Laboratories, Burlingame, CA). After being stained, the sections were dehydrated and mounted in Eukitt (Labonord, Templemars, France). In these double-stained sections, β-cells were stained in red, and apoptotic cells exhibited dark nuclei.

The analysis of the sections was performed using an Olympus BX40 microscope in conjunction with a video camera connected to a computer and using the Histolab software v. 7.2.7 (Microvision Instruments, Evry, France). The number of apoptotic cells, the surface area of the islets and the total pancreatic surface area were quantified in each stained section. Apoptosis was expressed as the number of apoptotic cells/total pancreas surface area (μm^2) multiplied by 10^7, and the β-cell mass was expressed as the percentage of the ratio between the β-cell surface area and the total pancreatic surface area.

Calculations and Statistical Analysis. The results are expressed as the mean ± SEM. Effects were assessed by Student's *t*-test. All calculations were performed with SPSS software.

References

[1] Griffiths-Jones, S., Saini, H.K., van Dongen, S., Enright, A.J. miRBase: tools for microRNA genomics. *Nucleic Acids Res.* . 2008*,36*,D154-8.
[2] Vorwerk, S., Ganter, K., Cheng, Y., Hoheisel, J., et al. Microfluidic-based enzymatic on-chip labeling of miRNAs. *N.Biotechnol.* . 2008*,25*,142-149.

[3] Huber, W., von Heydebreck, A., Sultmann, H., Poustka, A., Vingron, M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression*. Bioinformatics* . 2002*,18 Suppl 1*,S96-104.

[4] Smyth, G.K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments*. Stat.Appl.Genet.Mol.Biol.* . 2004*,3*,Article3.

[5] Dweep, H., Sticht, C., Pandey, P., Gretz, N. miRWalk - Database: Prediction of possible miRNA binding sites by "walking" the genes of three genomes*. J.Biomed.Inform.* . 2011.

[6] Huang da, W., Sherman, B.T., Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nat.Protoc.* . 2009*,4*,44-57.

[7] Movassat, J., Portha, B. Early administration of keratinocyte growth factor improves {beta}-cell regeneration in rat with streptozotocin-induced diabetes*. J.Endocrinol.* . 2007*,195*,333-340.

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GSPE Improves β-cell Functionality Under Lipotoxic Conditions Due to Its Lipid-Lowering Effect

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CHAPTER 4

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ABSTRACT

Procyanidins have positive effects on glucose metabolism in situations involving slightly disrupted glucose homeostasis, but it is not clear how procyanidins interact with β-cells. On this work, we evaluate the effects of procyanidins on β-cell functionality under an insulin-resistance situation. After 13 weeks of cafeteria diet, female Wistar rats were treated with 25 mg of grape seed procyanidin extract (GSPE)/kg of body weight for 30 days. To determine the possible mechanisms of action of procyanidins, INS-1E cells were separately incubated in high glucose, high insulin and high oleate media to reproduce the conditions the β-cells were subject to during the cafeteria diet feeding. *In vivo* experiments showed that chronic GSPE treatment decreased insulin production, since C-peptide levels and insulin protein levels in plasma were lower than those of cafeteria-fed rats, as were insulin and Pdx1 mRNA levels in the pancreas. GSPE effects observed *in vivo* were reproduced in INS-1E cells cultured with high oleate for 3 days. GSPE treatment significantly reduces triglyceride content in β-cells treated with high oleate and in the pancreas of cafeteria-fed rats. Moreover, gene expression analysis of the pancreas of cafeteria-fed rats revealed that procyanidins up-regulated the expression of Cpt1a and down-regulated the expression of lipid synthesis-related genes such as Fasn and Srebf1. Procyanidin treatment counteracted the decrease of AMPK protein levels after cafeteria treatment. Procyanidins cause a lack of triglyceride accumulation in β-cells. This counteracts its negative effects on insulin production, allowing for healthy levels of insulin production under hyperlipidemic situations.

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Introduction

Procyanidins have positive effects on glucose metabolism in situations of slightly disrupted glucose homeostasis [1], a property that makes these compounds very interesting as functional food ingredients. Part of this effect could be explained due to the activity of procyanidins on adipose cells [2], but in fact, in a rat cafeteria-diet model, grape seed procyanidins extract (GSPE)-treated animals had fewer instances of insulinemia and glycemia than did the cafeteria group. Literature analysis indicated that the mechanism of the interaction of procyanidins with β-cells is not completely understood [3]. On the other hand, we recently observed that, at some doses, procyanidins change β-cell functionality, modifying insulin synthesis and secretion under non-pathological situations [4], through their effects on membrane potentials.

A cafeteria diet allows for development of insulin resistance with hyperglycemia and hypertriglyceridemia conditions, and it is thus a good model for most syndrome X human pathologies [5]. Peripheral tissues play a key role in these pathologies, working together with pancreatic β-cells. In situations of insulin resistance, β-cells are in high glucose and high fatty acid conditions, and published studies have shown that prolonged exposure of pancreatic islets to elevated concentrations of fatty acids reduces insulin secretion *in vitro* [6,7]. This has also been implicated in the declining insulin secretory capacity of the β-cell, which accompanies the beginning of type 2 diabetes [8]. Like fatty acids, chronic hyperglycemia in β-cells causes defective insulin gene expression, diminished insulin content and defective insulin secretion [9]. While elevated levels of glucose or fatty acids can, by themselves, have detrimental effects on β-cell function in many experimental systems, the combination of both nutrients is synergistically harmful, and the term glucolipotoxicity has been coined to describe the phenomenon [10,11].

In the present study, our goal is to understand the relationship between procyanidins and insulin-producing cells under an insulin resistance situation. We first determine whether procyanidin extract could alleviate the deleterious effects of cafeteria diet on β-cell functionality *in vivo*. To analyze the biochemical mechanism of this postulated effect, we assess the actions of GSPE on β-cells cultured in high glucose, high insulin and high fatty acid media.

Materials and methods

Chemicals. According to the manufacturer, GSPE (Les Dérives Résiniques et Terpéniques, Dax, France) contained monomeric (16.6%), dimeric (18.8%), trimeric (16%), tetrameric (9.3%) and oligomeric procyanidins (5-13 units: 35.7%) and phenolic acids (4.22%).

Cell culture and treatment. INS-1E cells were kindly provided by Prof. Pierre Maechler, University of Geneva [12]. The cell line was cultured as previously described [13]. Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium). Three different models were assayed. 1) High glucose treatment: The cells were incubated with 25 mM glucose for 24 h with 5 or 25 mg/L of GSPE. 2) High insulin treatment: After 24 h of depletion, the cells were incubated for 24 h with 20 nM insulin (Novo Nordisk Pharma SA, Madrid, Spain) and with 1, 5 or 25 mg/L of GSPE. 3) High oleate treatment: Cells were cultured for 72 h with 0.4 mM oleate (stock solution: 10 mM oleate (Sigma-Aldrich, St. Louis, MO) dissolved in 12.5 % fatty acid-free BSA (Sigma-Aldrich, St. Louis, MO) [14] and during the last 24 h, cells were treated concomitantly with 25 mg GSPE/L.

Animal experimental procedures. For the cafeteria-fed animal study (6 animals per group), the animals were treated as previously described [2]**.** Briefly, female rats were divided into two groups: a control group fed with a standard diet (Panlab A03) and a cafeteria-fed group fed with a cafeteria diet and water plus the standard diet. After 13 weeks, obesity was induced in the animals and the cafeteria group was divided into two subgroups: i) cafeteria group of rats treated with a vehicle (sweetened condensed milk) and ii)

cafeteria + 25 group of rats treated with 25 mg of GSPE/kg of body weight (bw)*day. After 10 days of GSPE treatment, six animals from each group were sacrificed (Short treatment). After 30 days of GSPE treatment, the remaining six animals of each group were sacrificed (Long treatment). For the high-fat fed animal study (6 animals per group), the animals were treated as previously described [15]. Briefly, male rats were fed with a high-fat diet (control) or with a high-fat diet containing 1 mg of GSPE per gram of feed (approximately 30 mg GSPE/ kg of bw). After 19 weeks of treatment, animals were sacrificed. Blood was collected from all the animals using heparin and animal tissues were excised, frozen immediately in liquid nitrogen and stored at -80ºC until analysis. All the procedures were approved by the Experimental Animals Ethics Committee of the Rovira i Virgili University. Insulin and C-peptide plasma levels were assayed using ELISA methodology (Mercodia, Uppsala, Sweden) following the manufacturers' instructions. Glucose plasma levels were determined using an enzymatic colorimetric kit (GOD-PAP method from QCA, Amposta, Spain).

Glucose-stimulated insulin secretion. The secretory responses to glucose were tested in INS-1E cells as previously described [13]. Glucose stimulated insulin secretion (GSIS) was measured by Insulin ELISA (Mercodia, Uppsala, Sweden).

Triglyceride (TG) content. INS-1E cells were cultured in 12-well plates and treated with 0.4 mM oleate for 3 days. During the last 24 hours of oleate treatment, cells were incubated concomitantly with 25 mg/L of GSPE. Cells were collected in PBS containing 0.1% triton X-100 (Sigma-Aldrich, St. Louis, MO) and the solution was sonicated. TGs from the pancreas were extracted using the same buffer. TG content was determined using an enzymatic colorimetric kit (QCA, Amposta, Spain). Protein content of each sample was measured using the Bradford method [16] and was used to normalize the TG values.

Mitochondrial membrane potential ($\Delta\Psi_m$) and cell membrane potential measurements. Mitochondrial membrane potential ($\Delta\Psi_m$) and cell membrane potential were measured as described [12].

Western Blot. Protein was extracted from the whole frozen pancreas using RIPA lysis buffer (15 mM Tris-HCl, 165 mM NaCl, 0.5% Na-deoxycholate, 1% Triton X-100 and 0.1% SDS), with a protease inhibitor cocktail (1:1,000; Sigma-Aldrich, St. Louis, MO) and 1 mM PMSF. Total protein levels of the lysate were determined using the Bradford method [16]. Proteins were loaded and run through a 12 % SDS-polyacrylamide gel. Samples were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA), blocked at room temperature using 5% (wt/vol) non-fat milk in TTBS buffer (Tris-buffered saline [TBS], 0.5% [vol/vol] Tween-20) and incubated with rabbit AMPKα primary antibody (Cell Signaling Technology, Beverly, MA), or anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO). After washing with TTBS, blots were incubated with peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK). Blots were washed thoroughly in TTBS, followed by TBS after immunoblotting. Immunoreactive protein was visualized with ECL Plus Western blotting detection system (GE Healthcare, Buckinghamshire, UK) on the Alpha Innotech system with software version 6.0.2v (San Leonardo, CA). Densitometric analysis of immunoblots was performed using ImageJ 1.44p software; all proteins were quantified relative to the loading control.

Quantitative RT-PCR. Total RNA from pancreas, liver, kidney and white adipose tissue was extracted using TRIzol reagent following the manufacturer's instructions. Total RNA from INS-1E cells was isolated using an miRNeasy Mini Kit (Qiagen, Barcelona, Spain). cDNA from all the experiments was generated using the Applied Biosystems' kit and it was subjected to Quantitative RT-PCR amplification using Taqman Master Mix (Applied Biosystems, Foster City, CA). Specific Taqman probes (Applied Biosystems, Foster City, CA) were used for different genes: Rn01774648-g1 for insulin, Rn00565839-m1 for insulin degrading enzyme (Ide), Rn00755591_m1 for pancreatic duodenal homeobox 1 (Pdx1), Rn00561265-m1 for glucokinase, Rn01754856-m1 for mitochondrial uncoupling protein 2 (Ucp2), Rn00569117_m1 for fatty acid synthase (Fasn), Rn01495769_m1 for sterol regulatory element-binding

protein 1c (Srebf1), Rn00580702_m1 for carnitine palmitolitransferase-1a (Cpt1a) and Rn00440945_m1 for peroxisome proliferator-activated receptor γ (Pparγ). β-actin was used as the reference gene (Rn00667869-m1). Reactions were run on a quantitative RT-PCR 7300 System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Calculations and Statistical Analysis. Results are expressed as the mean ± SEM. Effects were assessed by Student's *t*-test. All calculations were performed with SPSS software.

Results

GSPE decreases insulin production.

For animals in which we previously induced damage by cafeteria-diet treatment for 13 weeks, 30 days of daily treatment with 25 mg GSPE/kg of bw improved glycemia and lowered insulinemia [2]. Peripheral effects were seen in the adipose tissue of these animals [2], and now we show that β-cell insulin production is lower, with an even stronger effect on mRNA levels (Table 1).

Table 1. Gene expression in the pancreas of cafeteria-fed rats treated with GSPE.

Gene	Cafeteria	Cafeteria + GSPE
Insulin	5.11 ± 1.4	1.48 ± 0.0*
Pdx1	4.07 ± 1.0	2.32 ± 0.3
Ucp2	1.64 ± 0.3	0.65 ± 0.1*
Cpt1a	1.63 ± 0.2	2.20 ± 0.3*
Fasn	0.75 ± 0.1	0.31 ± 0.0*
Srebf1	1.28 ± 0.1	1.03 ± 0.1

Rats were fed with a cafeteria diet for 13 weeks and then were orally treated with 25 mg GSPE/kg of bw for 30 days. Data are the mean ± SEM of six animals. *Indicates statistically significant differences between treatments (P ≤ 0.05).

The amount of insulin protein levels in the pancreas and the C-peptide levels in the plasma were also lower (Figure 1A and B, respectively). In fact, GSPE-treated rats had insulin gene expression and C-peptide levels similar to those of the control group. The strong effect on insulin synthesis agrees with the decrease in levels of the upstream insulin effector Pdx1 (Table 1), despite not statistically significant differences were observed. Must be highlighted that the Pdx1 mRNA levels from cafeteria group were not different compared with levels in the control group [17]. However, we did observe a decrease of Ucp2 gene expression (Table 1).

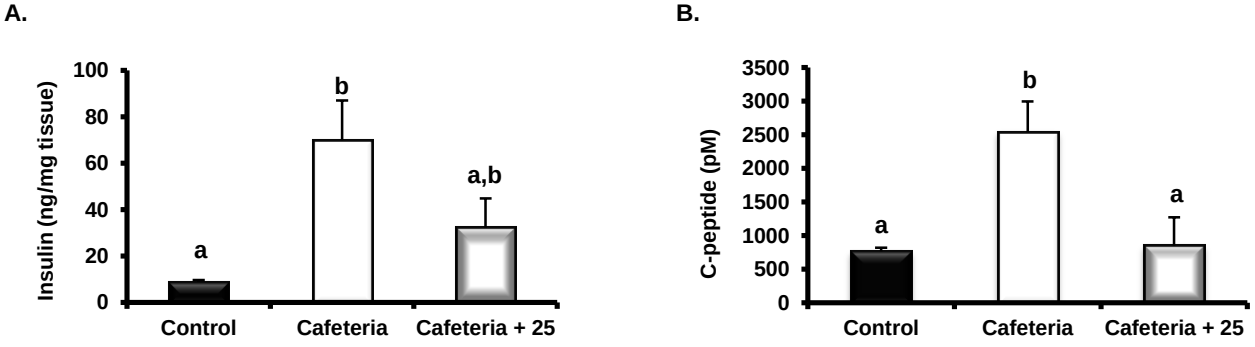

Figure 1. Insulin production after 30 days of GSPE treatment of cafeteria-fed animals. ***A.*** Pancreas insulin content. ***B.*** Peptide-C plasma levels. After 30 days of treatment, animals were sacrificed, and blood and pancreas samples were obtained. Pancreas insulin content and Peptide-C plasma levels were quantified by the ELISA method. Data are means ± SEM. Different letters indicate significantly different groups with *P*<0.05.

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Insulin plasma levels depend on insulin production but also on insulin clearance. In normal healthy animals, we have shown insulin-degrading enzyme (Ide) to be a target for GSPE [4]. However, although the cafeteria diet modifies the activity and expression of Ide in liver and white adipose tissue [17], GSPE treatment did not have any effects on insulin clearance (results not shown).

The effects of GSPE on glucose homeostasis are very dependent on the degree of damage [1]. Thus, to gain further evidence of the effects of GSPE on the pancreas as described above, we analyzed relevant data from other animal models. A similar study that used a shorter GSPE treatment of 10 days did not show a statistically significant effect on insulin mRNA, but there was a tendency towards decreased gene expression (Cafeteria: 1.07 ± 0.2; Cafeteria + 25: 0.76 ± 0.1; *vs*. Control: 1.16 ± 0.3). When we compared the insulin plasma levels to mRNA expression levels, there was a statistically significant increase due to GSPE treatment, suggesting a limited production versus the amount of circulating insulin (Cafeteria: 1.05 ± 0.3; Cafeteria + 25: 1.83 ± 0.2; *vs*. Control: 0.63 ± 0.2). This parameter was also clearly increased after 30 days of GSPE treatment (Cafeteria: 1.50 ± 0.4; Cafeteria + 25: 2.70 ± 0.7; *vs*. Control: 1.22 ± 0.3).

By contrast, an equivalent dose of GSPE simultaneously administrated with feed pellets in a high-fat diet (HF) to another group of rats did not cause any statistically significant change in insulin measurements. Despite a tendency towards lower mRNA levels (HF + 30: 0.94 ± 0.2; *vs*. HF: 1.32 ± 0.3), plasma insulin levels were unchanged (HF + 30: 5.20 ± 0.5; *vs*. HF: 4.77 ± 0.8), as was the ratio of plasma insulin to mRNA insulin (HF + 30: 5.95 ± 1.2; *vs*. HF: 5.46 ± 1.5). It must be highlighted that this third model showed only moderate signs of glucose homeostasis disruption [15]. By contrast, cafeteria animal models showed almost all the metabolic syndrome alterations: hyperglycemia, hyperinsulinemia [2] and increased plasma free fatty acids [18].

The effects of GSPE on insulin production can be explained through its action on lipid metabolism in β-cells.

β-cells of cafeteria animals live in a high glucose, high insulin and high FFA environment that affect their functionality [6,7,19]. We reproduced these three effects separately in cultured β-cells to identify where procyanidins act in limiting insulin production. High glucose medium for 24 hours provoked a very high decrease in insulin mRNA levels that was not counteracted by GSPE treatment (Control 11 mM glucose: 2.13 ± 0.0; 25 mM glucose + 5 mg GSPE/L: 1.01 ± 0.1; 25mM glucose + 25 mg GSPE/L: 0.96 ± 0.1; *vs*. Control 25 mM glucose: 1.00 ± 0.0). Glucokinase mRNA was also decreased by hyperglycemia, an effect that was statistically reinforced by concomitant treatment with 25 mg GSPE/L (Control 11 mM glucose: 1.38 ± 0.0; 25 mM glucose + 5 mg GSPE/L: 0.99 ± 0.0; 25mM glucose + 25 mg GSPE/L: 0.87 ± 0.0; *vs*. Control 25 mM glucose: 1.00 ± 0.0). High insulin treatment for 24 hours did not modify insulin mRNA levels, but concomitant GSPE treatment induced a tendency to increase insulin gene expression, being only statistically significant at 5 mg GSPE/L (20 nM insulin: 0.96 ± 0.1; 20 nM insulin + 1 mg GSPE/L: 1.06 ± 0.1; 20 nM insulin + 5 mg GSPE/L: 1.13 ± 0.1; 20 nM insulin + 25 mg GSPE/L: 1.06 ± 0.1; *vs*. Control: 1.00 ± 0.0)

Table 2 shows the effects of GSPE on high oleate culture medium. In this situation, there was an increase in insulin mRNA and GSPE limited this gene expression increase similar to what we observed in the *in vivo* studies. Glucokinase mRNA showed a similar pattern: levels were increased by oleate and GSPE limited the oleate effect. Pdx1 gene expression was unmodified by oleate treatment (Oleate: 0.97 ± 0.03; Oleate + GSPE: 0.96 ± 0.06; *vs*. Control: 1.00 ± 0.03). On the other hand, Ucp2 mRNA levels were up-regulated by oleate (Table 2), as was expected because Ucp2 expression is regulated in tandem with the level of FFA [20], and in isolated rat islets and INS-1 pancreatic β-cells, long term treatment with FFAs can increase Ucp2 mRNA [21,22]. However, in this case, GSPE did not mitigate the effects of oleate on Ucp2 mRNA levels.

Table 2. Gene expression in INS-1E cells treated with oleate and GSPE.

Gene	Control	Oleate	Oleate + GSPE
Insulin	1.00 ± 0.0	1.64 ± 0.1*	1.24 ± 0.1*,‡
Glucokinase	1.00 ± 0.0	1.20 ± 0.1*	1.08 ± 0.1
Ucp2	1.00 ± 0.0	1.86 ± 0.2*	1.71 ± 0.1*

INS-1E cells were cultured for 3 days in the absence of oleate or with 0.4 mM oleate (0.4 mM). During the last 24 h, the cells were cultured in the absence or presence of 25 mg GSPE/L. Data are the mean ± SEM. *Indicates a significant difference (P ≤ 0.05) *vs.* control group. ‡Indicates a significant difference (P ≤ 0.05) *vs.* oleate group.

Thus, out of all the conditions assayed for culture of β-cells, only hyperlipidemia reproduced the effects we had obtained *in vivo*, i.e., the cafeteria diet induced high insulin expression levels that could be counteracted by addition of procyanidins. Moreover, high oleate treatment altered insulin secretion, mainly basal secretion, but also the GSIS (Figure 2A), in agreement with previous publications [21,23].

GSPE treatment slightly improved the oleate effect on insulin secretion (Figure 2A). Therefore, GSPE seems to act on β-cell lipid metabolism to exert its bioactivity on insulin production.

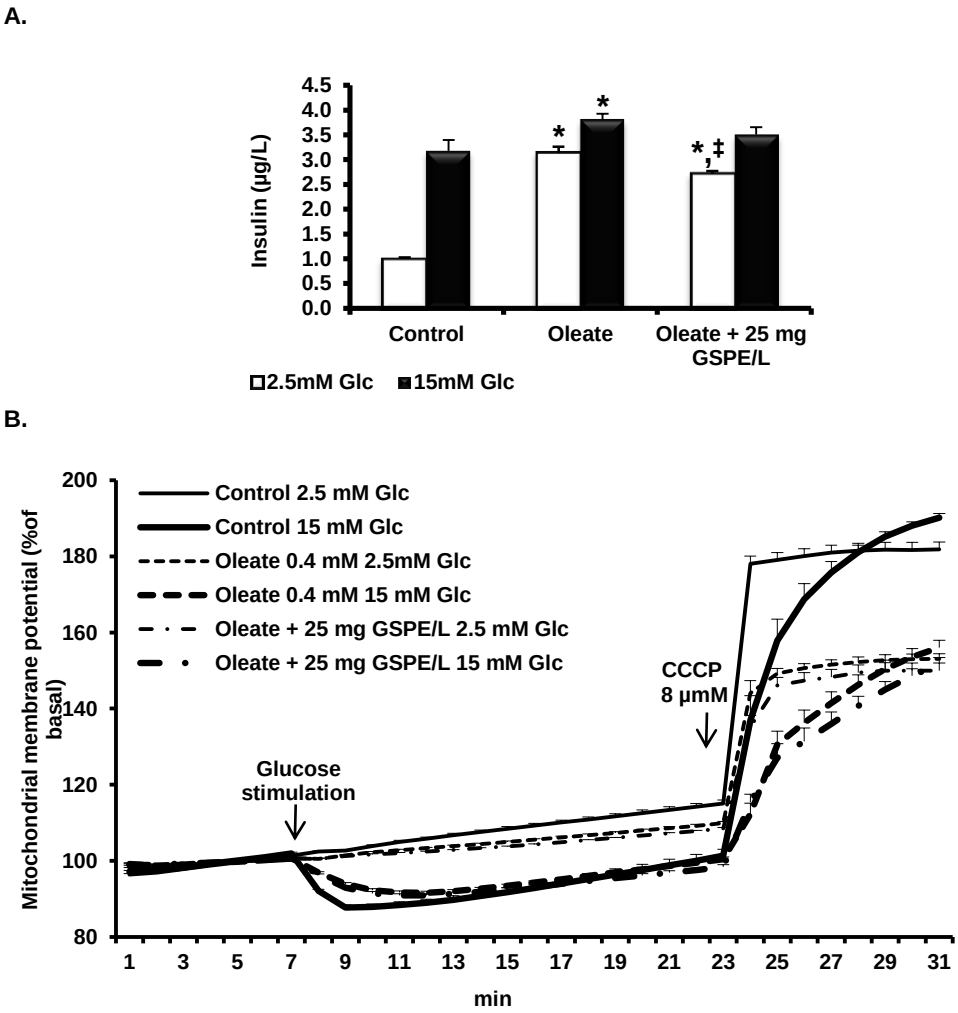

Figure 2. Effects of GSPE and oleate on insulin secretion. ***A.*** After oleate and GSPE treatment, cells were maintained for 2 h in RPMI w/o glucose medium and then cells were cultured with basal (2.5 mM) or stimulated (15 mM) glucose concentrations. Data shown are means ±SEM. *Indicates significantly different groups with *P*<0.05 *vs*. control group (same glucose concentration). ‡Indicates significantly different groups with *P*<0.05 *vs*. oleate group (same glucose concentration). ***B.*** Mitochondrial membrane potential was monitored with rhodamine 123 fluorescence. Hyperpolarization of $\Delta\Psi_m$ was induced with 15 mM glucose and after 10 min of glucose stimulation, depolarization was induced by FCCP.

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Mechanism of action of GSPE on β-cells under hyperlipidemic stress.

Data regarding how procyanidins affect β-cells is limited [3]. Working on an undamaged cell line (INS-1E), we have found that GSPE alters insulin secretion through its uncoupling action on cell membranes [4]. Oleate also uncouples mitochondrial plasma membrane potential [14,21]. To identify the target sites of GSPE on β-cells, we measured the mitochondrial membrane potential under oleate and GSPE treatment. We reproduced the expected uncoupling action induced by oleate, but GSPE did not reverse this effect (Figure 2b). Thus, GSPE must use other mechanisms to improve the function of damaged oleate cells.

One of the most obvious effects of GSPE is its ability to improve lipid metabolism [24]. Under oleate treatment, β-cells have higher levels of TG stores [25], so we measured the TGs accumulated in β-cells. Oleate treatment doubles the amount of TGs (2.05 ± 0.1) *vs*. control cells (1.00 ± 0.0), while GSPE slightly but statistically significantly decreases the amount of TGs in the cell (observed decrease: -0.096 ± 0.03).

We next checked the pancreas TG content of cafeteria-fed animals and found that GSPE reduced it significantly after 30 days of treatment (Figure 3). We also analyzed the differential expression of key genes that control lipid metabolism in the pancreas (Table 1).

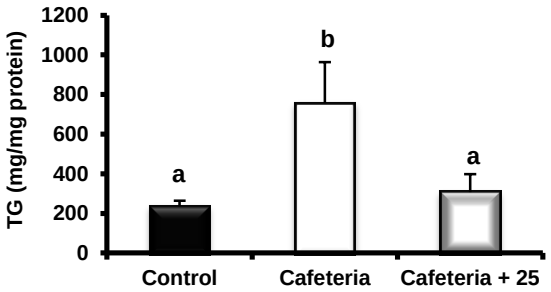

Figure 3. Effects of GSPE on TG content in the pancreas of cafeteria-fed rats. TGs from β-cells and pancreas tissue were quantified using an enzymatic colorimetric kit. Data shown are means ± SEM. Different letters indicate significantly different groups with $P<0.075$ *vs*. control.

We selected the Cpt1a gene, which is the key controller of free fatty acid oxidation [26], the Fasn gene, which is the key enzyme of *de novo* fatty acid synthesis [27] and the Srebf1 gene, a transcription factor that activates the expression of several genes involved in FFA and TG synthesis, as well as other components of the regulatory machinery of lipid metabolism [28]. Cafeteria-fed animals showed a slight increase in Cpt1a gene expression [17], suggesting an increase of β-oxidation, and GSPE treatment caused a higher increase in Cpt1a mRNA levels (Table 1). Fatty acid synthase levels, which were reduced by a cafeteria diet (submitted results), were significantly decreased with GSPE treatment as shown by Fasn gene expression (Table 1). GSPE treatment tended to reduce the mRNA levels of Srebf1 after 30 days of treatment (Table 1). These data agreed with the lipid-mobilization effect attributed to the GSPE.

To better understand how procyanidins modify β-cell functionality, we assessed whether the effects of GSPE on β-cells were mediated via AMP-activated protein kinase (AMPK). When we analyzed AMPK protein levels (Figure 4), we observed that a cafeteria diet produces a significant decrease in the levels of this protein in the pancreas, which was counteracted by GSPE treatment.

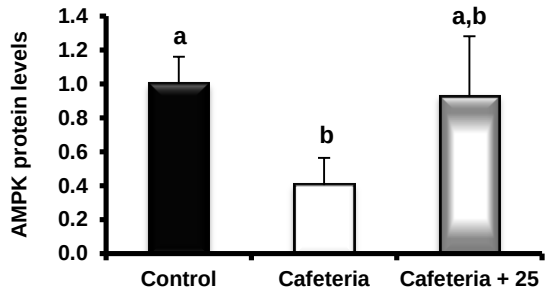

Figure 4. Effects of GSPE on AMPK protein levels in the pancreas of cafeteria-fed rats. AMPK protein levels were quantified by western blot analysis. The protein expression was quantified relative to the β-actin loading control using ImageJ 1.44p software. The data are presented as the mean ± SEM. Different letters indicate significantly different groups with $P<0.05$.

Discussion

Procyanidins have clear and well-defined beneficial, protective effects against most risk factors of metabolic syndrome, and they have been shown to have positive effects on glucose metabolism under situations of slightly disrupted glucose homeostasis [1]. We have previously shown that GSPE acts peripherally on adipose cells to improve glycemia, which leads to lower insulinemia in cafeteria-fed rats [2]. However, there is limited data regarding the effects of procyanidins on β-cells [3]. Taking into account that β-cells are responsible for maintaining glucose homeostasis by synthesizing and secreting insulin, the purpose of this study was to understand the effects of procyanidins on β-cell functionality under an insulin resistance situation.

Our results showed that rats fed with a cafeteria diet for 13 weeks and treated with 25 mg GSPE/kg of bw for 30 days had decreased insulin production. Studies with other flavonoids also showed reduced insulinemia. Ihm et al. showed that chronic intake of catechin for 12 weeks in the prediabetic stage significantly reduces insulin plasma levels [29]. This study was performed with the Otsuka Long-Evans Tokushima Fatty (OLETF) rat model, a distinct model of type 2 diabetes that has some characteristic features, such as late onset of hyperglycemia, hyperinsulinemia and obesity [30]. Similar to our results, the phenolic acids chlorogenic acid and caffeic acid administered with high-fat diet significantly lowered plasma insulin levels compared to the high-fat diet group [31]. In a similar way, a 4-week treatment with bitter melon extract, traditionally used as an antidiabetic, is effective for improving insulin resistance in a mouse model of Type 2 diabetes (animals fed with a high-fat diet for 12 weeks) by reducing blood glucose and insulin [32]. The authors suggested that the extract regulates the Ppars-ediated pathway, because thiazolidinedione (TZD), a synthetic Pparγ ligand that significantly increases insulin sensitivity via Pparγ, actually causes improved insulin sensitivity in a high-fat diet [33,34]. Insulin sensitivity is highly dependent on the peripheral actions of compounds. GSPE also proved to be effective at working through Pparγ in adipose tissue [2,35], but Pparγ also plays a role in pancreas tissue. We measured Pparγ expression in the pancreas of cafeteria-fed rats (Cafeteria: 1.50 ± 0.4; Cafeteria + 25: 1.21 ± 0.4; *vs*. Control: 1.15 ± 0.3) and we did not observe changes in Pparγ gene expression; but this might be due to very low levels of expression of this gene in the whole pancreas.

Cafeteria-diet is a good model to reproduce most syndrome X human pathologies [5]. It causes the development of an insulin resistance situation, with hyperglycemia and hypertriglyceridemia conditions and hyperinsulinemia. We tested several conditions to reproduce the effects *in vitro* that were observed *in vivo,* and found that only hyperlipidemia mimicked them. In fact, lipotoxicity is one of the major causes of β-cell dysfunction in type 2 diabetes. Prolonged exposure of β-cells to high levels of fatty acids can cause impairment in the expression of metabolic genes, leading to decreased glucose-stimulated insulin secretion [36,37], as we showed.

In this study, we observed that chronic exposure of INS-1E cells to the fatty acid oleate resulted in impaired mitochondrial response, lipid accumulation in the cells, and GSIS loss. GSPE treatment partially reversed the deleterious effects associated with lipid accumulation. Interestingly, the effects observed *in vitro* are correlated with the GSPE effects on cafeteria-fed rats, in which pancreatic TG accumulation and plasmatic insulin secretion (measured as C-peptide) are significantly reduced by the GSPE treatment vs. cafeteria-fed rats. In these animals, GSPE also significantly decreased levels of fatty acid synthase, suggesting reduced fatty acid synthesis. Furthermore, GSPE tended to reduce Srebf1. In fact, this effect on Srebf1 gene expression has also been observed in the white adipose tissue of cafeteria-fed rats [2] after 30 days of GSPE treatment and in the liver after 10 days of treatment [18]. Since Srebf1 activates the expression of acetyl-CoA, down-regulation of Srebf1 by GSPE could result in a lower concentration of malonyl-CoA in β-cells and, therefore, an increase of Cpt-1a [38]. We actually found increased gene expression of Cpt-1a, suggesting that the fatty acids that were present in the cytoplasm could be consumed via β-oxidation upon activation of the long fatty acid carrier Cpt1a, which carries the fatty acids through the mitochondrial membrane. A similar effect was observed in HepG2 cells treated with luteolin, one of the most common flavonoids [39]. Liu et al. have shown that luteolin decreases gene expression of Srebf1 and Fasn and increases Cpt1a gene expression in the absence and presence of palmitate and it enhances the phosphorylation of AMPK, leading to a decrease in intracellular lipid levels of HepG2 cells. AMPK, which plays a central role in regulating cellular metabolism and energy balance [40], is also activated by several other natural compounds, including resveratrol, epigallocatechin gallate, berberine and quercetin [41]. In MIN6 cells, berberine acutely increased AMPK activity and in high-fat diet-fed rats treated with berberine for 6 weeks, it decreased plasma glucose and insulin levels and improved the blood lipid profile [42]. We therefore assessed AMPK involvement in the effects of GSPE and found that a cafeteria diet significantly decreased AMPK protein levels in the pancreas, while GSPE treatment increased levels back up to the levels seen in the control group. It must be highlighted that total AMPK protein levels and AMPK phosphorylation levels follow the same tendency [43-45]. Interestingly, islets cultured with the AMPK activator 5-amino-4-imidazolecarboxamide riboside (AICAR) decreased the expression of Srebf1 and cellular TG content, effects that we observed in INS-1E after GSPE treatment [46]. Taken together, these observations show that GSPE promotes lipid mobilization in β-cells, favoring a negative energy balance. This effect is mediated through AMPK and it causes changes in insulin secretion.

In conclusion, we show that under situations of insulin resistance, chronic GSPE treatment (25 mg GSPE/Kg of bw) significantly decreases insulin production. The effects of GSPE on lipid-damaged β-cells can be explained through its lipid-lowering effect because the TG content in the pancreas was reduced, and procyanidin treatment also affected lipid oxidation through the up-regulation of Cpt1a gene expression and through lipogenesis, which down-regulated Fasn and Srebf1 gene expression. Moreover, GSPE treatment prevented the decrease in AMPK protein levels seen after cafeteria treatment.

References

[1] Pinent M, Cedó L, Montagut G, Blay M, Ardévol A. Procyanidins improve some disrupted glucose homoeostatic situations: an analysis of doses and treatments according to different animal models. Crit Rev Food Sci Nutr. 2011.
[2] Montagut G, Blade C, Blay M, Fernandez-Larrea J, Pujadas G, Salvado MJ, Arola L, Pinent M, Ardevol A. Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. J Nutr Biochem. 2010;21:961-967.
[3] Pinent M, Castell A, Baiges I, Montagut G, Arola L, Ardévol A. Bioactivity of Flavonoids on Insulin-Secreting Cells. Comprehensive Reviews in Food Science and Food Safety. 2008;7:299-308.
[4] Enciu AM, Popescu BO, Gheorghisan-Galateanu A. MicroRNAs in brain development and degeneration. Mol Biol Rep. 2011.

[5] Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, Newgard CB, Makowski L. Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome With Liver and Adipose Inflammation: Comparison to High-Fat Diet. Obesity (Silver Spring). 2011.
[6] Lupi R, Del Guerra S, Marselli L, Bugliani M, Boggi U, Mosca F, Marchetti P, Del Prato S. Rosiglitazone prevents the impairment of human islet function induced by fatty acids: evidence for a role of PPARgamma2 in the modulation of insulin secretion. Am J Physiol Endocrinol Metab. 2004;286:E560-7.
[7] Olofsson CS, Collins S, Bengtsson M, Eliasson L, Salehi A, Shimomura K, Tarasov A, Holm C, Ashcroft F, Rorsman P. Long-term exposure to glucose and lipids inhibits glucose-induced insulin secretion downstream of granule fusion with plasma membrane. Diabetes. 2007;56:1888-1897.
[8] Kashyap S, Belfort R, Gastaldelli A, Pratipanawatr T, Berria R, Pratipanawatr W, Bajaj M, Mandarino L, DeFronzo R, Cusi K. A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes. Diabetes. 2003;52:2461-2474.
[9] Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H. Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes. 2003;52:581-587.
[10] Poitout V, Robertson RP. Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. Endocrinology. 2002;143:339-342.
[11] Prentki M, Corkey BE. Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? Diabetes. 1996;45:273-283.
[12] Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P. Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. Endocrinology. 2004;145:667-678.
[13] Castell-Auvi A, Cedo L, Pallares V, Blay MT, Pinent M, Motilva MJ, Arola L, Ardevol A. Development of a coculture system to evaluate the bioactivity of plant extracts on pancreatic beta-cells. Planta Med. 2010;76:1576-1581.
[14] Frigerio F, Chaffard G, Berwaer M, Maechler P. The antiepileptic drug topiramate preserves metabolism-secretion coupling in insulin secreting cells chronically exposed to the fatty acid oleate. Biochem Pharmacol. 2006;72:965-973.
[15] Terra X, Pallares V, Ardevol A, Blade C, Fernandez-Larrea J, Pujadas G, Salvado J, Arola L, Blay M. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. J Nutr Biochem. 2011;22:380-387.
[16] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.
[17] Castell-Auví A, Cedó L, Pallarès V, Blay M, Ardévol A, Pinent M. The effects of a cafeteria diet on insulin production and clearance in rats. BJN. 2011. *In press*.
[18] Quesada H, del Bas JM, Pajuelo D, Diaz S, Fernandez-Larrea J, Pinent M, Arola L, Salvado MJ, Blade C. Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. Int J Obes (Lond). 2009;33:1007-1012.
[19] Robertson RP, Harmon JS. Diabetes, glucose toxicity, and oxidative stress: A case of double jeopardy for the pancreatic islet beta cell. Free Radic Biol Med. 2006;41:177-184.
[20] Dulloo AG, Samec S. Uncoupling proteins: their roles in adaptive thermogenesis and substrate metabolism reconsidered. Br J Nutr. 2001;86:123-139.
[21] Lameloise N, Muzzin P, Prentki M, Assimacopoulos-Jeannet F. Uncoupling protein 2: a possible link between fatty acid excess and impaired glucose-induced insulin secretion? Diabetes. 2001;50:803-809.
[22] Li LX, Skorpen F, Egeberg K, Jorgensen IH, Grill V. Induction of uncoupling protein 2 mRNA in beta-cells is stimulated by oxidation of fatty acids but not by nutrient oversupply. Endocrinology. 2002;143:1371-1377.
[23] Koshkin V, Wang X, Scherer PE, Chan CB, Wheeler MB. Mitochondrial functional state in clonal pancreatic beta-cells exposed to free fatty acids. J Biol Chem. 2003;278:19709-19715.
[24] Blade C, Arola L, Salvado MJ. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. Mol Nutr Food Res. 2010;54:37-59.

[25] Frigerio F, Brun T, Bartley C, Usardi A, Bosco D, Ravnskjaer K, Mandrup S, Maechler P. Peroxisome proliferator-activated receptor alpha (PPARalpha) protects against oleate-induced INS-1E beta cell dysfunction by preserving carbohydrate metabolism. Diabetologia. 2010;53:331-340.
[26] Prentki M, Vischer S, Glennon MC, Regazzi R, Deeney JT, Corkey BE. Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. J Biol Chem. 1992;267:5802-5810.
[27] Smith S. The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. FASEB J. 1994;8:1248-1259.
[28] Ferre P, Foufelle F. SREBP-1c transcription factor and lipid homeostasis: clinical perspective. Horm Res. 2007;68:72-82.
[29] Ihm SH, Lee JO, Kim SJ, Seung KB, Schini-Kerth VB, Chang K, Oak MH. Catechin prevents endothelial dysfunction in the prediabetic stage of OLETF rats by reducing vascular NADPH oxidase activity and expression. Atherosclerosis. 2009;206:47-53.
[30] Kim YK, Lee MS, Son SM, Kim IJ, Lee WS, Rhim BY, Hong KW, Kim CD. Vascular NADH oxidase is involved in impaired endothelium-dependent vasodilation in OLETF rats, a model of type 2 diabetes. Diabetes. 2002;51:522-527.
[31] Cho AS, Jeon SM, Kim MJ, Yeo J, Seo KI, Choi MS, Lee MK. Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. Food Chem Toxicol. 2010;48:937-943.
[32] Shih CC, Lin CH, Lin WL. Effects of Momordica charantia on insulin resistance and visceral obesity in mice on high-fat diet. Diabetes Res Clin Pract. 2008;81:134-143.
[33] Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, Komeda K, Satoh S, Nakano R, Ishii C, Sugiyama T, Eto K, Tsubamoto Y, Okuno A, Murakami K, Sekihara H, Hasegawa G, Naito M, Toyoshima Y, Tanaka S, Shiota K, Kitamura T, Fujita T, Ezaki O, Aizawa S, Kadowaki T. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. Mol Cell. 1999;4:597-609.
[34] Kuda O, Jelenik T, Jilkova Z, Flachs P, Rossmeisl M, Hensler M, Kazdova L, Ogston N, Baranowski M, Gorski J, Janovska P, Kus V, Polak J, Mohamed-Ali V, Burcelin R, Cinti S, Bryhn M, Kopecky J. N-3 Fatty Acids and Rosiglitazone Improve Insulin Sensitivity through Additive Stimulatory Effects on Muscle Glycogen Synthesis in Mice Fed a High-Fat Diet. Diabetologia. 2009;52:941-951.
[35] Pinent M, Blade MC, Salvado MJ, Arola L, Ardevol A. Intracellular mediators of procyanidin-induced lipolysis in 3T3-L1 adipocytes. J Agric Food Chem. 2005;53:262-266.
[36] Alstrup KK, Brock B, Hermansen K. Long-Term exposure of INS-1 cells to cis and trans fatty acids influences insulin release and fatty acid oxidation differentially. Metabolism. 2004;53:1158-1165.
[37] Unger RH. Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. Diabetes. 1995;44:863-870.
[38] Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, Auwerx J. Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. J Clin Invest. 2004;113:1408-1418.
[39] Liu JF, Ma Y, Wang Y, Du ZY, Shen JK, Peng HL. Reduction of lipid accumulation in HepG2 Cells by luteolin is associated with activation of AMPK and Mitigation of oxidative stress. Phytother Res. 2010.
[40] Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol. 2007;8:774-785.
[41] Hwang JT, Kwon DY, Yoon SH. AMP-activated protein kinase: a potential target for the diseases prevention by natural occurring polyphenols. N Biotechnol. 2009;26:17-22.
[42] Zhou L, Wang X, Shao L, Yang Y, Shang W, Yuan G, Jiang B, Li F, Tang J, Jing H, Chen M. Berberine acutely inhibits insulin secretion from beta-cells through 3',5'-cyclic adenosine 5'-monophosphate signaling pathway. Endocrinology. 2008;149:4510-4518.
[43] Sun Y, Ren M, Gao GQ, Gong B, Xin W, Guo H, Zhang XJ, Gao L, Zhao JJ. Chronic palmitate exposure inhibits AMPKalpha and decreases glucose-stimulated insulin secretion from beta-cells: modulation by fenofibrate. Acta Pharmacol Sin. 2008;29:443-450.
[44] Ma Y, Zhu MJ, Uthlaut AB, Nijland MJ, Nathanielsz PW, Hess BW, Ford SP. Upregulation of growth signaling and nutrient transporters in cotyledons of early to mid-gestational nutrient restricted ewes. Placenta. 2011;32:255-263.

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[45] Qin Y, Tian YP. Exploring the molecular mechanisms underlying the potentiation of exogenous growth hormone on alcohol-induced fatty liver diseases in mice. J Transl Med. 2010;8:120.
[46] Diraison F, Parton L, Ferre P, Foufelle F, Briscoe CP, Leclerc I, Rutter GA. Over-expression of sterol-regulatory-element-binding protein-1c (SREBP1c) in rat pancreatic islets induces lipogenesis and decreases glucose-stimulated insulin release: modulation by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Biochem J. 2004;378:769-778.

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Pancreatic Islet Proteome Profile in Zucker fatty Rats Chronically Treated with a Grape Seed Procyanidin Extract

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ABSTRACT

Grape seed procyanidins have been described to modify glucose metabolism and β-cell functionality through their lipid-lowering effects in a diet-induced obesity model. The objective of the present study was to evaluate the effects of chronically administrated grape seed procyanidin extract (GSPE) on the proteomic profile of isolated pancreatic islets from Zucker fatty (ZF) rats, a genetically-induced obesity model. To achieve this objective, an iTRAQ experiment was conducted, and 31 proteins were found to be differentially expressed in ZF rats treated with GSPE for two months compared to untreated ZF rats. Ten proteins were upregulated, and 21 were downregulated. Of these differentially expressed proteins, five subcategories of biological processes emerged: hexose metabolic processes, response to hormone stimulus, apoptosis and cell death, translation and protein folding, and macromolecular complex assembly. Gene expression analysis supported the role of the first three biological processes, concluding that GSPE limits insulin synthesis and secretion and tends to induce apoptosis, but these molecular changes are not sufficient to counteract the genetic background of the Zucker model at a physiological level.

Introduction

The increased prevalence of obesity has become a worldwide problem. Obesity is associated with insulin resistance and type 2 diabetes mellitus (T2DM), which has also reached epidemic proportions [1,2]. T2DM is a metabolic disorder characterized by hyperglycemia, altered lipid metabolism, and impaired insulin action in peripheral tissues [3,4]. T2DM is also associated with a deficient β-cell insulin-secretory response to glucose [3,4] and involves a combination of genetic and environmental or lifestyle factors [3,5]. While genetic background is responsible for insulin resistance and β-cell failure, weight gain and physical inactivity exacerbate these inherited metabolic abnormalities [5].

Procyanidins are the most abundant phenolic compounds in the human diet, and they are widely found in fruits, berries, beans, nuts, cocoa-based products, and wine [6,7]. Procyanidins are known to have protective effects against cardiovascular diseases, as they have antioxidant and anti-inflammatory properties and prevent atherosclerosis [6]. However, there is little information about procyanidins' effects on the endocrine pancreas, which is a key organ of nutrient metabolism [8]. We have recently described the effects of some doses of grape seed procyanidin extract (GSPE) on healthy animals [9]. Moreover, in a previous study by our group, GSPE was described to modify glucose metabolism by modulating plasma insulin levels and acting on peripheral tissues. The modifications were observed in the circumstance of obesity and mild insulin resistance induced by cafeteria diet [10]. In this model, the effects of GSPE on lipid-damaged β-cells can be explained by its lipid-lowering effect; procyanidins reduced the triglyceride content in the pancreas, stimulating β-oxidation and inhibiting lipid synthesis (submitted results).

Given these findings, the aim of the present study was to evaluate the effect of GSPE on the proteomic profile of the endocrine pancreas by utilizing a model of genetically-induced obesity (Zucker fatty rats). Zucker fatty rats are extensively used as a model of obesity and pre-diabetes and are characterized by insulin resistance and glucose intolerance. These rats are genetically obese, due to a mutation in the leptin receptor gene [11,12]. Under the influence of obesity and insulin resistance, β-cells are exposed to elevated glucose, insulin, and lipid levels. β-cells physiologically adapt to these conditions through increased β-cell mass and enhanced β-cell function [12,13]. We completed an iTRAQ experiment and identified proteins differentially expressed, which then we clustered, categorized according to Gene Ontology (GO) terms, and visualized into network context, in order to understand the proteome profile of isolated pancreatic islets from Zucker fatty rats chronically treated with GSPE.

Materials and methods

Reagents. According to the manufacturer, GSPE (Les Dérives Résiniques et Terpéniques, Dax, France) contained monomeric (16.6 %), dimeric (18.8 %), trimeric (16.0 %), tetrameric (9.3 %), and oligomeric procyanidins (5 to 13 units, 35.7 %), as well as phenolic acids (4.2 %).

Procedures. Five-week-old lean (10 animals, 113-135 g) and obese (20 animals, 129-170 g) female Zucker fa/fa rats were purchased from Charles River (Barcelona, Spain). The rats were housed in animal quarters at 22ºC with 12 h light/dark cycle and fed ad libitum with a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. After 1 week in quarantine, the treatment began as previously described [14]. Briefly, the lean control group (ZL) and ten randomly divided Zucker fatty rats (ZF) were treated with a vehicle (sweetened condensed milk diluted 1:6 with tap water). The other ten obese Zucker rats (ZF + GSPE) were treated daily with 35 mg of GSPE/kg of body weight (bw) dissolved in the vehicle. Every day at 8 am, food was withdrawn, and at 4 p.m., the vehicle (or treatment) was administrated by controlled oral intake with a syringe. At 5 p.m., the food was replaced. After two months of treatment, the animals were anesthetized using sodium pentobarbital (Sigma-Aldrich, St. Louis, MO) at 75 mg/kg of bw and were killed by abdominal aorta exsanguination. Blood was collected, and pancreatic islets were isolated from all of the animals. Insulin (Mercodia, Uppsala, Sweden), C-peptide (Mercodia, Uppsala, Sweden), and glucagon (Wako Chemicals, Neuss, Germany) plasma levels were assayed using ELISA methodology

following the manufacturers' instructions. Glucose (QCA, Amposta, Spain) and non-esterified fatty acids (NEFA) (Wako Chemicals, Neuss, Germany) plasma levels were determined using an enzymatic colorimetric kit. All procedures were approved by the Experimental Animals Ethics Committee of the Universitat Rovira i Virgili.

Islet isolation. Islets were obtained by collagenase digestion, as described previously [9]. Briefly, the rats were anesthetized, and the pancreas was infused with 7 mL of ice-cold collagenase P (Roche Diagnostics, Mannheim, Germany) solution (1 mg/mL) before removal and were incubated at 37ºC for 15 min. Islets were purified on a Histopaque gradient (Sigma-Aldrich, St. Louis, MO) and handpicked until a population of pure islets was obtained.

Proteome sample preparation and analysis. Islets were lysed with 100 µl of solution containing 8 M urea and 0.1 % ProteaseMAX™ Surfactant (Promega, Madison, WI). Following lysis, samples were centrifuged at 14000 rpm for 20 min to remove cell debris. Total protein content from the supernatants was determined by the Bradford method [15]. Equal amounts of protein from 1-3 rats of each experimental group were pooled, and 70 µg of each pool was reduced, alkylated, digested and labeled with a different 8-plex iTRAQ reagent, as described in the iTRAQ protocol (Applied Biosystems, Foster City, CA). Finally, all of the labeled samples were combined as a unique sample. Half of the unique sample (200 µg) was used to conduct isoelectric focusing. Peptides were focused at 5000 V until 12000 V/h. After focusing, the strip was divided into 15 pieces, and the peptides were extracted with three different solutions: 0.1 % trifluoroacetic acid (TFA); 50 % acetonitrile (ACN), 0.1 % TFA; and ACN 0.1 % TFA. The extracts were combined and concentrated into a volume of 9 µL. The other half of the sample was loaded onto a Reverse Phase Column (Gemini, 3 µm, C18 110 Å, Phenomenex, Torrance, CA) and peptides were separated in a 5-45 % linear gradient of solvent B (20 mM triethylamine in ACN) in 60 min at a flow rate of 0.15 ml/min. The fractions were analyzed by MALDI-TOF/TOF MS (4700 Proteomics analyzer, AB Sciex, Foster City, CA) and combined for a final amount of 15 fractions. Peptides contained in the fractions obtained after reversed-phase chromatography were separated by liquid chromatography and subjected to MS/MS analysis to sequence the peptides using an Ultimate Plus/Famos nano LC system (LC Packings, Amsterdam, The Netherlands) and a QSTAR XL hybrid quadrupole-TOF instrument (AB Sciex, Foster City, CA) equipped with a nano-electrospray ion source (Protana, Odense, Denmark). The samples were pre-concentrated on a 0.3 x 5 mm, 3 µm, C18 trap column from LC Packings PepMap (Dionex Company, Amsterdam, The Netherlands) at a flow rate of 40 µL/min, utilizing 0.1 % TFA as the mobile phase. After three minutes of pre-concentration, the trap column was automatically switched in-line with a 0.075 x 150 mm, 3 µm, Dionex C18 PepMap column from LC Packings (Amsterdam, The Netherlands). Mobile phases consisted of solvent A (0.1 % formic acid in water) and solvent B (0.1 % formic acid in 95 % ACN). Chromatographic conditions were a linear gradient from 95 % to 50 % solvent A in 30 min at a flow rate of 0.25 µL/min. The column outlet was directly coupled to a nano-eletrospray ion source (Protana, Odense, Denmark) using a 10-µm PicoTip EMITTER SilicaTip needle (New Objective, Massachusetts, USA). The positive TOF mass spectra were recorded on the QSTAR instrument using information-dependent acquisition (IDA). The TOF MS survey scan was recorded for mass range *m/z* 350 to 1800 followed by MS/MS scans of the three most intense peaks. Typical ion spray voltage was in the range of 2.5 to 3.0 kV, and nitrogen was used as collision gas. The spray positions and other source parameters were optimized with a tryptic digest of a protein standard mixture (LC Packings, Amsterdam, The Netherlands).

Database search. Search on SwissProt database (523,151 sequences and 184,678,199 residues) was performed using Mascot 2.2 in combination with the Mascot Daemon interface 2.2.2 (Matrix Science, Inc., Massachusetts, USA) (hppt://www.matrixscience.com) and the ProteinPilot™ 3.0 software (Applied Biosystems, Foster City, CA). Mascot.dll 1.6b25 and ABSciex.DataAccess.Wiff File DataReader.dll were used for importing data into Mascot and Protein Pilot, respectively. Mascot searches were performed with trypsin enzymatic specificity, allowing one missed cleavage and a tolerance on the mass measurement of

100 ppm in MS mode and 0.6 Da for MS/MS ions. Deamidation of Asparagine-Glutamine and oxidation of Methionine were used as variable modifications. Using ProteinPilot software is not necessary to fix mass tolerance or possible modifications because the Paragon algorithm used preset values. The results were represented as ZL/ZF and ZF+GSPE/ZF ratios, and each ratio has a P-value associated with it. The P-value is the probability that the iTRAQ ratio is different from 1 by chance. For protein identifications where no hit was found in the rat databases, protein homology search was done using the BLAST tool from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein with highest identity was considered. For the proteins identified, the cellular compartment, molecular function and biological process were assigned according to the DAVID (Database for Annotation, Visualization and Integrated Discovery) [16,17]. Additionally, the data were hierarchically clustered using the Cluster 3.0 software [18] (version 1.5), and the results were visualized with Java TreeView software [19] (version 1.1.6r2). The list of significantly regulated proteins by GSPE was further analyzed using the network building tool, Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc.), which uses the Ingenuity Pathways Knowledge Base. Hypothetical networks of proteins from our experiment and proteins from the Ingenuity database were built using the *de novo* network-building algorithm. IPA calculates a significance score for each network, where score=$-\log_{10}$(P-value). This score specifies the probability that the assembly of a set of proteins in a network could be generated randomly. A score of 3 indicates that there is a 1 in 1000 chance that the focus proteins are arranged together in a network due to random chance. Therefore, networks with scores of 3 or higher have a 99.9 % confidence of not being generated by random chance [20].

Apoptosis assay. Thirty pancreatic islets of ZL rats and 20 islets of ZF and ZF+GSPE were lysed. Oligonucleosomes in the cytosol, indicative of apoptosis-induced DNA degradation, were quantified using the Cell Death Detection kit ELISA^{PLUS} (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The absorbance value of the blank was subtracted from the values of the sample, and the results were normalized with the protein content of the islets, as analyzed by Bradford method [15].

Quantitative RT-PCR. Total RNA from isolated islets was extracted using the RNeasy Mini Kit (Qiagen, Barcelona, Spain), and cDNA was generated with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The cDNA from all of the experiments was subjected to quantitative Real-Time PCR amplification using the TaqMan Master Mix (Applied Biosystems, Foster City, CA). Specific TaqMan probes (Applied Biosystems, Foster City, CA) were used for each gene (Table 3), and β-actin was used as the reference gene (Rn00667869-m1). Reactions were run on a quantitative RT-PCR 7300 System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The relative mRNA expression levels were calculated using the ΔΔCt method.

Calculations and Statistical Analysis. Results are expressed as the mean ± SEM. Effects were assessed by Student's *t*-test. All calculations were performed with SPSS software.

Results

Effects of GSPE on the pancreatic islet proteomic profile.

A daily dose of 35 mg/kg of GSPE was administrated to ZF rats for two months. At the end of the treatment, plasma levels of glucose, insulin, C-peptide, glucagon and NEFA were quantified (Table 1). All of the parameters were significantly increased in ZF rats, but GSPE did not counteract this increase. A similar pattern was previously observed in the quantification of triglycerides [14].

Table 1. Plasmatic parameters

	ZL	ZF	ZF + GSPE
Glucose (mM)	7.2 ± 0.6 **a**	9.8 ± 0.5 **b**	9.9 ± 0.9 **b**
Insulin (µg/L)	1.0 ± 0.2 **a**	9.8 ± 0.6 **b**	10.0 ± 0.5 **b**
C-Peptide (nM)	0.7 ± 0.1 **a**	5.8 ± 0.3 **b**	5.6 ± 0.5 **b**
Glucagon (pg/mL)	166.4 ± 6.3 **a**	273.9 ± 33.2 **b**	265.7 ± 20.2 **b**
NEFA (mg/dL)	8.8 ± 0.8 **a**	29.3 ± 1.7 **b**	31.9 ± 3.5 **b**

Different letters indicate the statistically significant differences between treatments (P-Value ≤ 0.05).

In order to analyze the proteomic profile of the pancreatic islets after the treatment with GSPE, an iTRAQ experiment was conducted. The information from the iTRAQ experiment was analyzed using the ProteinPilot search algorithm against the SwissProt database, and a total of 84 proteins were identified. Regarding to the effect of the genetic background, 21 proteins that were differentially expressed in ZL vs. ZF, 18 were associated with a P-value ≤ 0.05, and 3 were associated with a P-value ≤ 0.1. Additionally, 4 of these differentially expressed proteins were upregulated, while the other 17 were downregulated (Table 2). Concerning the effect of the procyanidin treatment in the genetically obese rats 31 proteins were differentially expressed in ZF + GSPE rats when compared to ZF rats. Of these, 18 were associated with a P-value ≤ 0.05, and 13 were associated with a P-value ≤ 0.1; furthermore, 10 were upregulated, while 21 were downregulated (Table 2).

The proteins modified by GSPE were analyzed using DAVID according to the different categories in the GO classification. In the cellular component category, the highest proportion of differentially expressed proteins was cytosolic, and the classification based on the molecular functions revealed that the proteins were associated with structural molecular activity, catalytic activity, and binding functions. Upon analysis of the biological process, five subcategories were obtained: hexose metabolic, translation and protein folding, macromolecular complex assembly, response to hormone stimulus, and apoptosis and cell death (Figure 1 A-E).

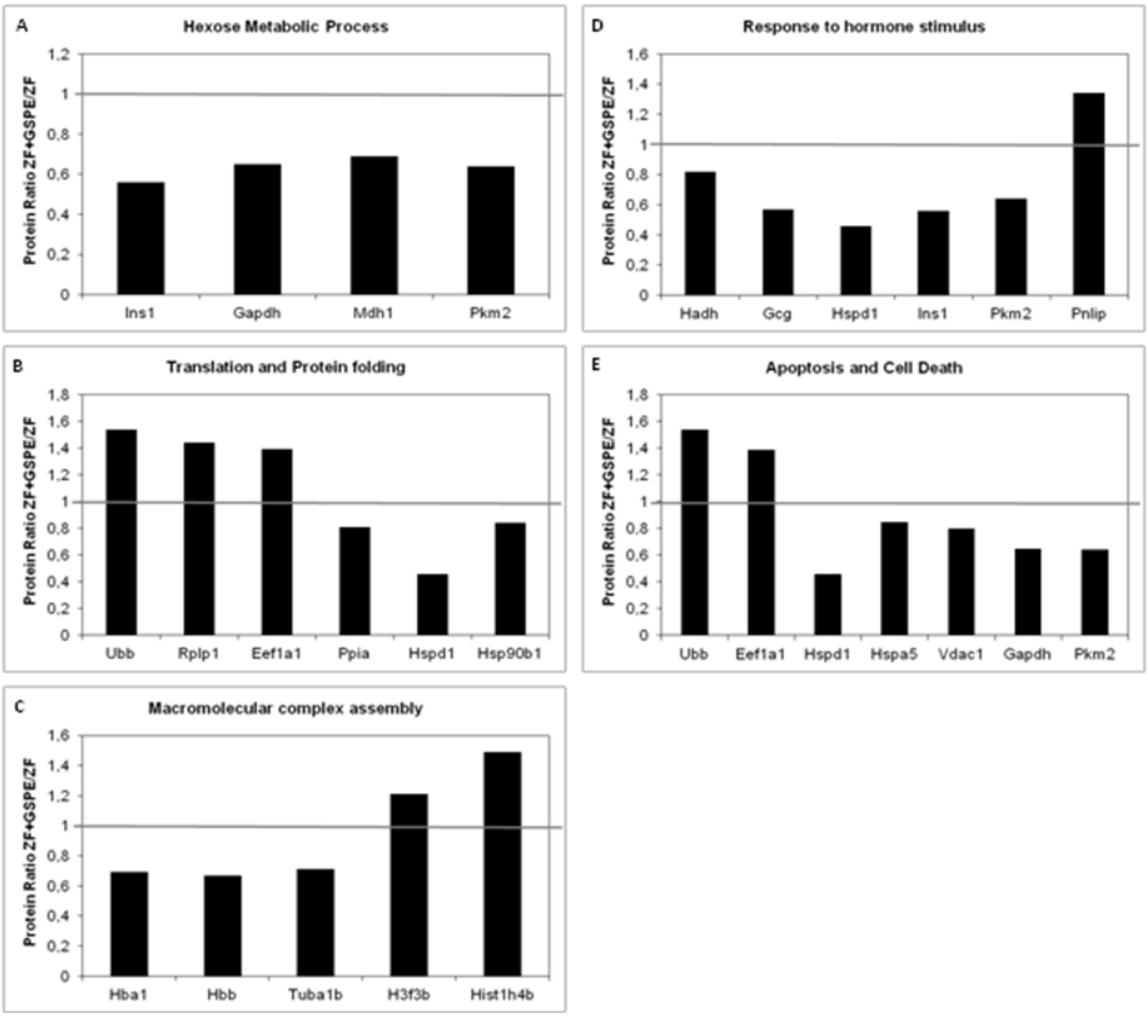

Figure 1. Biological processes of proteins whose expression was significantly modulated by GSPE treatment assessed using the DAVID database. The proteins involved in A) hexose metabolic process, B) translation and protein folding, C) macromolecular complex assembly, D) response to hormone stimulus, and E) apoptosis and cell death, are presented.

Table 2. Differentially Expressed Proteins[a]

Swiss-Prot Accession Number	Gene name	Protein name	Number of matched peptides	ZL / ZF	ZF+GSPE / ZF
P06761	Hspa5	78 kDa glucose-regulated protein	16	0.81 *	0.85 #
P10719	Atp5b	ATP synthase subunit beta. mitochondrial	9	0.93	0.85 #
P63259	Actg1	Actin. cytoplasmic type 8	28	0.64 **	0.89 *
P11598	Pdia3	Protein disulfide-isomerase A3	19	0.74 **	0.78 **
P02091	Hbb	Hemoglobin subunit beta-1	10	0.48 **	0.67 **
P00731	Cpa1	Carboxypeptidase A1	10	0.86 *	1.70 **
P00689	Amy2	Pancreatic alpha-amylase	4	2.01 **	1.22
P01946	Hba1	Hemoglobin subunit alpha-1/2	33	0.49 **	0.69 **
Q66HD0	Hsp90b1	Endoplasmin	14	0.65 **	0.84 **
P62804	Hist1h4b	Histone H4	18	1.71 **	1.49 **
P01322	Ins1	Insulin-1	5	0.75	0.56 **
Q9WVK7	Hadh	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	5	0.71 **	0.82 #
P06883	Gcg	Glucagon	6	2.94 **	0.57 **
O88989	Mdh1	Malate dehydrogenase, cytoplasmic	11	0.58 **	0.69 **
P62630	Eef1a1	Elongation factor 1-alpha 1	3	1.40	1.39 #
P27657	Pnlip	Pancreatic triacylglycerol lipase	6	1.06	1.34 *
P04797	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	8	0.91	0.65 *
Q6IMF3	Krt1	Keratin. type II cytoskeletal 1	6	0.98	0.27 **
Q6P9V9	Tuba1b	Tubulin alpha-1B chain	5	0.67 *	0.71 #
P19222	Cpa2	Carboxypeptidase A2	3	0.86	1.89 #
P11980	Pkm2	Pyruvate kinase isozymes M1/M2	3	1.01	0.64 #
P84245	H3f3b	Histone H3.3	5	1.07	1.21 #
P63039	Hspd1	60 kDa chaperonin	7	0.38 **	0.46 **
D3ZUL3	Col6a1	Collagen alpha-1(VI) chain	2	0.99	1.31 #
P10111	Ppia	Peptidyl-prolyl cis-trans isomerase A	5	1.06	0.81 #
P54316	Pnliprp1	Pancreatic lipase-related protein 1	5	0.70 #	1.22
Q05962	SLC25A4	ADP/ATP translocase 1	5	0.81	1.32 *
P19944	Rplp1	60S acidic ribosomal protein P1	3	0.74	1.44 #
P62989	Ubb	Polyubiquitin-B	2	1.14	1.54 #
P15087	Cpe	Carboxypeptidase E	4	0.96	0.61 **
P07338	Ctrb1	Chymotrypsinogen B	2	0.54 **	1.17
Q63617	Hyou1	Hypoxia up-regulated protein 1	2	0.55 #	0.74 *
Q8CIS9	Krt9	Keratin. type I cytoskeletal 9	6	0.58 **	0.20 **
P38983	Rpsa	40S ribosomal protein SA	2	1.50 *	1.29
P34139	Rab1A	Ras-related protein Rab-1A	2	0.52 **	1.10
Q9Z2L0	Vdac1	Voltage-dependent anion-selective channel protein 1	3	0.78 #	0.80 #

[a] SwissProt accession number, gene name, protein name, number of identified peptide sequences, fold regulation ZL vs. ZF, fold regulation ZF+GSPE vs. ZF. * P-Value ≤ 0.05; ** P-Value ≤ 0.01; # P-Value ≤ 0.1.

Clustering analysis of the differentially expressed proteins in the iTRAQ experiment.

The iTRAQ ratios of ZL/ZF and ZF+GSPE/ZF were used to perform hierarchical clustering of the differentially expressed proteins using the Cluster 3.0 software, and the results were visualized with Java TreeView software (Figure 2A). The values for the ZF group were assigned as 1 for all of the proteins. The hierarchical clustering revealed that the 36 proteins modified in ZL vs. ZF and/or ZF+GSPE vs. ZF were clustered in four expression patterns (Figure 2B). Two clusters exhibited a profile in which ZF+GSPE counteracted the effect of ZF: Down-Up in which the expression of 5 proteins was downregulated in ZF versus ZL, and the effect was counteracted by GSPE; and Up-Down, in which the expression of 12 proteins was upregulated in ZF versus ZL, and the effect was counteracted by GSPE. The major biological function affected in these clusters was apoptosis and cell death, with 31 % of the proteins being included in this category. Among these proteins, Pdia3, Hspd1, and Vdac1 are positive regulators of apoptosis included in the Up-Down cluster, and Eef1a1, involved in negative regulation of apoptosis, was found in the cluster Down-Up. The other biological processes with various proteins involved were translation and protein folding and macromolecular complex assembly. The other two clusters included proteins that showed a general profile in which ZF had no effects, though ZF + GSPE did: Down, in which the expression of 8 proteins was not modified in ZF versus ZL but was downregulated in ZF+GSPE versus ZF; and Up, in which the expression of 11 proteins was mainly not modified in ZF but was upregulated in ZF+GSPE versus ZF. Within the Down cluster, 50 % of the proteins (Ins1, Gapdh, Pkm2, and Cpe) were classified in the GO biological process of hexose metabolic process and response to hormone stimulus. The group Up was more heterogeneous, exhibiting effects of GSPE in proteins classified into diverse biological processes.

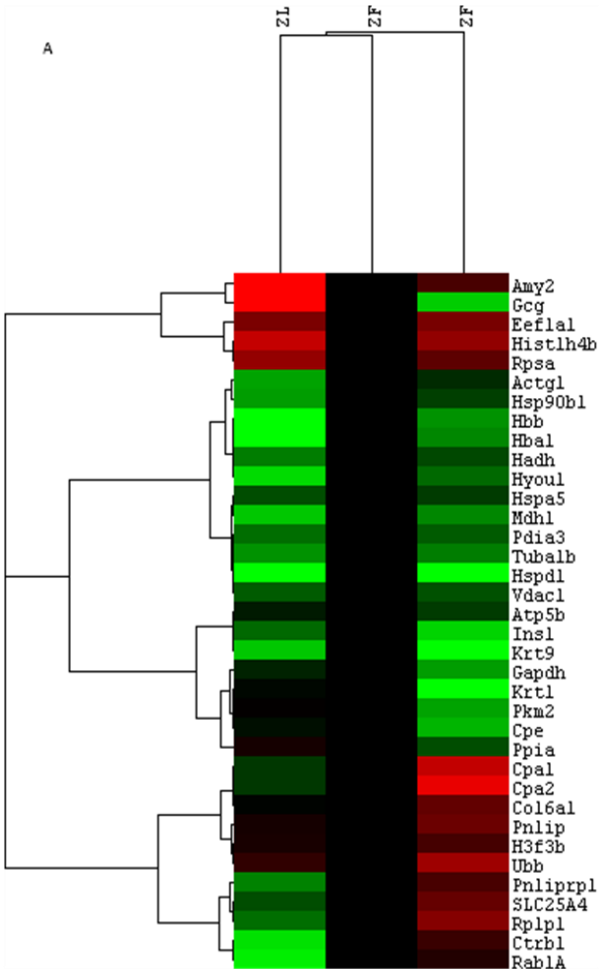

Figure 2. A) Hierarchical clustering for differentially expressed proteins performed using the Cluster 3.0 program [18]. Red gradients represent upregulated proteins, and green gradients represent downregulated proteins compared to ZF rats (which ratio is 1, represented in black).

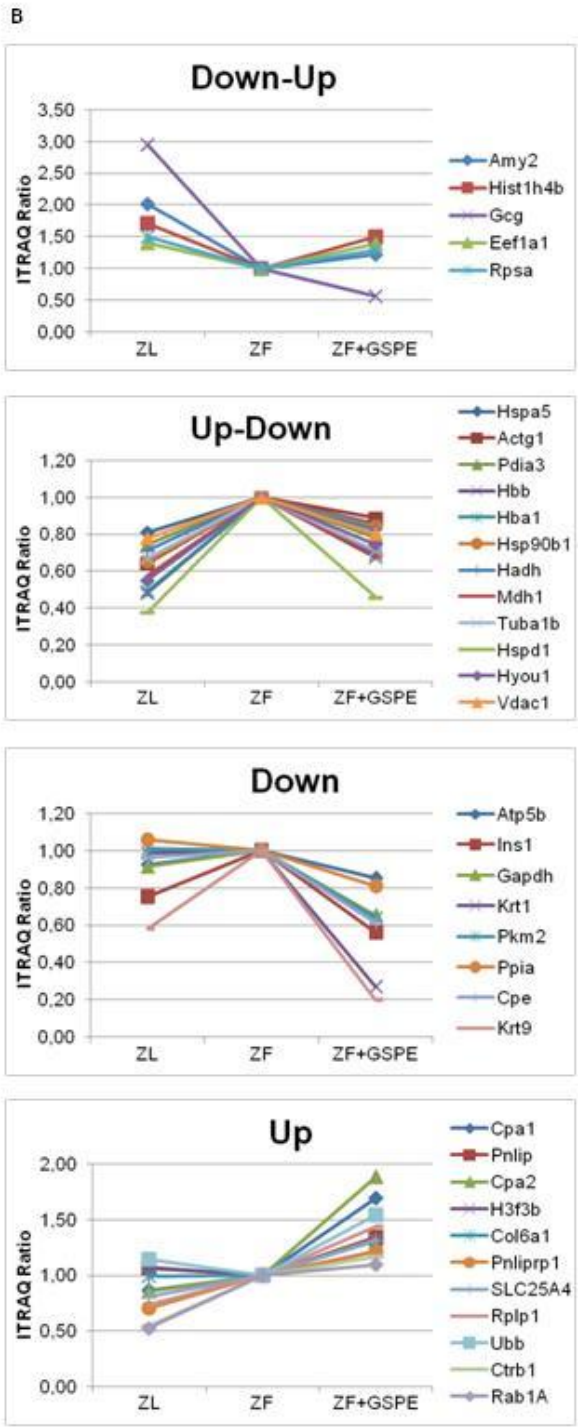

Figure 2. B) Six different clusters were derived from the analysis and are represented in different graphics.

Network analysis of differentially expressed proteins in ZF rats treated with GSPE.

The 31 proteins differentially expressed in ZF+GSPE rats were imported into the Ingenuity Pathway Analysis software and were mapped to two different protein networks in the Ingenuity database (Figure 3A and B). Network A had a score of 48 (P-value = 10^{-48}) and included the following top functions: free radical scavenging, cell function and maintenance and cellular compromise. Network B had a score of 25 (P-Value = 10^{-25}) and included the following top functions: drug metabolism, lipid metabolism and molecular transport.

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THE EFFECTS OF GRAPE SEED PROCYANIDIN EXTRACT ON INSULIN SYNTHESIS AND SECRETION
Anna Castell Auvi
DL:T. 271-2012

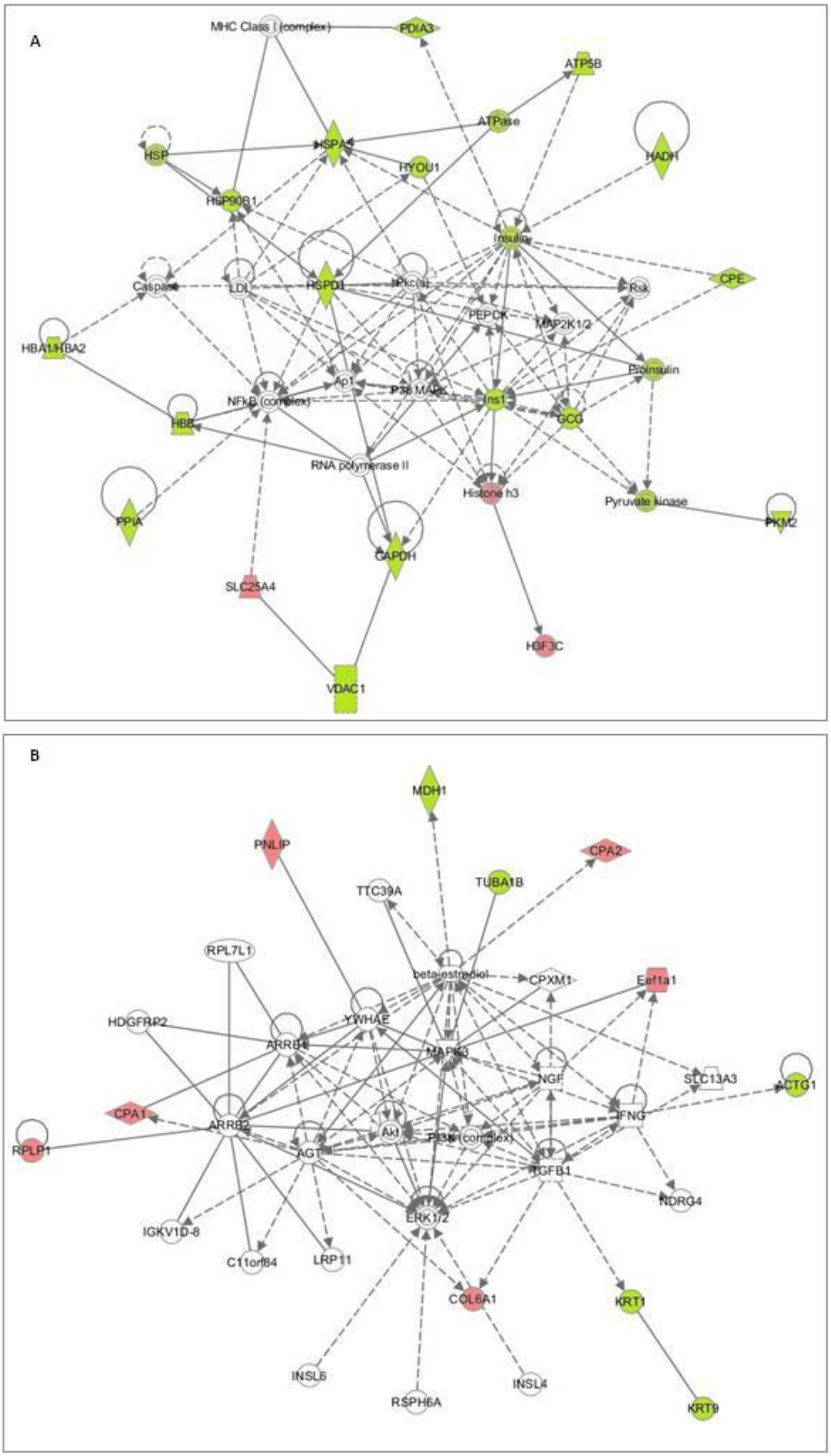

Figure 3. Hypothetical networks associated with the proteins differentially expressed following treatment of GSPE in Zucker Fatty rats generated by Ingenuity Pathway Analysis software. In the analysis, two networks were generated, A and B. Proteins are represented as nodes with different shapes that represent different functional type of proteins. Upregulated proteins are shown in red, and downregulated proteins in green. Proteins depicted in white are proteins from the Ingenuity database. The relationship between proteins is represented as a line, and the arrowheads indicate the direction of the interaction.

Apoptosis and expression analysis in pancreatic islets.

Given that one of the subcategories obtained in the biological process analysis using DAVID was apoptosis and cell death, the apoptosis levels in the isolated islets were determined (Figure 4). The results indicate that apoptosis was increased in the islets from ZF and ZF+GSPE rats compared to the islets from ZL rats. No significant difference in apoptosis was observed between ZF and ZF+GSPE, but GSPE treatment counteracted the effects of ZF on gene expression of the anti-apoptotic marker Bcl-2 and the proliferation marker Cyclin D2 (Table 3).

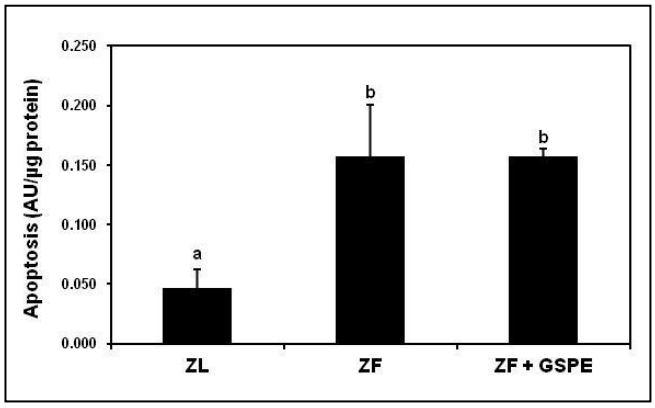

Figure 4. Apoptosis of the isolated islets assessed using the cell death apoptosis kit (Roche) that quantifies the histone-complexed DNA fragments. The results were normalized with the quantity of protein in the islets. The data are shown as the mean ± SEM of 5 ZL, 7 ZF and 6 ZF+GSPE rats. Different letters indicate groups significantly different (P-value ≤ 0.05).

Conversely, considering that another subcategory obtained in the DAVID analysis was hexose metabolic processes and that insulin and glucagon were modified at the protein level in the proteomic experiment, the gene expression levels of insulin and glucagon were also assayed. Additionally, the expression of other genes involved in the insulin synthesis, secretion and degradation pathways were assayed (Table 3). The gene expression of insulin and glucagon followed the same profile that was obtained in the proteomic experiment. Glucagon protein levels were significantly downregulated in ZF compared to ZL and in ZF+GSPE compared to ZF; the mRNA levels of this gene followed the same tendency. Similarly, insulin protein levels were downregulated in ZF+GSPE compared to ZF. Insulin gene expression was upregulated in ZF compared to ZL, and GSPE counteracted this effect. The same profile was seen in the expression of the transcription factor that regulates insulin gene expression, that is, Pdx-1; in one of the regulators of insulin secretion, that is, glucokinase; and in a protein that can alter the insulin secretion process, that is, Ucp2 (Table 3).

Table 3. Islets gene expression

Gene Name	Commercial reference	ZL	ZF	ZF + GSPE
Ins2	Rn01774648-g1	1.0 ± 0.1 **a**	3.2 ± 0.2 **b**	1.8 ± 0.4 **a**
Pdx1	Rn00755591-m1	1.0 ± 0.1 **ab**	1.3 ± 0.1 **b**	0.9 ± 0.2 **a**
Pparg	Rn00440945-m1	1.1 ± 0.3 **a**	2.4 ± 0.3 **b**	1.9 ± 0.2 **ab**
Gck	Rn00561265-m1	1.0 ± 0.0 **ab**	1.4 ± 0.1 **b**	1.0 ± 0.1 **a**
Ucp2	Rn01754856-m1	1.1 ± 0.2 **a**	2.1 ± 0.2 **b**	1.6 ± 0.2 **ab**
Bcl2	Rn99999125_m1	1.2 ± 0.3 **ab**	1.3 ± 0.1 **a**	0.9 ± 0.1 **b**
Bax	Rn01480160_g1	0.9 ± 0.2 **a**	1.1 ± 0.0 **a**	1.0 ± 0.2 **a**
Ratio Bcl2/Bax		0.9 ± 0.1 **a**	1.3 ± 0.1 **b**	1.0 ± 0.1 **a**
Ddit3	Rn01458526_m1	1.4 ± 0.5 **a**	1.4 ± 0.3 **a**	1.7 ± 0.4 **a**
Ccnd2	Rn01492401_m1	1.0 ± 0.0 **a**	1.4 ± 0.1 **b**	0.8 ± 0.1 **a**

Mki67	Rn01451446_m1	1.0 ± 0.1 **a**	2.2 ± 0.3 **b**	1.6 ± 0.2 **b**
Gcg	Rn00562293_m1	1.1 ± 0.2 **a**	0.7 ± 0.1 a**b**	0.5 ± 0.1 **b**
Ppy	Rn00561768_m1	1.0 ± 0.1 **a**	0.7 ± 0.2 a**b**	0.5 ± 0.1 **b**

Different letters indicate the statistically significant differences between treatments (P-Value ≤ 0.05).

Discussion

Procyanidins are flavonoids with well-known antioxidant and anti-inflammatory activity that protect against cardiovascular and metabolic diseases. However, the effects of procyanidins on glucose metabolism and the endocrine pancreas are poorly investigated [8]. Since obesity is associated with insulin resistance, ZF rats are a useful model to analyze the effects of natural compounds in pancreatic islets under conditions of insulin resistance. Therefore, the aim of the present study was to analyze the effect of GSPE on the proteomic profile of isolated pancreatic islets of Zucker fatty rats. For this purpose, we carried out a proteome analysis, and we obtained 31 proteins differentially expressed in islets isolated from ZF+GSPE rats compared to ZF rats. These differentially expressed proteins provide information that aids our understanding of the effects of procyanidins on a genetically induced pre-diabetic model. To understand better the changes induced by GSPE, we also analyzed the effect of the genetic obese background by comparing ZF and ZL rats. A similar proteome analysis had been previously performed [21]. Clustering analysis revealed that GSPE effects counteracted the action of the obesity-related genetic mutation in ZF rats for approximately half of the proteins. The main effects were related to processes involving apoptosis and cell death followed by translation and protein folding and macromolecular complex assembly, suggesting an improvement in the genotype-induced dysfunction.

Our main objective was to identify the role of GSPE on the ZF pancreatic islet proteome. The GO analysis using DAVID allowed us to identify the biological processes that involve proteins that are expressed differently following GSPE treatment. One subcategory obtained in the biological process analysis was apoptosis and cell death. Procyanidins have been shown to modulate apoptosis in other tissues/cell lines. These compounds have been found to be pro-apoptotic in cancer cell lines. Procyanidins from hops decreased the cell viability of human colon cancer HT-29 cells [22], and dimer procyanidins produced significant cytotoxicity in numerous human cancer cell lines [23]. Conversely, antiapoptotic activity has been suggested for procyanidins in non-cancer cells. Grape procyanidins inhibited the damage induced by ethanol and carbon tetrachloride in rat hepatocytes [24], and they protected against cardiac cell apoptosis via the induction of endogenous antioxidant enzymes [25]. Previous experiments by our group showed that GSPE modulates apoptosis markers in the pancreas (submitted results); therefore, we were interested in analyzing this subcategory in more detail. We checked apoptosis and apoptosis markers in the pancreatic islets. GSPE did not counteract the increased apoptosis levels in pancreatic islets of ZF rats. The mRNA levels of the antiapoptotic marker Bcl-2 were decreased by GSPE treatment, as well as the ratio Bcl-2/Bax, which counteracts the effects of ZF. The expression of Cyclin D2 (a marker of proliferation), which was increased in the ZF rats, was also decreased by GSPE treatment. However, GSPE modulated some proteins involved in the apoptotic process. GSPE treatment increased expression of Eef1a1, which mediates cytoskeletal changes during cell death [21,26]; this treatment also decreased the levels of chaperones involved in endoplasmatic reticulum (ER) homeostasis and the unfolded protein response (UPR) following ER stress (Hspd1 [27], Hspa5 [28], and Hsp90b1 [29,30]). Procyanidins also decreased Cpe, which positively controls β-cell survival via effects on ER stress [31,32], and Vdac1, the mitochondrial element of the cell-death pathway [33]. Most of these proteins are included in the clusters in which GSPE counteracts the effects of ZF genotype. With all of this information, the effects of GSPE on cell death and apoptosis are not clearly understood. However, using ZF rats as a reference for apoptosis, GSPE would tend to improve the process, as procyanidins counteract apoptotic markers at the gene and protein level, although they do not induce changes in the final apoptosis levels.

Another biological process identified in the functional analysis of the differentially expressed proteins induced by GSPE was translation and protein folding. The ER is the organelle where the protein folding takes place, and beta cells are very sensitive to disruptions in ER homeostasis. When ER exceeds its folding capacity ER stress occurs, and this activates the UPR, which mitigates stress. ER stress seems to be one of the molecular mechanisms of beta-cell dysfunction contributing to diabetes [34]. Analysis of differentially expressed proteins in Zucker diabetic rats compared to ZL rats showed a reduction of Hspd1/Hspe1 chaperone complex [21]. We found that GSPE decreased protein levels of the chaperones Hspd1, Hsp90b1, and Hspa5, proteins that promote protein folding and degradation [35]; and of protein disulfide-isomerase A3 (Pdia3), involved in ER-associated degradation. Chaperones Hspa5 and Hsp90b1, as well as Pdia3, were also decreased in the beta-cell line INS-1E when severe ER stress was induced or when they were treated with high glucose, indicating a defective UPR [33,36,37]. GSPE treatment also increased Polyubiquitin-B (Ubb), a molecule that targets misfolded proteins for degradation [35]. Taken together these results could point out to an alteration of the response to ER stress due to GSPE. On the other hand, according to clustering analysis, GSPE reversed the effects of ZF rats in this biological process. And GSPE increased the levels of the 60S acidic ribosomal protein P1 (Rplp1) and Eef1a1, which could indicate an activation of protein biosynthesis by the treatment. So our results could also indicate that the level of misfolded proteins is lower in ZF rats treated with GSPE and that the UPR is unnecessary. A reason of such reduced amount of misfolded proteins could be a lower insulin production by GSPE, considering that the high insulin production in β-cell due to insulin resistance is a cause of ER stress and activation of UPR.

In the functional analysis using the DAVID server, we also identified hexose metabolic process and response to hormone stimulus that were modified at the protein level by GSPE treatment. Although we found that these biological processes were targets of procyanidins in the clustering analysis, we did not detect changes due to the fa/fa genotype. These results suggest that the effects of GSPE do not directly counteract the effects of the genetic background and that procyanidin actions go beyond modifying these processes. Insulin was one of the proteins centrally located in network A, confirming its crucial role in the proteome profile of pancreatic islets. These results reinforce our previous studies that describe procyanidins as modulators of glucose homeostasis [10,38] and insulin metabolism [9,10]. The reduced insulin protein and gene expression in the islets confirm GSPE as repressor of insulin production, as was previously suggested [9]. The downregulation of insulin production is also supported by the action of GSPE as a repressor of Pdx1 [39] gene expression, as we observed in previous studies [9] (and submitted results), and as a repressor of Cpe protein levels [40]. The proteome profile study also uncovered proteins involved in insulin secretion that were targets of GSPE. The energetic production necessary to secrete insulin was decreased, and this result suggests the inhibitory effect of GSPE on the protein levels of two enzymes involved in the glycolytic pathway, Pkm2 and Gapdh. Protein levels of Atp5b, a member of ATP synthase protein complex, also decreased following GSPE treatment [41]. Reduced expression of a mitochondrial metabolic enzyme, malate dehydrogenase, can also contribute to decreased insulin secretion [42]. One of the key regulators of insulin secretion is glucokinase [43]. Although glucokinase protein levels were not modified by GSPE treatment, gene expression analysis showed that procyanidins inhibited glucokinase mRNA levels. Therefore, our data suggests that GSPE was able to affect insulin secretion. GSPE limits insulin synthesis and secretion, as we have found in other assayed animal models, i.e., healthy animals [9] and cafeteria-fed animals (submitted results).

Conclusion

The present study demonstrates that chronically administrated GSPE modulates the proteomic profile of β-pancreatic islets from Zucker Fatty rats. Procyanidins modulate proteins involved in insulin synthesis and secretion. Procyanidins also alter the protein or gene expression levels of other factors involved in apoptosis. However, the molecular changes induced by GSPE are not sufficient to counteract the genetic background of the Zucker model at a physiological level. In addition, the proteome analysis has provided

new information about the procyanidin mechanism of action and identified translation, protein folding and macromolecular assembly as biological processes that are targeted by procyanidins.

Acknowledgments

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References

[1] Kahn, S. E. et al. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature.* 2006, *444* (7121), 840-846.
[2] Qatanani, M.; Lazar, M. A. Mechanisms of obesity-associated insulin resistance: many choices on the menu. *Genes & Development.* 2007, *21* (12), 1443 -1455.
[3] Baggio, L. L.; Drucker, D. J. Therapeutic approaches to preserve islet mass in type 2 diabetes. *Annu. Rev. Med.* 2006, *57*, 265-281.
[4] Nolan, C. J. et al. Type 2 diabetes across generations: from pathophysiology to prevention and management. *The Lancet.* 9, *378* (9786), 169-181.
[5] DeFronzo, R. A.; Abdul-Ghani, M. A. Preservation of β-cell function: the key to diabetes prevention. *J. Clin. Endocrinol. Metab.* 2011, *96* (8), 2354-2366.
[6] Rasmussen, S. E. et al. Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Mol Nutr Food Res.* 2005, *49* (2), 159-74.
[7] Crozier, A. et al. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat Prod Rep.* 2009, *26* (8), 1001-1043.
[8] Pinent, M. et al. Bioactivity of flavonoids on insulin-secreting cells. *Compr. Rev. Food. Sci. Food Saf.* 2008, *7* (4), 299-308.
[9] Castell-Auví, A. et al. Procyanidins modify insulinemia by affecting insulin production and degradation. *JNB.* 2011, *in press*.
[10] Montagut, G. et al. Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. *J. Nutr. Biochem.* 2010, *21* (10), 961-967.
[11] Berthiaume, N. et al. Development of insulin resistance and endothelin-1 levels in the Zucker fatty rat. *Metabolism.* 2003, *52* (7), 845-849.
[12] Augstein, P.; Salzsieder, E. Morphology of pancreatic islets: a time course of pre-diabetes in Zucker fatty rats. *Methods Mol. Biol.* 2009, *560*, 159-189.
[13] Jetton, T. L. et al. Mechanisms of compensatory beta-cell growth in insulin-resistant rats: roles of Akt kinase. *Diabetes.* 2005, *54* (8), 2294-304.
[14] Pajuelo, D. et al. Improvement of mitochondrial function in muscle of genetically obese rats after chronic supplementation with proanthocyanidins. *J. Agric. Food Chem.* 2011, *59* (15), 8491-8498.
[15] Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, *72*, 248-254.
[16] Huang, D. W. et al. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009, *4* (1), 44-57.
[17] Dennis, G., Jr et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 2003, *4* (5), P3.
[18] de Hoon, M. J. L. et al. Open source clustering software. *Bioinformatics.* 2004, *20* (9), 1453 -1454.
[19] Saldanha, A. J. Java Treeview—extensible visualization of microarray data. *Bioinformatics.* 2004, *20* (17), 3246 -3248.
[20] Ingenuity Systems, Inc. Ingenuity Pathways Analysis highlights novel molecular mechanisms of

immune system regulation. Application Note 0704.
[21] Han, D. et al. Detection of differential proteomes associated with the development of type 2 diabetes in the Zucker rat model using the iTRAQ technique. *J. Proteome Res.* 2011, *10* (2), 564-577.
[22] Chung, W.-G. et al. Hop proanthocyanidins induce apoptosis, protein carbonylation, and cytoskeleton disorganization in human colorectal adenocarcinoma cells via reactive oxygen species. *Food Chem. Toxicol.* 2009, *47* (4), 827-836.
[23] Actis-Goretta, L. et al. Cytotoxic effects of digalloyl dimer procyanidins in human cancer cell lines. *J. Nutr. Biochem.* 2008, *19* (12), 797-808.
[24] Zhong, J.-Y. et al. Inhibitory effects of grape procyanidins on free radical-induced cell damage in rat hepatocytes in vitro. *World J. Gastroenterol.* 2007, *13* (19), 2752-2755.
[25] Du, Y. et al. Grape seed polyphenols protect cardiac cells from apoptosis via induction of endogenous antioxidant enzymes. *J. Agric. Food Chem.* 2007, *55* (5), 1695-1701.
[26] Borradaile, N. M. et al. A Critical Role for Eukaryotic Elongation Factor 1A-1 in Lipotoxic Cell Death. *Molecular Biology of the Cell.* 2006, *17* (2), 770 -778.
[27] Parcellier, A. et al. Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochem. Biophys. Res. Commun.* 2003, *304* (3), 505-512.
[28] Laybutt, D. R. et al. Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia.* 2007, *50*, 752-763.
[29] Reddy, R. K. et al. The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca(2+)-binding and antiapoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis. *J. Biol. Chem.* 1999, *274* (40), 28476-28483.
[30] Bando, Y. et al. GRP94 reduces cell death in SH-SY5Y cells perturbated calcium homeostasis. *Apoptosis.* 2004, *9* (4), 501-508.
[31] Jeffrey, K. D. et al. Carboxypeptidase E mediates palmitate-induced beta-cell ER stress and apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 2008, *105* (24), 8452-8457.
[32] Johnson, J. D. Proteomic identification of carboxypeptidase E connects lipid-induced beta-cell apoptosis and dysfunction in type 2 diabetes. *Cell Cycle.* 2009, *8* (1), 38-42.
[33] Ahmed, M. et al. Mitochondrial proteome analysis reveals altered expression of voltage dependent anion channels in pancreatic β-cells exposed to high glucose. *Islets.* 2010, *2* (5), 283-292.
[34] Fonseca, S. G. et al. Stress hypERactivation in the β-cell. *Islets.* 2010, *2* (1), 1-9.
[35] Kriegenburg, F. et al. Molecular chaperones in targeting misfolded proteins for ubiquitin-dependent degradation. *The FEBS Journal [Internet].* [cited 2012 Jan 11] 2011,. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22177318
[36] D'Hertog, W. et al. Novel insights into the global proteome responses of insulin-producing INS-1E cells to different degrees of endoplasmic reticulum stress. *J. Proteome Res.* 2010, *9* (10), 5142-5152.
[37] Maris, M. et al. High glucose induces dysfunction in insulin secretory cells by different pathways: a proteomic approach. *J. Proteome Res.* 2010, *9* (12), 6274-6287.
[38] Pinent, M. et al. Procyanidins improve some disrupted glucose homeostatic situations: an analysis of doses and treatments according to different animal models. *Crit.Rev.Food Sci.Nutr.* 2011, *in press*.
[39] Docherty, H. M. et al. Relative contribution of PDX-1, MafA and E47/beta2 to the regulation of the human insulin promoter. *Biochem. J.* 2005, *389* (Pt 3), 813-820.
[40] Hutton, J. C. Insulin secretory granule biogenesis and the proinsulin-processing endopeptidases. *Diabetologia.* 1994, *37 Suppl 2*, S48-56.
[41] Boyer, P. D. The ATP synthase--a splendid molecular machine. *Annu. Rev. Biochem.* 1997, *66*, 717-749.
[42] Jitrapakdee, S. et al. Regulation of insulin secretion: role of mitochondrial signalling. *Diabetologia.* 2010, *53* (6), 1019-1032.
[43] Matschinsky, F. M. Regulation of pancreatic beta-cell glucokinase: from basics to therapeutics. *Diabetes.* 2002, *51 Suppl 3*, S394-404.

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IV. GENERAL DISCUSSION

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The endocrine pancreas is a key organ in metabolic control. Because it follows enteric absorption, this organ might be a target of procyanidins and other flavonoids. However, little data on the effects of procyanidins on the pancreas can be found in the literature, as we highlighted previously [1] and revised in the introduction of this doctoral thesis.

Previous work of our research group suggests that procyanidins act on the endocrine pancreas [2]. Our present work was designed to verify and understand this action. It was necessary to more concretely describe the effects of procyanidins on β-cell functionality by studying the modulation of the insulin synthesis and secretion processes by grape seed procyanidin extract (GSPE) in different physiological states. To achieve our objectives, we used *in vitro* models of β-cells, mouse insulinoma MIN6 cells and rat insulinoma INS-1E cells; and *in vivo* models of non-pathological rats, cafeteria diet-fed rats and genetically obese Zucker fatty (ZF) rats.

Our research group is focused on the identification and description of new bioactive compounds with potential protective/preventive properties against several diseases for functional food design. Extracts from natural plant sources are good candidates for formulating and developing new functional foods, and when working with extracts, it is necessary to develop tools that will produce reliable results. It is also important to note that the European Union recommends restricting animal experimentation as much as possible.

Taking together all of these considerations, we designed a tool to evaluate the bioactivity of natural extracts on β-cells using an *in vitro* model (**Chapter 1**). We previously described a coculture system of Caco-2 and HepG2 cells to study the bioactivity of these extracts on hepatocytes [3]. The coculture system was designed with human epithelial Caco-2 cells seeded onto culture inserts and INS-1E cells grown on the base of the well. This method reproduced the physiological situation in which the molecules present in the extracts are absorbed, and in some cases metabolised, across the intestinal epithelium barrier to the pancreas. Although this tool allows us to work with complex mixtures of compounds and to reproduce the processes that these compounds suffer in the epithelium cells, we did not use this system in our current study because of its culture time limitations. While there is a phenotypic adaptation of the cells to the new culture situation in the coculture system, which might reproduce the *in vivo* situation, we found that it is better to coculture the cells for only a short time. This finding restricted the use of this system; it has been not useful for 24 h treatments, this being the period of time necessary to treat the cells to reproduce the chronic effects seen in the *in vivo* models. However, our coculture system is a new tool that could be useful for other applications. In this respect, the same approach with hepatocytes is more robust and can be used for many types of experiments [3].

To overcome the limitations that *in vitro* models present, the most suitable alternative to obtain physiological relevant data is to work with *in vivo* models that allow the study of concepts in a whole body system. Therefore, we analysed the physiological effects of GSPE on β-cell functionality in non-pathological animals and in models with damaged β-cells.

To address whether different GSPE treatments modify β-cell functionality in non-pathological models, *i.e.*, healthy animals (**Chapter 2**), we designed four different *in vivo* experiments using Wistar rats. Two experiments were chronic (21 or 36 days) dose-response treatments that were directed to determine the most effective dose. The GSPE doses ranged from 2.5 to 50 mg GSPE/kg of body weight (bw). The third experiment was a chronic treatment (45 days) with 25 mg GSPE/kg of bw, and the final experiment was an acute treatment (1 h) with a high dose of GSPE (1 g GSPE/kg of bw). Islets were isolated in the last two experiments, which allowed us to obtain more information on the effects of GSPE on β-cell functionality by analysing the islet insulin secretion response after stimulation with glucose.

These groups of animals showed peculiar insulin plasma level and HOMA index profiles when the results from all animal groups are represented according to the GSPE dose ingested. Whereas the lowest

and highest doses of GSPE did not affect insulinaemia, moderate doses, such as 315 mg GSPE/kg of bw * days of treatment, increased insulin plasma levels and a higher dose, 900 mg GSPE/kg of bw * days of treatment, decreased insulinaemia. In addition, the HOMA index values followed the same tendency, showing that the changes in insulin did not provoke significant changes in glycaemia. These data emphasise that the efficacy of procyanidins depends on the dose and time of treatment.

The effects of GSPE on insulinaemia may be due to modifications in insulin production (synthesis and/or secretion) and/or insulin removal. In fact, our data indicate that procyanidins have a tendency to reduce insulin biosynthesis, as shown by the insulin and PDX1 mRNA levels. Otherwise, we are able to explain the increase in insulin plasma levels at some moderate doses with the decrease in insulin removal. The expression of insulin-degrading enzyme (IDE) in the liver, the primary player in insulin clearance, at a dose of 315 mg GSPE/kg of bw * days of treatment decreased. These results identify IDE as a target of procyanidins for the first time. In fact, there is little information in the literature about the transcriptional regulation of IDE. In the available studies, insulin and PPARγ have been identified as modulators of IDE gene expression in hepatocytes and neurons, respectively [4,5]. Thus, procyanidins are insulinomimmetic in other tissues, and GSPE could modulate IDE gene expression in a similar manner as insulin.

Functional foods are foods and food components that provide a health benefit beyond basic nutrition. Functional foods are not designed to be drugs to correct or improve a disrupted function, but are expected to delay or avoid some initial signs of pain, allowing better health. The ability to delay or limit type 2 diabetes mellitus (T2DM) in Western societies is being analysed using this approach [6-8]. This multifactorial disease results from the interaction of environmental factors and genetic predisposition, leading to two abnormalities: insulin resistance and β-cell dysfunction. During the long persistent silent phase, known as prediabetes, that precedes the onset of T2DM, hyperinsulinaemia appears to compensate for insulin resistance. Hyperglycaemia then develops with progressive impaired β-cell function [9]. Several animal models have been used to study β-cell dysfunction, which can develop from other diseases to T2DM. In this doctoral thesis, we examined two animal models. First, we used a diet-induced obesity model in which animals were fed a cafeteria diet; and in the second experiment, we studied genetically induced obesity with ZF rats.

The cafeteria diet model has been used as a robust model because it is a good reproduction of the diet of Western society, where the prevalence of overweight and obesity has increased drastically in the last decades [10]. Because we induce the damage, this animal model is very useful to assay the possible action of functional foods to prevent or improve the dysfunctionality. The cafeteria diet model consists of feeding the animals a substantial amount of salt, sugar and fat to promote voluntary hyperphagia, producing a rapid increase in weight gain and fat pad mass. In addition, the animals fed this type of diet reach a prediabetic state, showing insulin resistance and impaired β-cell function [10,11]. We previously described that these animals have ectopic lipid accumulation in the pancreas and increased insulin clearance activity mediated by IDE [12].

In our study, obesity was induced by feeding the animals a cafeteria diet for 13 weeks (**Chapter 4**). Then, the animals were divided into two groups: the cafeteria diet group and the cafeteria + 25 GSPE group, with animals fed the cafeteria diet concomitantly with 25 mg GSPE/kg of bw for 30 days. A study on the effects of a cafeteria diet on insulin production and clearance confirmed that treatment of rats with a cafeteria diet, for 17 weeks in our case, mimicked a prediabetic state [12]. Chronic GSPE treatment was able to improve hyperinsulinaemia [2]. Studies with other flavonoids also showed the effects of these phenolic compounds on reducing insulinaemia [13-15]. This decrease was due, first of all, to decreased insulin production; GSPE-treated animals have decreased insulin gene expression, as well as decreased expression of one of its major regulators, PDX1. Moreover, the pancreatic insulin content and insulin secretion of these animals was lower. The action of procyanidins on insulin resistance leads the cells to

achieve the levels of insulin production and secretion observed in the control rats. Therefore, our results suggest that procyanidins are able to prevent the cafeteria diet-induced dysfunction in β-cells.

The data presented are in accordance with previous results related to the effects of procyanidins mimicking some of the physiological effects of insulin [16]. Analysis of the same experiment with cafeteria-fed animals reported that procyanidins stimulate glucose uptake in peripheral tissues, with less insulin necessary to induce this uptake [2]. In this regard, the present study shows that GSPE targets the β-cells that are repressing insulin synthesis and secretion to adapt the insulin plasma levels to the physiological demands.

As described above, we also used a genetically induced obesity model, ZF rats, to evaluate the effectiveness of the extract under this genetically programmed dysfunction. The ZF rat is a model of obesity and prediabetes that is characterised by a point mutation in the leptin receptor. This mutation leads to impaired signalling of the leptin receptor, which results in hyperphagia, insulin resistance, hyperinsulinaemia, hyperlipoproteinaemia and obesity [17,18]. These rats become glucose intolerant but do not develop T2DM.

To assess the effects of procyanidins on β-cell functionality in the ZF model, we designed an experiment with three animal groups: a control lean group, a control ZF group (animals with the mutation in the leptin gene) and an experimental ZF + 35 mg GSPE/kg of bw group (Zucker fatty rats treated with GSPE) (**Chapter 5**). We worked with five- to six-week old rats, and the period of treatment was 10 weeks. We selected younger animals that guaranteed the genetically obese recessive phenotype but were not strongly damaged because we were expecting mild GSPE action [19]. As hypothesised, our results indicate that in a situation of obesity-related insulin resistance with a failure of β-cell compensation due to genetic factors, procyanidins are not effective enough to prevent β-cell dysfunction.

The analysis of insulin plasma levels demonstrates that procyanidins, at the dose and treatment time studied, cannot counteract hyperinsulinaemia. Therefore, these flavonoids are not able to modify insulin secretion (C-peptide levels). However, insulin production is decreased, as shown by the insulin mRNA and protein levels in isolated islets. In addition, procyanidins also reduce the expression of the β-cell master gene PDX1, which controls insulin gene expression. These results suggest that although procyanidins affect β-cell gene expression, the action of GSPE is not strong enough to show observable effects in insulin physiological levels. Therefore, GSPE cannot counteract the strong metabolic disruption resulting from the studied genetic factor.

After describing the effects of GSPE on insulin synthesis and secretion in non-pathological and insulin resistant models, we then analysed the mechanism of action of GSPE. We isolated islets from the rats treated with GSPE and used the β-cell line INS-1E after rejecting MIN6 cells mainly because the GSIS of the MIN6 cells was not very consistent.

A common trait found throughout our studies is that the procyanidin extract tested caused a reduced GSIS. When isolated islets from rats were treated acutely or chronically with GSPE, we found that the procyanidin extract significantly decreased the capacity of the islets to respond to glucose stimulus. Some of these GSPE effects in these islets could result from its effect on insulin biosynthesis because insulin and PDX1 mRNA levels were downregulated. It must be highlighted that the total amount of procyanidins reached at the end of the treatment was similar, but the analysis of procyanidin content in the plasma from both groups showed differences in concentration and composition, as could be expected due to their different treatments (**Chapter 2)**. We found the same effect when we treated INS-1E cells chronically with GSPE (**Chapter 2**) at the mRNA and protein level.

Effects on mRNA expression control insulin synthesis, but an important step that controls insulin secretion is the cell energetic pathway from glucose entry to insulin secretion. Thus, we next evaluated whether some of the points in this pathway could be targets of GSPE (Figure 16). GSPE increases

glucose uptake in β-cells under high glucose conditions, although it decreases the expression of the key effectors of glucose uptake, GLUT2 and glucokinase. In addition, procyanidins impair glucose-induced mitochondrial hyperpolarisation, decrease ATP synthesis and alter cellular membrane potential. These results note that GSPE acts through decreasing the ability of β-cells to secrete insulin in response to glucose entry by uncoupling the entire process.

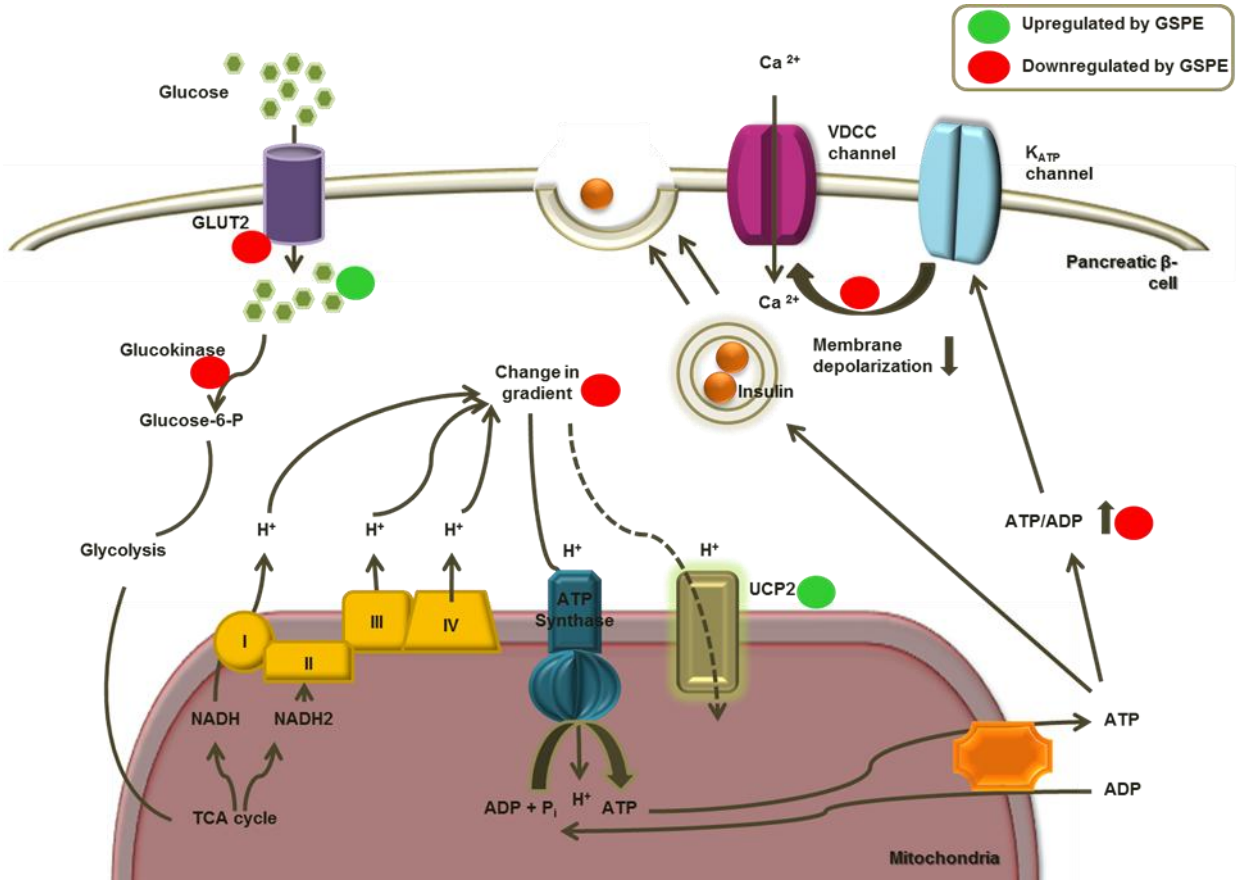

Figure 16. Effects of GSPE on the insulin secretion process.

To identify GSPE targets in β-cells, we developed a study to create a microRNA (miRNA) profile for islets isolated from the animals treated chronically (45 days) with 25 mg GSPE/kg of bw to support the previous results (**Chapter 3**). MiRNAs play a key role in the regulation of metabolic processes in diabetes [20], and some authors have shown that flavonoids modulate miRNA expression [21-23]. In this work, we found four miRNAs that demonstrated significantly different expression in GSPE-treated islets. This is the first time, to our knowledge, that pancreatic miRNAs have been described as procyanidin targets. Because the information on the function of the identified miRNAs is lacking, we performed an *in silico* analysis to understand the roles of these miRNAs in the pancreas through their predicted and validated targets. The *in silico* prediction studies suggest that ion transport and response to stimuli, such as hormones and organic substances, including glucose, might be among the pathways affected by the miRNAs modulated by GSPE. Therefore, the effects that we have described on islet functionality, such as the modulation of cell and mitochondrial membrane permeability, might be due in part to the effects of GSPE on modifying miRNA expression patterns. In fact, the involvement of other miRNAs has been described in the regulation of pancreas functionality by modulating membrane proteins [24,25]. The main targets of GSPE in non-pathological β-cells are involved in mitochondrial and cellular membrane permeability. We did not discard the possibility of a direct GSPE role in insulin synthesis because according to published data [26], insulin accumulation in the cell does not seem to be responsible for the inhibition of insulin mRNA expression.

As we observed in the non-pathological *in vitro* and *in vivo* models, GSPE targets β-cells by repressing their insulin production and secretion capacity; this effect was also observed in cafeteria diet-fed animals. To evaluate if the previously described GSPE target from the non-pathological models also worked in an impaired β-cell environment, we incubated INS-1E cells in a high glucose, high insulin and high oleate medium, separately (**Chapter 4**). We reproduced the GSPE effects observed in the cafeteria diet *in vivo* model by culturing the β-cells with high oleate for three days. In this culture condition, GSPE counteracted the increase observed in the insulin mRNA levels after oleate incubation and slightly improved the effect of oleate on insulin secretion. These results suggest that GSPE acts on β-cell lipid metabolism to exert its bioactivity on insulin synthesis and secretion.

Next, we analysed the accumulation of triglycerides (TGs) in β-cells cultured with oleate and in the pancreas of cafeteria diet-fed animals. Although the GSPE effects observed *in vitro* were moderate, the results obtained *in vivo* indicated that the effects of GSPE on impaired β-cells are mediated by its ability to diminish lipid accumulation in pancreas; the GSPE-treated animals had the same amount of TGs in their pancreas as the control groups. Therefore, GSPE might act by up-regulating the expression of Cpt1a, which increases β-cell oxidation, and downregulating the expression of lipid synthesis-related genes, such as Fasn and Srebf1.

Moreover, we have described AMPK as a GSPE target because procyanidin treatment counteracts the decrease in AMPK protein levels due to the cafeteria diet. AMPK plays a central role in regulating cellular metabolism, and its activity is influenced by changes in the AMP/ATP ratio [27]. In addition, AMPK has been identified as a target of other natural compounds [28]. Our results show that in damaged β-cells, GSPE also diminishes the accumulation of TGs.

Once we described the effects of GSPE in non-pathological conditions and with a cafeteria diet, we studied the more damaged *in vivo* model. As described above, although GSPE represses insulin gene expression in islets isolated from ZF rats treated with GSPE, the effects of procyanidins are not sufficient to counteract the characteristic hyperinsulinaemia of this model. In this sense, we observed similar effects *in vitro* in INS-1E-cells cultured under conditions of hyperglycaemia and hyperinsulinaemia (**Chapter 2**), in which GSPE was unable to improve β-cell functionality. Similar effects were expected in INS-1E cells cultured with palmitate because this saturated fatty acid causes considerable negative effects on β-cell function [29].

The proteomic study of the islets isolated from the chronically treated rats (**Chapter 5**) gave us a more complete picture of the effects of GSPE on pancreatic islets. A total of 31 proteins were differentially expressed between ZF rats treated with GSPE and the ZF rat control group; 10 of the proteins were upregulated and the other 21 were downregulated. Gene Ontology (GO) analysis revealed that these proteins are associated with hexose metabolic processes, translation and protein folding, macromolecular complex assembly, response to hormone stimulus and apoptosis and cell death. Interestingly, insulin was identified as one of the differentially expressed proteins. GSPE treatment significantly reduced the insulin protein in islets, which is in accordance with the results on insulin gene expression. However, the effects observed in the mRNA and protein levels in islets are not observed in insulin plasma. Moreover, GSPE treatment decreases the protein levels of carboxypeptidase E (CPE), an important enzyme in the conversion of proinsulin to insulin [30]. These results support the direct effect of GSPE on insulin synthesis as suggested above. As a part of insulin production, the insulin secretion process is also a target of GSPE in ZF rats. Procyanidin treatment downregulates the protein levels of two of the enzymes involved in the glycolytic pathway, PKM2 (pyruvate kinase isozymes M1/M2) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and of a member of ATP synthase protein complex, ATP5B [31]. This effect is in accordance with the GSPE action on ATP synthesis observed in non-damaged β-cells (**Chapter 2**), suggesting that procyanidin treatment in ZF rats might affect ATP synthesis and thus insulin secretion, although the C-peptide levels were not altered by this treatment.

All of the data discussed above allow us to form a hypothesis of the GSPE mechanism of action on β-cells (Figure 17).

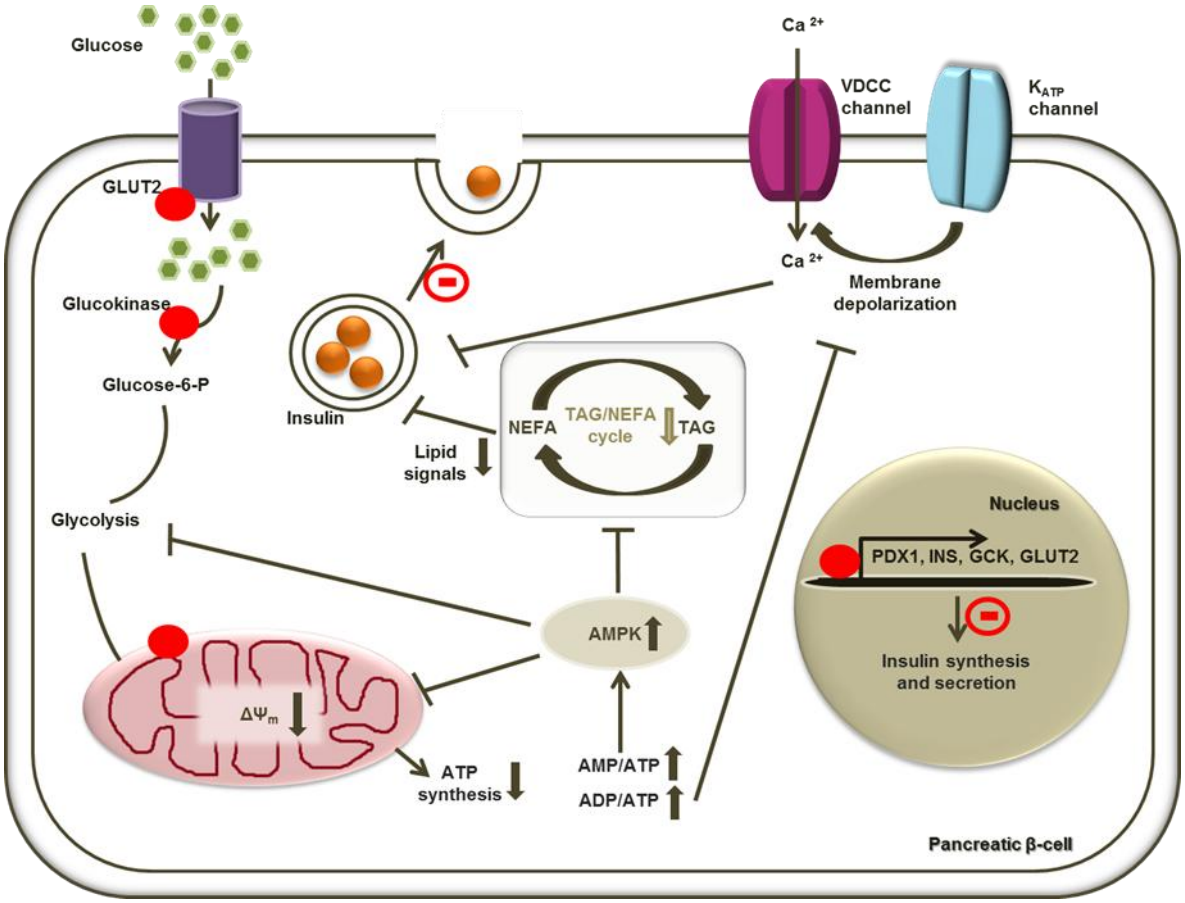

Figure 17. Hypothesis of GSPE action on pancreatic β-cells.

According to our hypothesis and the results obtained in this study, procyanidins reduce the mitochondrial membrane polarisation, producing a decrease in the rate of ATP synthesis. A consequent increase in the AMP/ATP ratio may result in AMPK activation, and an increased ADP/ATP ratio limits the closure of the K_{ATP} channels (indicated by cell membrane potential), therefore triggering the Ca^{2+} pathway for glucose-induced insulin secretion. In addition, as has been previously reported [32], upregulation of AMPK activity increases lipid oxidation and reduces TGs accumulation in β-cells. This effect produces a decrease in the TG/NEFA cycle, producing a diminution in the generation of lipid signalling molecules involved in the amplification pathways of GSIS [33].

Moreover, procyanidins modify the gene expression of proteins involved in the insulin synthesis and secretion processes. GSPE inhibits the expression of PDX1, a master β-cell gene that regulates insulin, glucokinase and GLUT2 [34,35], producing a decrease in the mRNA levels of these genes. These procyanidin effects on gene expression lead to decreased insulin production and secretion.

In conclusion, GSPE modulates β-cell functionality in non-pathological states and could be used as a bioactive compound to limit β-cell dysfunction under high-palatable diets. Otherwise, at the assayed doses, its action is not sufficient to counteract a genetically induced metabolic disruption.

References

[1] Pinent, M., Castell, A., Baiges, I., Montagut, G., et al. Bioactivity of Flavonoids on Insulin-Secreting Cells. *Comprehensive Reviews in Food Science and Food Safety*. 2008*,7*,299-308.

[2] Montagut, G., Blade, C., Blay, M., Fernandez-Larrea, J., et al. Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. *J.Nutr.Biochem.* 2010*,21*,961-967.

[3] Castell-Auví, A., Motilva, M.J., Macià, A., Torrell, H., et al. Organotypic co-culture system to study plant extract bioactivity on hepatocytes. *Food Chem.* 2010*,122*,775-781.

[4] Pivovarova, O., Gogebakan, O., Pfeiffer, A.F., Rudovich, N. Glucose inhibits the insulin-induced activation of the insulin-degrading enzyme in HepG2 cells. *Diabetologia*. 2009*,52*,1656-1664.

[5] Du, J., Zhang, L., Liu, S., Zhang, C., et al. PPARgamma transcriptionally regulates the expression of insulin-degrading enzyme in primary neurons. *Biochem.Biophys.Res.Commun.* 2009*,383*,485-490.

[6] Li-Chan, E.C., Huang, S.L., Jao, C.L., Ho, K.P., Hsu, K.C. Peptides Derived from Atlantic Salmon Skin Gelatin as Dipeptidyl-Peptidase IV Inhibitors. *J.Agric.Food Chem.* 2012.

[7] Udani, J.K., Singh, B.B., Singh, V.J., Barrett, M.L. Effects of Acai (Euterpe oleracea Mart.) berry preparation on metabolic parameters in a healthy overweight population: a pilot study. *Nutr.J.* 2011*,10*,45.

[8] Hirai, S., Takahashi, N., Goto, T., Lin, S., et al. Functional food targeting the regulation of obesity-induced inflammatory responses and pathologies. *Mediators Inflamm.* 2010*,2010*,367838.

[9] DeFronzo, R.A. Pathogenesis of type 2 diabetes mellitus. *Med.Clin.North Am.* 2004*,88*,787-835, ix.

[10] Sampey, B.P., Vanhoose, A.M., Winfield, H.M., Freemerman, A.J., et al. Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome With Liver and Adipose Inflammation: Comparison to High-Fat Diet. *Obesity (Silver Spring)*. 2011.

[11] Rolls, B.J., Rowe, E.A., Turner, R.C. Persistent obesity in rats following a period of consumption of a mixed, high energy diet. *J.Physiol.* 1980*,298*,415-427.

[12] Castell-Auví, A., Cedó, L., Pallarès, V., Blay, M., et al. The effects of a cafeteria diet on insulin production and clearance in rats. *BJN*. 2011.

[13] Ihm, S.H., Lee, J.O., Kim, S.J., Seung, K.B., et al. Catechin prevents endothelial dysfunction in the prediabetic stage of OLETF rats by reducing vascular NADPH oxidase activity and expression. *Atherosclerosis*. 2009*,206*,47-53.

[14] Cho, A.S., Jeon, S.M., Kim, M.J., Yeo, J., et al. Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. *Food Chem.Toxicol.* 2010*,48*,937-943.

[15] Shih, C.C., Lin, C.H., Lin, W.L. Effects of Momordica charantia on insulin resistance and visceral obesity in mice on high-fat diet. *Diabetes Res.Clin.Pract.* 2008*,81*,134-143.

[16] Pinent, M., Blay, M., Blade, M.C., Salvado, M.J., et al. Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. *Endocrinology*. 2004*,145*,4985-4990.

[17] Cockburn, B.N., Ostrega, D.M., Sturis, J., Kubstrup, C., et al. Changes in pancreatic islet glucokinase and hexokinase activities with increasing age, obesity, and the onset of diabetes. *Diabetes*. 1997*,46*,1434-1439.

[18] Liu, Y.Q., Jetton, T.L., Leahy, J.L. beta-Cell adaptation to insulin resistance. Increased pyruvate carboxylase and malate-pyruvate shuttle activity in islets of nondiabetic Zucker fatty rats. *J.Biol.Chem.* 2002*,277*,39163-39168.

[19] Pinent, M., Cedo, L., Montagut, G., Blay, M., Ardevol, A. Procyanidins improve some disrupted glucose homeostatic situations: an analysis of doses and treatments according to different animal models. *Crit.Rev.Food Sci.Nutr.* in press.

[20] Herrera, B.M., Lockstone, H.E., Taylor, J.M., Ria, M., et al. Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes. *Diabetologia*. 2010*,53*,1099-1109.

[21] Arola-Arnal, A., Blade, C. Proanthocyanidins Modulate MicroRNA Expression in Human HepG2 Cells. *PLoS One*. 2011*,6*,e25982.

[22] Murase, T., Misawa, K., Minegishi, Y., Aoki, M., et al. Coffee polyphenols suppress diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice. *Am.J.Physiol.Endocrinol.Metab.* 2011*,300*,E122-33.

[23] Boesch-Saadatmandi, C., Loboda, A., Wagner, A.E., Stachurska, A., et al. Effect of quercetin and its metabolites isorhamnetin and quercetin-3-glucuronide on inflammatory gene expression: role of miR-155. *J.Nutr.Biochem.* 2011*,22*,293-299.

[24] Sun, L.L., Jiang, B.G., Li, W.T., Zou, J.J., et al. MicroRNA-15a positively regulates insulin synthesis by inhibiting uncoupling protein-2 expression. *Diabetes Res.Clin.Pract.* 2011*,91*,94-100.

[25] Pullen, T.J., da Silva Xavier, G., Kelsey, G., Rutter, G.A. miR-29a and miR-29b Contribute to Pancreatic {beta}-Cell-Specific Silencing of Monocarboxylate Transporter 1 (Mct1). *Mol.Cell.Biol.* 2011*,31*,3182-3194.

[26] Leibiger, I.B., Leibiger, B., Berggren, P.O. Insulin signaling in the pancreatic beta-cell. *Annu.Rev.Nutr.* 2008*,28*,233-251.

[27] Hardie, D.G. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat.Rev.Mol.Cell Biol.* 2007*,8*,774-785.

[28] Hwang, J.T., Kwon, D.Y., Yoon, S.H. AMP-activated protein kinase: a potential target for the diseases prevention by natural occurring polyphenols. *N.Biotechnol.* 2009*,26*,17-22.

[29] Hovsepyan, M., Sargsyan, E., Bergsten, P. Palmitate-induced changes in protein expression of insulin secreting INS-1E cells. *J.Proteomics*. 2010*,73*,1148-1155.

[30] Hutton, J.C. Insulin secretory granule biogenesis and the proinsulin-processing endopeptidases. *Diabetologia*. 1994*,37 Suppl 2*,S48-56.

[31] Boyer, P.D. The ATP synthase--a splendid molecular machine. *Annu.Rev.Biochem.* 1997*,66*,717-749.

[32] da Silva Xavier, G., Leclerc, I., Salt, I.P., Doiron, B., et al. Role of AMP-activated protein kinase in the regulation by glucose of islet beta cell gene expression. *Proc.Natl.Acad.Sci.U.S.A.* 2000*,97*,4023-4028.

[33] Nolan, C.J., Madiraju, M.S., Delghingaro-Augusto, V., Peyot, M.L., Prentki, M. Fatty acid signaling in the beta-cell and insulin secretion. *Diabetes*. 2006*,55 Suppl 2*,S16-23.

[34] Waeber, G., Thompson, N., Nicod, P., Bonny, C. Transcriptional activation of the GLUT2 gene by the IPF-1/STF-1/IDX-1 homeobox factor. *Mol.Endocrinol.* 1996*,10*,1327-1334.

[35] Watada, H., Kajimoto, Y., Miyagawa, J., Hanafusa, T., et al. PDX-1 induces insulin and glucokinase gene expressions in alphaTC1 clone 6 cells in the presence of betacellulin. *Diabetes*. 1996*,45*,1826-1831.

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V. CONCLUSIONS

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1. **The coculture system designed with Caco-2 and INS-1E cells is a suitable method to assay the bioactivity of natural extracts of unknown bioavailability on β-cells for short treatment times.**

2. **Procyanidins modulate β-cell functionality.**

   In non-pathological models, GSPE acts on insulin synthesis, secretion and/or degradation. The efficacy of procyanidins depends on the dose and the time of treatment.

   GSPE treatment partially disrupts cafeteria diet-induced β-cell dysfunction by counteracting the increase in insulin production and secretion provoked by this diet.

   Although GSPE modulates β-cell gene expression, its effect on Zucker fatty model, at the dose and period of treatment tested, is not enough to counteract the physiological dysfunctions caused by the genetic mutation.

3. **The main target sites of GSPE in β-cells are mitochondrial and cellular membrane permeability. Under lipotoxic conditions, GSPE improves β-cell functionality, thus preventing lipid damage.**

4. **The effects of GSPE observed on membrane permeability might be due in part to its effects on modifying the microRNA profile.**

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VI. ANNEXE

UNIVERSITAT ROVIRA I VIRGILI
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1. ABBREVIATION LIST

ACH	Acetylcholine
ACN	Acetonitrile
ACTG1	Actin cytoplasmic type 8
AD	Alzheimer disease
AICAR	Activator 5-amino-4-imidazolecarboxamide riboside
AKT1	v-akt murine thymoma viral oncogene homolog 1
AMPK	AMP-activated protein kinase
AMPKA2	AMP-activated protein kinase, alpha 2
AMY2	Pancreatic alpha-amylase
ATCC	American tissue culture collection
ATF	Activating transcription factor 1
ATP2A2	ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2
ATP5B	ATP synthase subunit beta. mitochondrial
BAX	BCL2-associated X protein
BCAT2	Branched chain amino-acid transaminase 2
BCL2	B-cell CLL/lymphoma 2
Bw	Body weight
$[Ca^{2+}]_c$	Cytosolic free calcium
$[Ca^{2+}]_i$	Intracellular free calcium
CBG	Cytosolic β-glucosidase
CCK	Cholecystokinin
CCKAR	CCKA receptor
CDX3	Caudal type homeobox 3
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CEBP	CCAAT/enhancer binding protein
CFTR	Cystic fibrosis transmembrane conductance regulator
CNS	Central nervous system
COL6A1	Collagen alpha-1(VI) chain
CPA1/2	Carboxypeptidase A1/2
CPE	Carboxypeptidase E
CpG	Cytosine-guanosine dinucleotide
CPT1A	Carnitine palmitolitransferase-1A
CRE	cAMP response element
CREB	cAMP responsive element binding protein
CSE	Consecutive signal enhancement
CTRB1	Chymotrypsinogen B
DAG	Diacylglycerol
DAVID	Database for annotation, visualization and integrated discovery
DMEM	Dulbecco's modified minimum essential medium
E2	17β-estradiol
ECG	Epicatechin gallate
EEF1A1	Elongation factor 1-alpha 1
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
EGP	Endogenous glucose production
ELK1	ELK1, member of ETS oncogene family
ER	Endoplasmic reticulum
ESR1	Estrogen receptor 1
FASN	Fatty acid synthase
FCCP	p-trifluoromethoxyphenylhydrazone
FFA	Free fatty acid
FOS	FBJ murine osteosarcoma viral oncogene homolog

FOXO1	Forkhead box O1
FOXA2	Forkhead box A2
FPG	Fasting plasma glucose levels
GAB1	GRB2-associated binding protein 1
GABA	Gamma-aminobutyric acid
$GABA_AR$	type A GABA receptors
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCG	Glucagon
GDH	Glutamate dehydrogenase
GI	Glycemic index
GIP	Glucose-dependent insulinotropic polypeptide
GlcNAc	N-acetylglucosamine
GLIS3	GLIS family zinc finger 3
GLP1	Glucagon-like peptide 1
GLP1R	GLP1 receptor
GLRX1	Glutaredoxin 1
GLUT1/2/4	Glucose transporters 1/2/4
GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide
GO	Gene ontology
GPCR	G-protein-coupled receptors
GRB2	Growth factor receptor-bound protein 2
GRM5	Glutamate receptor, metabotropic 5
GRP	Gastrin releasing peptide
GRTA	Geniom real time analyzer
GSIS	Glucose-stimulated insulin secretion
GSK·	Glycogen synthase kinase 3
GSPE	Grape seed procyanidin extract
H3F3B	Histone H3.3
HADH	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial
HBA1	Hemoglobin subunit alpha-1/2
HBB	Hemoglobin subunit beta-1
HBP	Hexosamine biosynthetic pathway
HDAC1/2	Histone deacetylase 1
HF	High fat diet
HIST1H4B	Histone H4
HNF1A	Hepatocyte nuclear factor 1 homeobox A
HNF4A	Hepatocyte nuclear factor 4 homeobox A
HSP90B1	Endoplasmin
HSPA5	78 kDa glucose-regulated protein
HSPD1	60 kDa chaperonin
HYOU1	Hypoxia up-regulated protein 1
IAPP	Islet amyloid polypeptide
IDA	Information-dependent acquisition
IDE	Insulin-degrading enzyme
IFG	Impaired fasting glucose
IGF1/2	Insulin-like growth factor 1/2
IGFR1/2	IGF receptors ½
IGT	Impaired glucose tolerance
INS1/2	Insulin gene-1/2
INSRA/B	Insulin receptor type A or B
IPA	Ingenuity Pathway Analysis
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin resistance
IRS	Insulin receptor substrate
ISL1	ISL LIM homeobox 1

iTRAQ	Isobaric tags for relative and absolute quantitation
K_{ATP} channel	ATP-sensitive potassium channel
KLF11	Kruppel-like factor 11
KRBH	Krebs-Ringer bicarbonate HEPES buffer
KRT1	Keratin. type II cytoskeletal 1
KRT9	Keratin. type I cytoskeletal 9
K_v channels	Voltage dependent K^+ channels
LC-CoA	Long-chain Co-A
LDH	Lactate dehydrogenase
LPH	Lactase phloridizin hydrolase
MAFA	v-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK	Mitogen-activated protein kinase
MAZ	MYC-associated zinc finger protein (purine-binding transcription factor)
MDH1	Malate dehydrogenase, cytoplasmic
MECP2	Methyl CpG binding protein 2
MEKK	Mitogen-activated protein kinase kinase
MIDD	Maternally inherited diabetes with deafness
MiRNA	MicroRNA
MODY	Maturity-onset diabetes of the young
mSOS	Guanine nucleotide exchange factor
MTORC1	Mechanistic target of rapamycin (serine/threonine kinase) complex 1
MYC	v-myc myelocytomatosis viral oncogene homolog
NEFA	Non-esterified fatty acids
NEUROD1	Neurogenic differentiation 1
NFAT	Nuclear factor of activated T-cells
NFKB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGT	Normal glucose tolerance
NKX2-2	NK2 homeobox 2
NKX6-1	NK6 homeobox 1
NOD mice	Non-obese diabetic mice
NPY	Neuropeptide Yare
NR0B2	Nuclear receptor subfamily 0, group B, member 2
NT	Neurotensin
OGT	O-linked N-acetylglucosaminyl transferase
OGTT	Oral glucose tolerance test
OLETF	Otsuka Long-Evans Tokushima Fatty
PACAP	Pituitary adenlyate cyclase activating polypeptide
PASK	PAS domain containing serine/threonine kinase
PAX4/6	Paired box 4/6
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PDI	Protein disulfide isomerase
PDIA3	Protein disulfide-isomerase A3
PDPK1	3-phosphoinositide dependent protein kinase-1
PDX1	Pancreatic duodenal homeobox 1
PI3K	Phosphoinositide-3-kinase
PKA	Protein kinase A
PKM2	Pyruvate kinase isozymes M1/M2
PLC	Phospholipase C
PLCB	Phospholipase C, beta
PLCG	Phospholipase C, gamma
PNLIP	Pancreatic triacylglycerol lipase
PNLIPRP1	Pancreatic lipase-related protein 1
PP	Polypeptide
PPARG	Peroxisome proliferator-activated receptor gamma

PPIA	Peptidyl-prolyl cis-trans isomerase A
PPRE	Peroxisome proliferator-response element
PRKC	Protein kinase C
PRKCA/E	Protein kinase C, alpha/epsilon
PRKG	Protein kinase, cGMP-dependent
PTB	Polypyrimidine tract-binding protein
PTPs	Protein tyrosine phosphatises
PURA	Purine-rich element binding protein A
RAB1A	Ras-related protein Rab-1A
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RAPGEF4	Rap guanine nucleotide exchange factor 4
ROS	Reactive oxygen species
RP	Reserve pool
RPLP1	60S acidic ribosomal protein P1
RPS6KB2	Ribosomal protein S6 kinase, 70kDa, polypeptide 2
RPSA	40S ribosomal protein SA
RRP	Readily releasable pool
RXR	Retinoid X receptor
SAPE	Streptavidin-phycoerythrin
SAPK	Stress-activated protein kinase
SAT	Subcutaneous adipose tissue
SCS	Succinyl-CoA synthetase
SGLT1	Sodium glucose cotransporter 1
SH2/3	SRC homology domain 2/3
SHC1	(Src homology 2 domain containing) transforming protein 1
SH2B2	SH2B adaptor protein 2
SHP2	Protein-tyrosine phosphatase containing SH2 domains
SLC8A1	Solute carrier family 8 (sodium/calcium exchanger), member 1
SLC17A6/7	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6/7
SLC25A4	ADP/ATP translocase 1
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SRE	Serum response element
SRF	Serum response factor
SUR1	Sulfonylurea receptor 1
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA cycle	Tricarboxylic acid cycle
TCF3	Transcription factor 3
TEER	Trans-epithelium electrical resistance
TFA	Trifluoroacetic acid
TFAM	Mitochondrial transcription factor A
TG	Triglycerides
TGFA	Transforming growth factor alpha
TRPM	Thermosensitive transient receptor potential
TTBS	Tris-buffered saline plus tween-20
TUBA1B	Tubulin alpha-1B chain
TXN1	Thioredoxin
TZD	Thiazolidinedione
UCP2	Mitochondrial uncoupling protein 2
UNC13	Unc-13 homolog A
UBB	Polyubiquitin-B
UPR	Unfolded protein response
UTR	Untranslated region
VAT	Visceral adipose tissue

VDAC1	Voltage-dependent anion-selective channel protein 1
VDCC	Voltage-dependent Ca^{2+} channels
VIP	Vasoactive intestinal polypeptide
ZDF	Zucker diabetic fatty rat
ZF	Zucker fatty rats
ZL	Zucker lean rats
βIRKO	β-cell–specific insulin receptor knockout
$\Delta\Psi_m$	Mitochondrial membrane potential

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Publications

1. **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M.; Motilva, M.J.; Garcia-Vallvé, S.; Pujadas, G.; Maechler, P.; Ardévol, A. Procyanidins modify insulinaemia by affecting insulin production and degradation. *Journal of Nutritional Biochemistry* **2012**, *In press*.

2. Pallarès, V.; Calay, D.; Cedó, L.; **Castell-Auví, A.**; Raes, M.; Pinent, M.; Ardévol, A.; Arola, L.; Blay, M. Enhanced anti-inflammatory effect of resveratrol and EPA in treated endotoxin-activated RAW 264.7 macrophages. *British Journal of Nutrition* **2012**, Jan 6:1-12.

3. **Castell-Auví, A.***; Cedó, L.*; Pallarès, V.; Blay, M.; Ardévol, A.; Pinent, M. The effects of a cafeteria diet on insulin production and clearance in rats. *British Journal of Nutrition* **2011**, Dec 12:1-8.

4. Pallarès, V.; Calay, D.; Cedó, L.; **Castell-Auví, A.**; Raes, M.; Pinent, M.; Ardévol, A.; Arola, L.; Blay, M. Additive, antagonistic and synergistic effects of procyanidins and polyunsaturated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopoysaccharide. *Nutrition* **2011**, Dec 12.

5. **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M.; Motilva, M.J.; Arola, L.; Ardévol, A. Development of a coculture system to evaluate the bioactivity of plant extracts on pancreatic β-cells. *Planta Medica* **2010**,76:1-6.

6. **Castell-Auví, A.**; Motilva, M.J.; Macià, A.; Torrell, H.; Bladé, C.; Pinent, M.; Arola, L.; Ardévol, A. Organotypic co-culture system to study plant extract bioactivity on hepatocytes. *Food Chemistry* **2010**, 122:775-781.

7. Pinent, M.; **Castell, A.**; Baiges, I.; Montagut, G.; Arola, L.; Ardévol, A. Bioactivity of flavonoids on insulin-secreting cells. *Comprehensive reviews in food science and food safety* **2008**, 7:299-308.

8. **Castell-Auví, A.***; Cedó, L.*; Movassat, J.; Portha, B.; Sánchez-Cabo, F.; Pallarès, V.; Blay, M.; Ardévol, A.; Pinent, M. Procyanidins modulate microRNA expression in pancreatic islets. *Molecular Nutrition & Food Research*, submitted.

9. **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M.; Ardévol, A. GSPE improves β-cell functionality under lipotoxic conditions due to its lipid-lowering effect. *Journal of Nutritional Biochemistry*, submitted.

10. Cedó, L.*; **Castell-Auví, A.***; Pallarès, V.; Ubaida Mohien, C.; Baiges, I.; Blay, M.; Ardévol, A.; Pinent, M. Pancreatic islet proteome in Zucker fatty rats chronically treated with a grape seed procyanidins extract. *Journal of Proteome Research*, submitted.

11. Guasch, L.; Sala, E.; **Castell-Auví, A.**; Cedó, L.; Liedl, K.; Wolber, G.; Muehlbacher, M.; Mulero, M.; Pinent, M.; Ardévol, A.; Pujadas, G.; Garcia-Vallvé, S. Identification of PPARgamma partial agonists of natural origin (I): Development of a virtual screening procedure and *in vitro* validation. *PloS ONE*, submitted.

Contribution to congresses

1. **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M.; Ardévol, A. Procyanidins modify β-functionality. Its effectiveness on insulinaemia depends on the physiological situation. *Advanced Technologies & Treatments for Diabetes*. Barcelona, Spain, **2012**.

2. Pallarès, V.; Cedó, L.; **Castell-Auví, A.**; Pinent, M.; Ardévol, A.; Blay, M. Efecto de las procianidinas de pepita de uva (GSPE) sobre inflamación en ratas Zucker fa/fa. *XXXIV Congreso de la Sociedad Española de Bioquímica y Biología Molecular*. Barcelona, Spain, **2011**.

3. Pinent, M.; Ardévol, A.; Arola, L.; Bladé, C.; Blay, M.; Arola, A.; **Castell-Auví, A.**; Cedó, L.; Fernández-Larrea, J.; García-Vallvé, S.; Mulero, M.; Pujadas, G.; Salvadó, M.J. El páncreas como diana de los efectos de las procianidinas de la uva. *XXXIV Congreso de la Sociedad Española de Bioquímica y Biología Molecular*. Barcelona, Spain, **2011**.

4. Cedó, L.; **Castell-Auví, A.**; Pallarès, V.; Blay, M.; Ardévol, A.; Pinent, M. GSPE effects on apoptosis of pancreatic β-cells in conditions of glucotoxicity. *36th FEBS Congress*. Torino, Italy, **2011**.

5. Pallarès, V.; Calay, D.; Cedó, L.; **Castell-Auví, A.**; Raes, M.; Pinent, M.; Ardévol, A.; Arola, L.; Blay, M. Synergistic effects of polyphenols and polyunsaturated fatty acids (PUFAs) on RAW 264.7 macrophages activated by lipopolysaccharide (LPS). *36th FEBS Congress*. Torino, Italy, **2011**.

6. Cedó, L.; **Castell-Auví, A.** Pallarès, V. Blay, M.; Ardévol, A.; Pinent, M. GSPE inhibits proliferation of pancreatic β-cells. *4th International Congress on Prediabetes and the Metabolic Syndrome*. Madrid, Spain, **2011**.

7. Ardévol, A.; **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M. Procyanidins affect insulinaemia due to their effects on insulin synthesis and insulin degradation sensitivity depends on the physiological situation. *4th International Congress on Prediabetes and the Metabolic Syndrome*. Madrid, Spain, **2011**.

8. Cedó, L.; **Castell-Auví, A.**; Pallarès, V.; Blay, M.; Ardévol, M.; Pinent, M. GSPE effects on proliferation an apoptosis on pancreatic β-cells in genetically obese rats. *4th Congress of the International Society of Nutrigenetics/Nutrigenomics*. Pamplona, Spain, **2010**.

9. Pallarès, V.; **Castell-Auví, A.**; Cedó, L.; Pinent, M.; Ardévol, A.; Blay, M. A pararine loop between adipocytes and machrophages in adipose tissue: An in vitro model to study obesity-induced inflammation. *4th Congress of the International Society of Nutrigenetics/Nutrigenomics*. Pamplona, Spain, **2010**.

10. Ardévol, A.; Matas, A.; **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M. Screening of the effects of grape seed procyanidins (GSPE) on different adipose tissue depots in Zucjker fa/fa rats. *7th Nutrigenomics Conference-NuGOweek 2010*. Glasgow, United Kingdom, **2010**.

11. Cedó, L.; **Castell-Auví, A.**; Pallarès, V.; Blay, M.; Ardévol, A.; Pinent, M. GSPE effects on proliferation and apoptosis markers in pancreas and mesenteric adipose tissue. *XXV International Conference on Poliphenols*. Montpellier, France, **2010**.

12. **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M.; Motilva, M.J.; Arola, L.; Ardévol, A. Development of co-culture system to evaluate the bioactivity of plant extracts on pancreatic beta cells. *NuGOweek 2009*. Montecatini, Italy, **2009**.

13. **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pujadas, G.; Pinent, M.; Arola, L.; Ardévol, A. GSPE modify insulin-degrading enzyme expression. *Bioactive food components, mitochondrial function and health*. Palma Mallorca, Spain, **2009**.

14. **Castell-Auví, A.**; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M.; Garcia-Vallvé, S.; Ardévol, A. GSPE modify insulin synthesis and secretion in β-cell. *3rd International Congress on Prediabetes and the Metabolic Syndrome*. Nice, France, **2009**.

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