EFFECTS OF GRAPE SEED PROCYANIDIN EXTRACT ON PROLIFERATION AND APOPTOSIS IN PANCREATIC CELLS

Lidia Cedó Giné

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Effects of Grape Seed Procyanidin Extract on Proliferation and Apoptosis in Pancreatic Cells

Doctoral Thesis

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FAIG CONSTAR que aquest treball, titulat "**Effects of Grape Seed Procyanidin Extract on Proliferation and Apopotosis in Pancreatic Cells**", que presenta Lídia Cedó Giné 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.

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La directora de la tesi doctoral

Dra. Montserrat Pinent Armengol

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Als meus pares,

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"Les idees són com les estrelles, no arribaràs mai a tocar-les amb les mans, però si com el mariner en el desert de les aigües, les tries com a guia i les segueixes, aconseguiràs el teu destí."

Carl Schurz

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ABBREVIATIONS

ANT	Adenine nucleotide translocase
APAF-1	Apoptosis protease-activating factor 1
ATF	Activating transcription factor
BAD	Bcl-2 agonist of cell death
BAK	Bcl-2 antagonistic killer
BAX	Bcl-2 associated X protein
Bcl-2	B cell lymphoma-2 protein
Bcl-xL	Bcl-extra long
BH	Bcl-2 homology
BID	BH3-interacting domain death agonist
BIK	Bcl-2-interacting killer
BIM	Bcl-2-interacting mediator of cell death
BMF	Bcl-2-modifying factor
BOK	Bcl-2-related ovarian killer
BOO	Bcl-2 homolog of ovary
bw	Body weight
CDK	Cyclin-dependent kinase
CIP	Cdk interacting protein
CK	Creatine kinase
DAVID	Database for annotation, visualization and integrated discovery
DISC	Death-inducing signalling complex
EGCG	Epigallocatechin gallate
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
FFA	Free fatty acid
GLP-1	Glucagon-like peptide 1
GSPE	Grape seed procyanidin extract
HDL	High-density lipoprotein
HGF	Hepatocyte growth factor
HK2	Hexokinase-2
HRK	Hara-kiri
HSP	Heat shock protein
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL	Interleukin
IMM	Inner mitochondrial membrane
IMS	Mitochondrial intermembrane space

IRE1	Inositol requiring ER-to-nucleus signal kinase 1
IRS	Insulin receptor substrate protein
iTRAQ	Isobaric tag for relative and absolute quantitation
JNK	c-Jun NH_2-terminal kinase
KIP	Kinase inhibitory protein
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia factor-1
miRNA	microRNA
MMP	Mitochondrial membrane potential
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
NF-κB	Nuclear factor-kappa B
NGN-3	Neurogenin-3
NO	Nitric oxide
NOXA	Named for “damage”
OMM	Outer mitochondrial membrane
PBR	Peripheral-type benzoadiazepine receptor
PDX1	Pancreatic duodenal homeobox-1
PERK	PKR-like ER kinase
PI3K	Phosphatidylinositol 3-kinase
PiC	Phosphate carrier protein
PP	Pancreatic polypeptide
pRb	Retinoblastoma-associated protein
PUMA	p53 up-regulated modulator of apoptosis
ROS	Reactive oxygen species
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TNF	Tumor necrosis factor
UPR	Unfolded protein response
VDAC	Voltage-dependent anion channel
VLDL	Very low-density lipoprotein

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RESUM

Les procianidines són compostos fenòlics abundants en plantes i vegetals. S'ha demostrat que aquests compostos bioactius tenen efectes beneficiosos per la salut, entre els quals destaquen les seves propietats antiinflamatòries i antioxidants. També s'ha vist que participen en l'homeòstasi dels lípids i la glucosa.

En un estudi previ realitzat en el grup de recerca, es van avaluar els efectes d'un extracte de procianidines de pinyol de raïm (GSPE) en un model de resistència a la insulina induït per l'alimentació de rates femelles amb una dieta de cafeteria. Es va veure que el GSPE reduïa l'índex HOMA-IR i els nivells d'insulina plasmàtica, suggerint una millora de la resistència a la insulina en teixits perifèrics. A més a més, aquests resultats semblaven indicar que les procianidines podrien estar afectant el pàncrees, el principal òrgan responsable de l'homeòstasi dels nutrients, ja sigui millorant la funcionalitat o la massa de les cèl·lules β pancreàtiques. De fet, aquesta tesi doctoral s'ha dut a terme en paral·lel amb una altra, en la qual es va concloure que les procianidines actuen en el pàncrees modulant la síntesi, secreció i degradació de la insulina.

Els individus amb diabetis del tipus 2 presenten hiperglucèmia i un metabolisme lipídic alterat, juntament amb resistència a la insulina, disfunció de les cèl·lules β i disminució de la massa β. Tot i que determinats factors genètics hi estan implicats, la diabetis del tipus 2 està estretament lligada a l'obesitat, i ambdós patologies estan assolint proporcions d'epidèmia a nivell mundial. En els primers estadis de la resistència a la insulina, la massa β s'incrementa per compensar la hiperglucèmia. Tot i així, quan les cèl·lules β ja no són capaces de compensar l'augment de la demanda d'insulina, la massa β es veu reduïda degut a un augment de l'apoptosi.

A més a més, considerant el pàncrees, l'adenocarcinoma pancreàtic és un dels càncers més agressius, caracteritzat per una elevada resistència al tractament. L'acumulació d'alteracions genètiques resulta en un augment del creixement cel·lular i de la proliferació i en una inhibició de l'apoptosi.

D'aquesta manera, l'obtenció d'informació sobre els compostos naturals amb efectes beneficiosos sobre la proliferació i l'apoptosi en les cèl·lules pancreàtiques, processos estretament lligats i alterats en les malalties mencionades anteriorment, és de gran interès.

Els efectes de les procianidines sobre la proliferació i l'apoptosi han estat molt estudiats en diferents tipus cel·lulars. En línies cel·lulars de càncer, les procianidines baixen els nivells de

proliferació i incrementen l'apoptosi, actuant com a anticarcinogèniques. En altres tipus cel·lulars, les procianidines actuen com a eina terapèutica, protegint les cèl·lules del dany induït per factors ambientals o químics, disminuint l'apoptosi i estimulant el creixement cel·lular. Tot i així, existeix poca informació relativa als efectes de les procianidines en el pàncrees.

Per tant, aquesta tesi doctoral es va centrar en l'estudi dels efectes de les procianidines sobre la proliferació i l'apoptosi de les cèl·lules pancreàtiques, avaluant la modulació d'aquests processos en situacions fisiològiques o patològiques. Per assolir els nostres objectius, vam utilitzar models *in vivo* de rates sanes, de rates amb obesitat induïda per la dieta i rates amb obesitat induïda genèticament; i models *in vitro*, usant la línia cel·lular d'insulinoma de rata INS-1E i d'adenocarcinoma de pàncrees MIA PaCa-2.

La hiperglucèmia postprandial i la dislipèmia són factors comuns que tenen lloc prèviament al desenvolupament de la diabetis del tipus 2. L'exposició crònica a un ambient hiperglucèmic i a elevades concentracions d'àcids grassos causa la disfunció de les cèl·lules β pancreàtiques i la mort cel·lular, fenòmens anomenats glucotoxicitat i lipotoxicitat, respectivament. D'aquesta manera, quan vam exposar les cèl·lules INS-1E a elevades concentracions de glucosa i palmitat, ambdós nutrients van incrementar l'apoptosi. Quan, en aquestes condicions, les cèl·lules es van tractar amb GSPE, l'extracte va incrementar els efectes pro-apoptòtics de l'elevada glucosa, sense modificar la situació de lipotoxicitat. L'apoptosi induïda pel GSPE en situacions d'hiperglucèmia involucra la via intrínseca de l'apoptosi.

In vivo, vam continuar l'estudi previ realitzat en rates femelles amb obesitat induïda per una dieta de cafeteria, i vam veure que GSPE modulava els marcadors d'apoptosi en el pàncrees d'aquestes rates, però els efectes eren dependents de la dosi i el període de tractament. Tot i així, el tractament semblava que tendia a contrarestar l'augment de l'apoptosi de les rates alimentades amb dieta de cafeteria. En canvi, quan els efectes de l'extracte es van analitzar en rates mascle, GSPE incrementava un marcador pro-apoptòtic, suggerint un increment de l'apoptosi en les rates tractades amb l'extracte. D'aquesta manera, es conclou que la modulació dels marcadors d'apoptosi per part del GSPE en rates alimentades amb dieta de cafeteria és dependent de la dosi, el període de tractament i/o el gènere.

Pel que fa als efectes de GSPE sobre la proliferació, quan les cèl·lules β pancreàtiques es van exposar a elevats nivells de glucosa i insulina, els quals indueixen la proliferació, i nivells alts de palmitat, el qual inhibeix la proliferació, l'extracte va mostrar un clar efecte antiproliferatiu. Aquests efectes antiproliferatius són probablement a causa de les molècules d'alt pes molecular, les quals no es poden absorbir a l'intestí, de manera que cal tenir-ho en compte en el moment de comparar els efectes obtinguts *in vitro* amb els possibles efectes *in vivo*. De fet, en els experiments de rates alimentades amb una dieta de cafeteria, el GSPE no va modificar els marcadors de proliferació analitzats.

Com a model d'obesitat induïda genèticament, es van utilitzar rates *Zucker Fatty*. Quan aquestes rates es van tractar crònicament amb GSPE, tot i que l'extracte contrarestava l'expressió de marcadors d'apoptosi i proliferació en comparació amb les rates obeses no tractades, els canvis moleculars induïts per les procianidines no van ser suficients per contrarestar l'efecte genètic de les rates *Zucker Fatty* a un nivell fisiològic, ja que tant l'apoptosi com els nivells plasmàtics de glucosa i insulina eren tan elevats com en les rates control. En aquest experiment, també es va analitzar el perfil proteic dels illots realitzant un estudi de proteòmica. Un dels processos biològics en els quals les proteïnes modificades per GSPE estaven involucrades era l'apoptosi i la mort cel·lular. Els nivells de la majoria de les proteïnes incloses en aquest grup contrarestaven els efectes del genotip *Zucker Fatty*, de la mateixa manera que es va observar en els marcadors d'expressió gènica. Per tant, tenint en compte les rates *Zucker Fatty* com a referència d'apoptosi, el GSPE tendia a millorar aquest procés, tot i que no va induir canvis als nivells finals d'apoptosi.

Un cop analitzats els efectes de GSPE en les cèl·lules β en situacions patològiques, es van avaluar els seus efectes en situacions fisiològiques. El tractament de les cèl·lules INS-1E amb GSPE no va modificar ni l'apoptosi ni la proliferació d'aquestes cèl·lules. Aquests resultats *in vitro* coincideixen amb els observats *in vivo*, en els quals, el tractament crònic de rates amb GSPE no va modificar ni l'apoptosi ni la massa β. En aquest experiment, el perfil de microRNA també es va analitzar, ja que alguns microRNA s'ha vist que poden regular la funció pancreàtica, incloent la regulació de la síntesi i la secreció de la insulina i l'apoptosi. Tot i que vam trobar que els microRNAs dels illots pancreàtics eren diana de les procianidines, els modificats per l'extracte no estan involucrats en els processos de proliferació i apoptosi, fet que confirma el fet que el GSPE no altera aquests processos en condicions fisiològiques.

Finalment, una altra situació patològica en la qual la proliferació i l'apoptosi estan alterats en cèl·lules pancreàtiques és en càncer, en el qual la proliferació està incrementada i l'apoptosi inhibida. D'aquesta manera, vam analitzar els efectes del GSPE en la línia cel·lular d'adenocarcinoma pancreàtic MIA PaCa-2 i vam veure que l'extracte inhibia la proliferació cel·lular i incrementava l'apoptosi, procés mediat per la modulació de proteïnes de la família de la Bcl-2 i per la despolarització de la membrana mitocondrial, implicant la via intrínseca de l'apoptosi. En aquest cas, els components de l'extracte amb més activitat antiproliferativa i pro-apoptòtica també van ser identificats. Tant l'epigal·locatequina gal·lat com l'àcid gàl·lic foren els components amb efectes antiproliferatius més elevats, però, considerant que la concentració d'àcid gàl·lic en l'extracte és més de 40 vegades més elevat que la d'epigal·logatequina gal·lat, es va considerar l'àcid fenòlic com un dels components de l'extracte responsables dels efectes observats. De la mateixa manera que el GSPE, l'àcid gàl·lic modulava l'expressió de proteïnes de la família de la Bcl-2 i promovia la despolarització de la membrana mitocondrial.

En aquest estudi, es van utilitzar dues aproximacions per tal d'apropar-nos a una situació *in vivo*, ja que un cop ingerides, no tots els components de les procianidines són absorbides a l'intestí.

I. INTRODUCTION

1. The pancreas

The pancreas is an organ that weights 70-150 grams and is 15-25 cm in length. It is located in the upper central region of the abdominal cavity, behind the lower surface of the stomach (Figure 1). The pancreas is connected to the duodenum by the ampulla of Vater, where the main pancreatic duct joins with the common bile duct (Figure 2a).[1] In rodents, the shape of the pancreas is less defined than in humans, and invertebrates do not have a pancreas.[1]

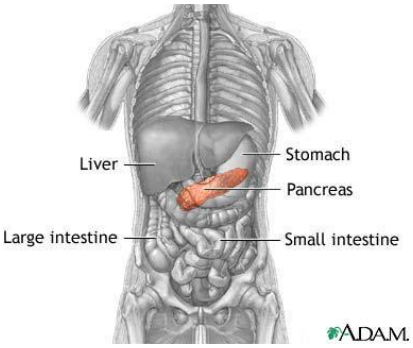


Figure 1. Pancreas in situ.[2]

The pancreas performs both exocrine and endocrine functions. The bulk of the pancreas is exocrine, containing acinar cells (70-90%) and ductal cells (5-25%) at a ratio that depends on the species. Endocrine cells comprise only 3-5% of the pancreas.[3] The mesenchymal cell compartment includes pancreatic fibroblasts and endothelial cells in addition to vascular smooth muscle and stellate cells.[4]

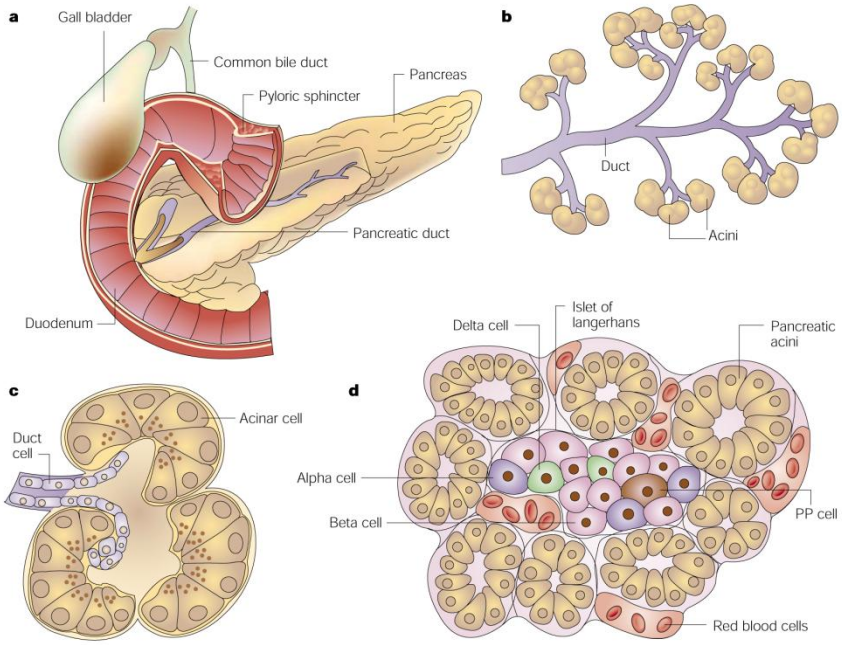


Figure 2. a) Anatomy of the human pancreas. b) The exocrine pancreas. c) A single acinus. d) A pancreatic islet embedded in exocrine tissue.[5]

The exocrine pancreas is a lobulated and branched acinar gland. The secretory cells are grouped into acini (Figure 2b and c) and are responsible of the production of at least 22 different digestive enzymes, including proteases, amylases, lipases, and nucleases. These enzymes facilitate nutrient digestion and absorption.[1,6] Ductal cells line the channels that transport these secreted enzymes to the gastrointestinal tract.[6]

The endocrine pancreas is embedded in the exocrine tissue and contains aggregates of several cell types that form the islets of Langerhans. The hormone-secreting cell types include the following: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, pancreatic-polypeptide (PP)-producing cells, and ghrelin-producing ε-cells (Figure 2d). Finely tuned regulation of hormone release is achieved by coordinated interactions between the islet cells and the vascular environment, establishing hormonal homeostasis.[7,8]

The structural organisation of the various cell types within the islets is species-dependent, which is thouth to be functionally significant.[9] Rodent islets are typically composed of a central core of β-cells, representing 60-80% of the cells, surrounded by smaller numbers of the other cell types (15-20% of α-cells, less than 10% of δ-cells, and less than 1% of PP-cells) (Figure 3A).[7,8] Core-mantle segregation of islet cells is useful for aiding homologous contacts between β-cells, which subsequently improves insulin secretion. Furthermore, the characteristic islet architecture may also serve to facilitate interactions between different islet hormones via interstitial or vascular routes.[10]

In contrast, some publications claim that in humans the different cell types are randomly distributed throughout the islet (Figure 3B), which contains 50% β-cells, 40% α-cells, 10% δ-cells, and few PP-cells.[8] However, it has also been reported that α-cells in human islets maintain a mantle position around β-cells, but α-cells are also found along vessels that penetrate and branch inside the islets. In this case, islet cells are organised into a trilaminar plate with one layer of β-cells sandwiched between two α-cell-enriched layers, folded with different degrees of complexity and bordered by vessels on both sides.[10] This architecture suggests enhanced paracrine interactions in the human islets.[7] Heterologous contacts predominate in human islets, suggesting that glucagon is a more potent regulator of insulin secretion in human islets compared to rodent islets.[10]

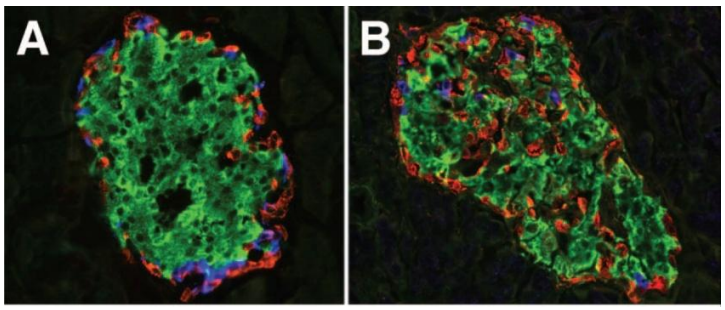


Figure 3. Histological section of a mouse (A) and a human (B) pancreas stained for islet hormones (insulin, green; glucagon, red; somatostatin, blue). The micrograph on the left shows the typical rodent core-mantle arrangement of β and non-β cells, and the micrograph on the right demonstrates an intermingling between β and non-β cells. Magnification was (A) x20 and (B) x40.[11]

1.2. Maintenance of β-cell mass

The β-cell mass is determined by the number and the size of the pancreatic β-cells.[12] It plays an essential role in determining the amount of insulin that is secreted to maintain the body's glucose levels within a narrow range.[13] Accordingly, the β-cell mass is dynamic and adjusts to meet the changes in metabolic demand, both under physiological and pathological conditions.[14] The β-cell mass increases throughout life and maintains a linear correlation with body weight.[14] Moreover, an expanded β-cell mass has been observed in several other conditions characterised by increased insulin demand, such as the neonatal period, in obesite individuals, during pregnancy, and in insulin resistance (Figure 4).[15] The β-cell mass doubles during pregnancy as a result of both β-cell hyperplasia and hypertrophy, and during the postpartum period, the rate of β-cell apoptosis increases to ensure a rapid return to normal levels of β-cell mass.[16] This mass also increases in obesity and insulin resistance, but the extent of this adaptative increase is modest in humans when compared to changes in obese rodents (≈0.5-fold versus ≈10-fold).[17] In humans, this increased β-cell mass is thought to occur via an increase in β-cell replication and neogenesis combined with β-cell hypertrophy. A small increase in β-cell apoptosis has also been observed in non-diabetic obesity, but this apoptosis is counterbalanced by an increase in the other processes, resulting in a net increase in β-cell mass.[18]

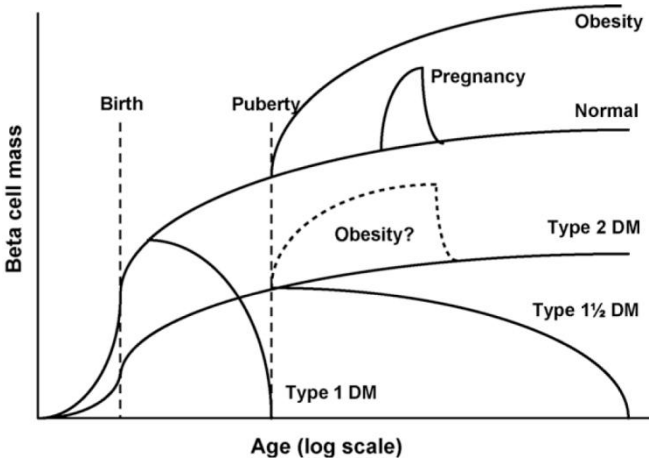


Figure 4. A general illustration of the changes in β-cell mass under physiological and pathological conditions.[15]

β-cell mass increases via β-cell neogenesis, proliferation, and hypertrophy. β-cell differentiation from precursor cells gives rise to the initial β-cells of an organism during embryogenesis, but differentiation declines in the postnatal stages.[12,18] β-cell proliferation, or replication capacity, is highest in infancy, coinciding with the postnatal β-cell mass expansion, and the ability of β-cell regeneration declines soon after (Figure 5B).[15,17,18] However, some expansion continues at very slow rates throughout life, and proliferation is responsible for the postnatal expansion and homeostatic maintenance of this cell population, rather than differentiation of adult stem cells not expressing insulin.[17,19] The proliferation of differentiated β-cells can be as high as 7% in neonatal rats and mice.[20] Hypertrophy of the cells allows for sustained amplification of gene expression without cell division.[21]

β-cell mass decreases with β-cell death, which occurs primarily through apoptosis, and β-cell atrophy. β-cell apoptosis occurs at very low rates during embryogenesis, but there is a transient burst at weaning, which may be associated with islet remodelling and/or changes in β-cell maturation. During adulthood, β-cell apoptosis also normally occurs at very low rates.[12,18] β-cell atrophy describes a decrease in the cell size, and atrophy increases with age (Figure 5B).[12] β-cell mass reduction is a central aspect in the development of both type 1 and type 2 diabetes mellitus (Figure 4 and 5B).[14]

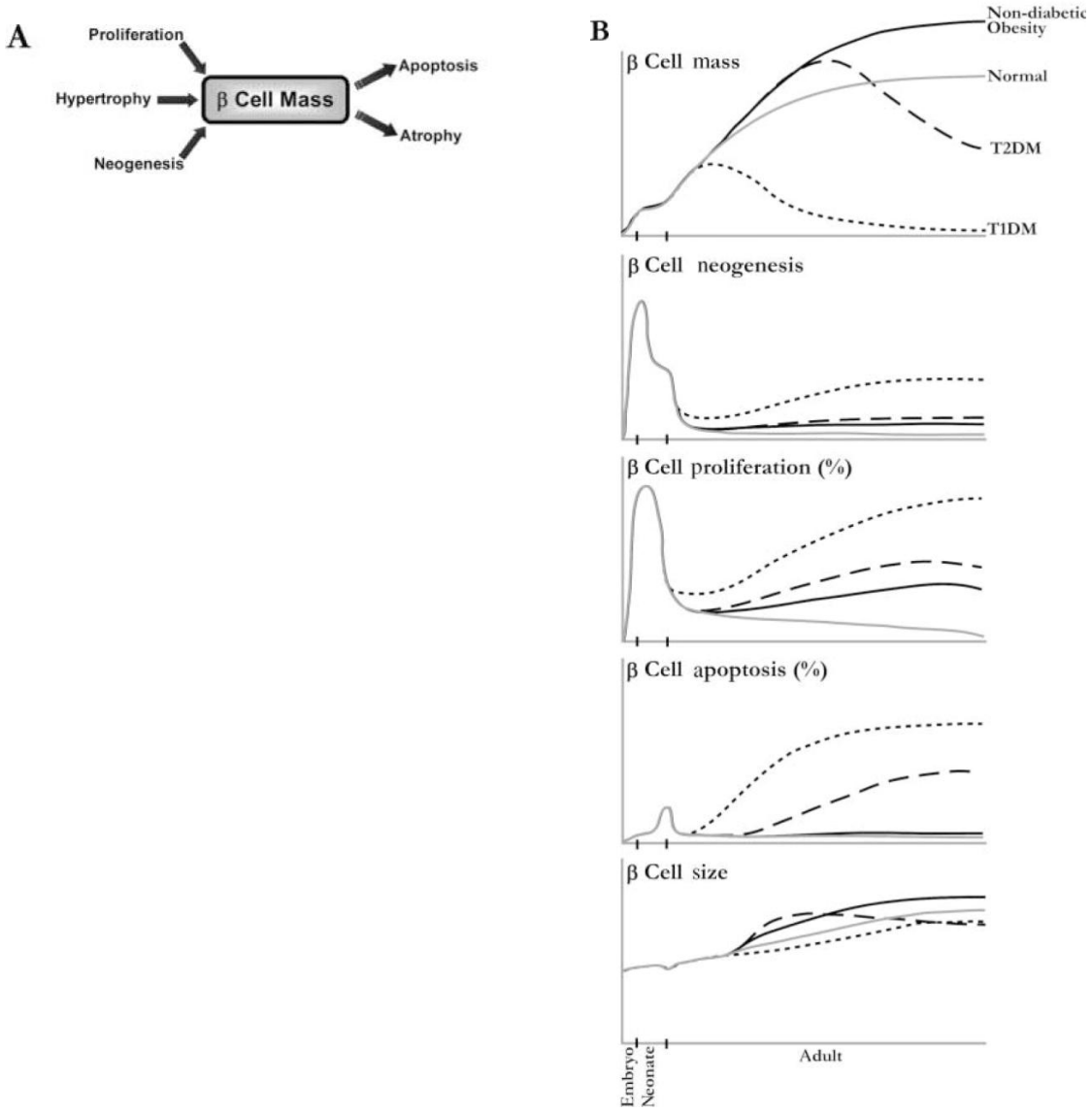


Figure 5. β-cell mass dynamics. A) β-cell proliferation, neogenesis, and hypertrophy increase β-cell mass, while β-cell apoptosis and atrophy decrease β-cell mass. B) Graphs represent approximate changes in these processes over the course of a lifetime in normal individuals (gray solid line), in non-diabetic obesite individuals (black solid line), in Type 2 diabetes mellitus (T2DM) individuals (black dashed line), and in Type 1 diabetes mellitus (T1DM) individuals (black dotted line).[12]

In adult rats, β-cells have an estimated life-span of approximately 60 days.[21] Under normal conditions, approximately 0.5% of adult β-cells undergo apoptosis, but this is balanced by β-cell replication and, to a lesser extent, β-cell neogenesis.[18] Thus, the β-cell mass remains relatively

constant under physiological conditions during adult life.[22] In humans, where studying physiological β-cell development and turnover is more difficult than in rodents, there are indications that similar phenomena occur.[22]

Depending on the specific condition analysed, β-cell apoptosis, replication, size, and neogenesis differentially contribute to the remodelling of the endocrine pancreas, and it is the balance of these processes that determines whether β-cell mass is eventually increased or decreased (Figure 5A).[14]

It is important to note that measuring dynamic changes in β-cell mass is technically difficult, and subtleties can be overlooked. This is one of the reasons for discrepancies in the literature. Markers of cell division, such as Ki-67, correspond to a small and transient window in the cell cycle and may underestimate the incidence of β-cell replication. Likewise, apoptotic cells are efficiently cleared by macrophages *in vivo*; thus the extent of β-cell apoptosis, especially when analysed in *ex vivo* pancreatic sections, may be under-represented. Pancreatic β-cell neogenesis (often measured as the abundance of insulin-positive cells in the pancreatic ductal epithelium) is relatively rare, and its detection requires analysis of multiple pancreatic sections. Moreover, without specific markers for “precursor β-cells”, it is difficult to determine if “insulin-positive cells” actually mature into fully differentiated β-cells or are alternative cell types that have been misdiagnosed. Such studies are particularly challenging in humans, where retrograde analyses from autopsy specimens cannot be performed, and pancreatic biopsies are difficult to obtain. In addition, pancreatic specimens from patients with T2DM often represent only the end stages of the disease.[18]

1.2.1. β-cell neogenesis

During embryonic development of the pancreas, new insulin-producing β-cells are generated from transient endocrine progenitor cells. These progenitor cells emerge from the pancreatic epithelium (future pancreatic ducts), differentiate into various hormone-producing cells and then coalesce to form the islets of Langerhans.[23] The embryonic development of islet β-cells is critically dependent on the function of the basic helix-loop-helix transcription factor **neurogenin-3** (NGN-3). Genetic lineage-tracing studies and the phenotype of Ngn3-null mice demonstrate that all islet hormone-producing endocrine cell types are derived from NGN-3-expressing progenitors.[24] NGN-3 functions primarily as an activator of gene transcription in endocrine progenitor cells and enhances the expression of the lineage-committed transcription factors required for the differentiation of these cells into each of the endocrine cell subtypes. NGN-3 expression decreases as the endocrine hormones that define the endocrine lineages are expressed.[25] Conversely, **PDX1** (pancreatic duodenal homeobox-1) has critical regulatory roles in early pancreas development upstream of NGN-3 and in mature β-cells.[24] In the study of β-cell neogenesis, NGN-3 is used as a marker for neogenesis, whereas PDX1 is used as a marker for the precursor or the progenitor cells within the ducts.

After birth, the principal mode of new β-cell formation shifts to proliferation of terminally differentiated insulin-positive β-cells.[26] Moreover, it is possible that this shift is not irreversible and that under certain conditions, the embryonic program is reactivated. It has been proposed that

adult pancreatic ducts function as “facultative progenitor cells”, which, under specific injury conditions, can give rise to new β-cells via recapitulation of the embryonic pathway in a process termed “**neogenesis**”.[27] In this case, mature duct cells may transiently regain a more pluripotent, and less differentiated, phenotype after replication. External stimuli, as soluble factors of matrix components, can then direct differentiation to endocrine, acinar, or mature duct phenotypes.[21] It has also been proposed that new β-cells are derived from other sources such as transdifferentiated acinar cells,[28] spleen cells,[29] or other bone marrow-derived cells[23].

The evidence for β-cell neogenesis in adults comes from morphological studies that induce damage to all or part of the pancreas in rodents, such as partial pancreatomy, cellophane wrapping, duct ligation, and aberrant expression of γ-interferon in β-cells. The most common result in these studies is the appearance of insulin-positive cells in the duct epithelium. However, other cell types, including acinar cells, centroacinar cells, intercalated duct cells, and δ-cells, have been proposed to transdifferentiate into β-cells.[30]

In humans, the study of β-cell neogenesis has been limited, and studies on β-cell regeneration rely heavily on culturing cells and tissues *in vitro*, and on the examination of post-mortem pancreases; therefore, the question of whether neogenesis occurs in humans remains unanswered.[30] A larger population of extra-islet β-cells have been observed in human adult pancreases, and these cells are scattered throughout the exocrine tissue, suggesting that a neogenic mechanism could be present even in normal physiological states.[31] However, this finding relies on a morphological study and does not directly demonstrate neogenesis.[4]

1.2.2. β-cell proliferation

The cell cycle is a complex process involved in the growth and proliferation of cells. It involves numerous regulatory proteins that direct cells trough a specific sequence of events that culminate in mitosis and the production of two daughter cells. The cell cycle is divided into G_1, S, G_2, and M phases. In the G_1 phase, the cell is preparing for DNA synthesis. S phase cells are synthesising DNA, and cells in G_2 phase prepare for mitosis, or M phase (Figure 6). Cells in G_0 phase are not in the cycle but have the potential to divide; G_0 also includes terminally differentiated cells.[32]

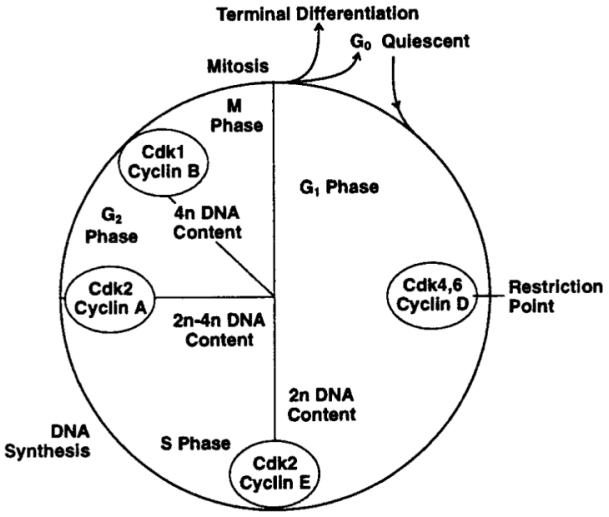


Figure 6. Schematic drawing of normal cell cycle progression.[32]

Normal cell replication and growth are dependent on and governed by the precise control of cell entry, passage, and exit from the cell cycle. This series of events is initiated by **cyclins** and **cyclin-dependent kinases** (CDKs). D-type cyclins are the first to emerge following mitogenic stimulation, while CDKs are constitutively expressed and are the cell cycle entry regulators (Figure 7). The induction of D-type cyclins may result in the formation of different cyclin D-CDK complexes, which are considered to be the checkpoint of mitogenic stimulation. These complexes lead to the phosphorylation and deactivation of the retinoblastoma-associated protein (pRb), causing release of the E2F transcription factor. E2F can then initiate the expression of genes whose activities are required for DNA synthesis (Figure 7). Cyclins E and A are E2F-regulated genes, and they are both required to catalyse the G_1/S transition in normal cells. Cyclin E forms a complex with its catalytic partner, CDK2, and collaborates with the Cyclin D-CDK4 complex to completly phosphorylate pRb. The activity of the cyclin E-CDK2 complex peaks at the G_1/S transition, and cyclin E is then degraded and replaced by cyclin A. The activated cyclin D-CDK4 complex also promotes cell cycle progression by sequestering the cyclin E-CDK2 and cyclin A-CDK2 complex inhibitors ($p21^{CIP}$, $p27^{KIP}$, and $p57^{KIP}$), which increases CDK2-associated activity in late G_1 and S phase.[33]

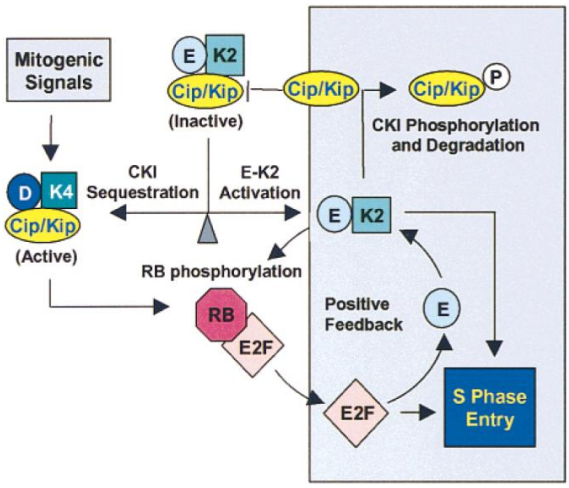


Figure 7. Regulation of the G_1/S transition.[34]

There are three D-type cyclins, designated as D1, D2, and D3. In pancreatic islets, cyclin D1 and cyclin D2 are important regulators of β-cell proliferation, whereas cyclin D3 is expressed at low levels.[35] In the case of β-cells, cyclin D2[36] and CCK4[37] are the two critical players in the progression of quiescent adult β-cells to the G_1/S stage of the cell cycle.[13,23,38] CDK6, the functional analogue of CDK4, can also form complexes with D-cyclins, but there is an apparent lack of CDK6 expression in murine islets.[15]

CDK inhibitors also play a contributing role in β-cell proliferation; they control cell cycle entry by binding to and inhibiting CDKs, which in turn, phosphorylate and activate cyclins, leading to cell cycle entry.[30] CDK inhibitors belong to two protein families. The INK4 (inhibitor of CDK4) family, which includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ specifically inhibits CDK4 and CDK6. The CIP/KIP (Cdk interacting protein/Kinase inhibitory protein) family includes $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, and these proteins affect the activities of cyclin D-, E-, and A-dependent kinases.[34] Specific CDK inhibitors control progression at distinct phases in the cell cycle. In humans, the

major tissue-specific CDK inhibitor in adult β-cells is p57. In the mouse, p57 is not expressed in adult β-cells, though p27 is present at high levels.[30] Some CDK inhibitors, such as p16, are highly expressed in the adult and inhibit β-cell replication. This observation is consistent with the idea that β-cells have the highest replication capacity during the postnatal period.[15] Under certain circumstances, however, p21 and p27 can also function as an assembly factor in promoting the binding of CDK4 with D-type cyclins.[39]

In the regulation of proliferation in β-cells, D-type cyclins are responsive to extra-cellular stimulation. **Glucose** is one of the regulators of proliferation in β-cells (Figure 8). Glucose is taken up by β-cells, which secrete insulin in amounts appropriate for the respective blood glucose concentration.[40] Exposure to glucose enhances β-cell proliferation, implicating cyclin D2 in this process. Some have suggested that glucose infusion increases the overall levels of cyclin D2, which is then responsible for cell cycle entry of quiescent β-cells.[41] However, other reports demonstrated that cyclin D2 is always expressed at high levels in pancreatic β-cells.[38] Some reports have suggested that cyclin D2 is found in the cytoplasm and is localised to the nucleus upon replication.[41] However, others have reported that cyclin D2 is constitutively localised in the nucleus of most β-cells and that glucose metabolism controls cyclin D2 levels during β-cell replication, causing down-regulation of cyclin D2 mRNA and protein during the S, G_2, and M phases of the cell cycle. Because β-cells need cyclin D2 for replication, the decrease in β-cell cyclin D2 during cell division and immediately after mitosis prevents cells from re-entering the cell cycle. Thus, cyclin D2 appears to have a permissive role and is necessary but not sufficient for β-cell replication.[38,42]

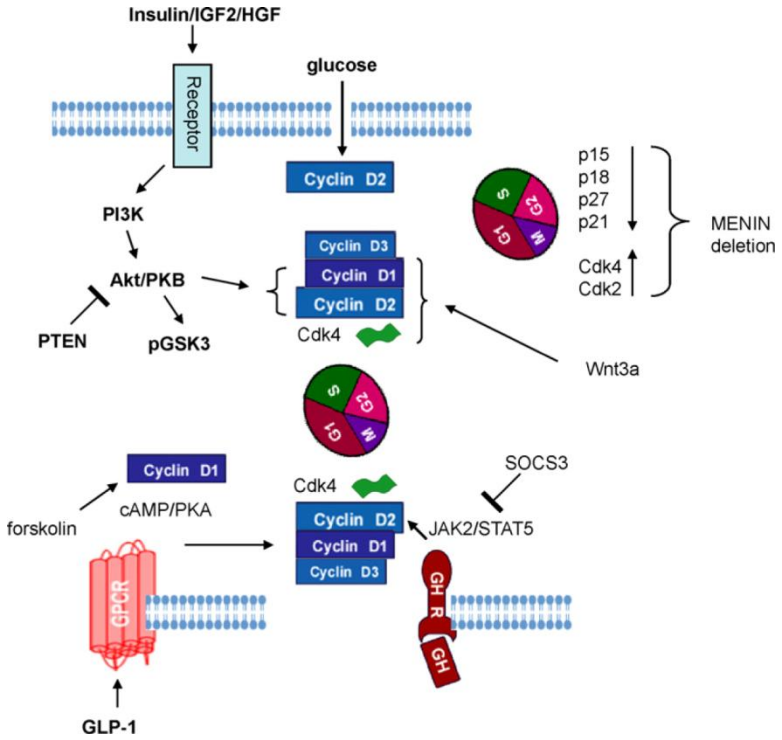


Figure 8. Schematic summary of factors involved in the regulation of the expression of D-type cyclins and CDKs in β-cells.[15]

Glucose and **insulin** metabolism are tightly linked, and any increase in blood glucose is accompanied by an increase in insulin secretion, which is also a potential β-cell mitogen (Figure 8). It has been suggested that insulin, rather than glucose, could be the critical factor controlling β-cell proliferation and mass.[23,43] Upon insulin binding to its receptors (insulin receptor substrate: IRS), two major pathways can be activated, the phosphatidylinositol 3-kinase (PI3K) pathway[15] and the Raf-1/ERK (extracellular signal-regulated kinase) cascade.[44,45] Survival signals produced following PI3K activation are mediated by the AKT family of serine/threonine protein kinases.[46] AKT (also known as protein kinase B) induces β-cell proliferation by modulating cyclin D1 and cyclin D2 levels; changes in these G_1 components result in the activation of CKD4. [47] Raf-1 also appears to participate in β-cell proliferation. Insulin-activated Raf-1 phosphorylates MAPK (mitogen-activated protein kinase) kinase and the upstream kinase activator of ERK.[44,45]

In addition to the importance of insulin and glucose metabolism, other systemic regulators of β-cell proliferation and mass have been identified (Figure 8).[23] Growth hormone, hepatocyte growth factor (HGF), prolactin, glucagon-like peptide 1 (GLP-1), and exendin (GLP-1 receptor agonist) are known insulinotropic factors and positively affect pancreatic islet growth.[15] Prolactin acts as a β-cell mitogen during pregnancy.[18,23]

All β-cells have similar replicative potentials, and no sub-population of replication-privileged cells exists. In addition, β-cells are dramatically less likely to re-enter the cell cycle following division, and they enter a prolonged “refractory period” during which they cannot divide again. Aging lengthens the refractory period for β-cells, and β-cell injury and an increased rate of glucose metabolism shorten the post-mitosis refractory period and increase the likelihood of individual β-cells to divide again. It has been estimated that a single β-cell undergoes only two or three replications over the course of its life time.[42]

1.2.3. β-cell apoptosis

Apoptosis, also known as programmed cell death, plays an important role in a variety of biological events including normal development, maintenance of tissue homeostasis, aging, removal of unwanted and harmful cells, and immune regulation.[48,49] Characteristic apoptotic features include cell membrane blebbing, reduction of cellular volume (pyknosis), chromatin condensation and DNA fragmentation, nucleus condensation (karyorhexis), and maintenance of an intact plasma membrane. The process finally ends with the engulfment of the apoptotic cell by macrophages or neighbouring cells, thereby avoiding an inflammatory response in surrounding tissues.[50,51] Apoptosis is distinct from necrosis, in which the cell suffers a major insult that leads to a loss of membrane integrity, swelling, and disruption of the cell. During necrosis, the cellular contents are released into the cellular environment, which results in damage of surrounding cells and a strong inflammatory response in the tissue.[50]

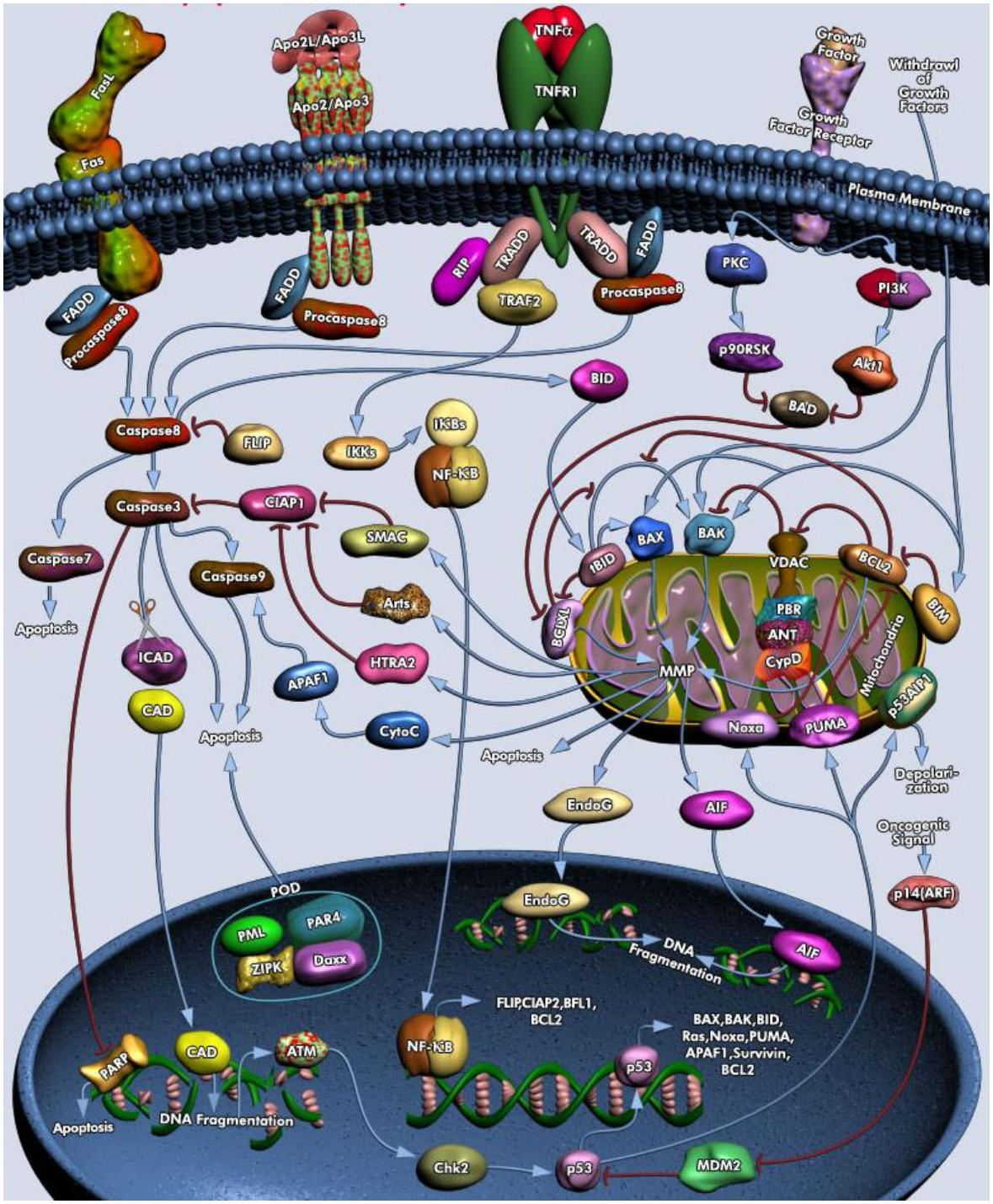


Figure 9. Cellular apoptosis pathways. © 2009 QIAGEN.

In mammals, there are two major apoptotic pathways (Figure 9):

· The extrinsic pathway, also known as the receptor-mediated pathway, is activated by apoptotic stimuli that include extrinsic signals such as the binding of death inducing ligands to cell surface receptors, such as FAS-L, tumour necrosis factor receptor (TNFR), and TNF-related apoptosis-inducing ligand (TRAIL) receptors.[50] Death ligand stimulation results in oligomerisation of the receptors and recruitment of the adaptor protein Fas-associated death domain (FADD) and procaspase-8 (and procaspase-10 in humans).

These factors form a death-inducing signalling complex (DISC), which results in the autoactivation of procaspase-8. Caspase-8 can directly activate effector caspases, including procaspase-3, -6, and -7, which function as downstream effectors of the cell death program.[49,50]

· The intrinsic pathway, also termed the mitochondrial pathway, is initiated following intrinsic signals that include DNA damage induced by irradiation or chemicals, growth factor deprivation, and the presence of unfolded proteins or oxidative stress. Generally, intrinsic signals initiate apoptosis via altering the outer mitochondrial membrane (OMM) permeability. Cytochrome c is released from the mitochondria, and it binds to apoptosis protease-activating factor 1 (APAF-1). Binding of these factors triggers the oligomerisation of this complex and leads to the recruitment of procaspase-9, generating an intracellular DISC-like complex called the "apoptosome". Within the apoptosome, caspase-9 is activated, leading to processing of caspase-3.[50,52,53]

The two apoptosis pathways converge on caspase-3, which induces ICAD (inhibitor of caspase-activated DNase) cleavage. Cleavage releases CAD, an endonuclease that induces DNA fragmentation.[50,54] Furthermore, caspase-8 from the extrinsic pathway cleaves BID, a Bcl-2 family protein, which also increases the permeabilization of the OMM and the subsequent processes mentioned above, further linking the death receptor and the APAF-1/caspase-9 pathways (Figure 9).[51,55] The last steps of apoptosis include packaging of cell contents into apoptotic bodies and phagocytosis.[50]

A complex regulatory network tightly controls cellular apoptosis. Pro-survival signals enhance the expression and/or activity of anti-apoptotic regulatory molecules, thereby balancing the activation of pro-apoptotic factors. Pro-apoptotic factors can counteract these inhibitory molecules when apoptosis is necessary.[50]

Caspases are apoptosis regulatory molecules and are aspartate-specific cysteine proteases that are synthesised in the cell as inactive zymogens. Caspase activation is induced by proteolytic cleavage. There are 15 caspases in mammals, 11 of which are expressed in humans (caspases 1-10 and 14), but not all of these caspases have been implicated in apoptosis. They have been divided into upstream or initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12, and -13) and downstream or effector caspases (caspase-3, -6, -7, and -14). Initiator caspases activate themselves and the effector caspases, leading to the progression of the proteolytic cascade.[49,55] In addition, caspases also cleave other apoptotic effectors and structural proteins, such as lamins (the major cytoskeletal structural component of the nucleus), actin, or proteins involved in DNA synthesis and repair; cleavage of these proteins is necessary for disassembling cells undergoing apoptosis.[55]

A pivotal event in the mitochondrial pathway is **mitochondrial outer membrane permeabilisation** (MOMP). MOMP is mainly mediated and controlled by Bcl-2 family members. Once MOMP occurs, it initiates cell death through either 1) the release of molecules involved in apoptosis or 2) the loss of mitochondrial functions essential for cell survival.[50]

There are approximately 20 **Bcl-2 family members** in mammals, and they are characterised by the presence of distinct and conserved sequence motifs known as Bcl-2 homology (BH) domains.

These domains are designated as BH1, BH2, BH3, and BH4 and are important for heterodimeric interactions between members of this family. Bcl-2 family proteins are divided into three groups according to the BH domain that they contain (Table 1).[49,56,57]

Table 1: Members of the Bcl-2 family.[49,56,57]

Bcl-2 family member	Role	Receptor domains	Members
Bcl-2-like survival factors	anti-apoptotic	BH1, BH2, BH3 and BH4	Bcl-2, Bcl-xL, Bcl-W, A1, MCL-1*, BOO, Bcl-B
BH3-only death proteins	pro-apoptotic	BH3	BIM, BAD, BID, BIK, BMF, HRK, NOXA, PUMA
BAX-like death factors	pro-apototic	BH1, BH2, and BH3	BAX, BAK, BOK

* MCL-1 has only three domains (BH1-3).

The pro-survival Bcl-2 family members have a hydrophobic carboxy-terminal domain (TM) that helps to anchor the protein to the cytoplasmic face of the OMM, the nuclear envelope, and the endoplasmatic reticulum (ER) membrane.[49,56] These Bcl-2 proteins are involved in maintaining homeostasis and preventing mitochondrial outer membrane permeabilisation by neutralising the activity of pro-apoptotic members of the Bcl-2 protein family (Figure 11).[49,50]

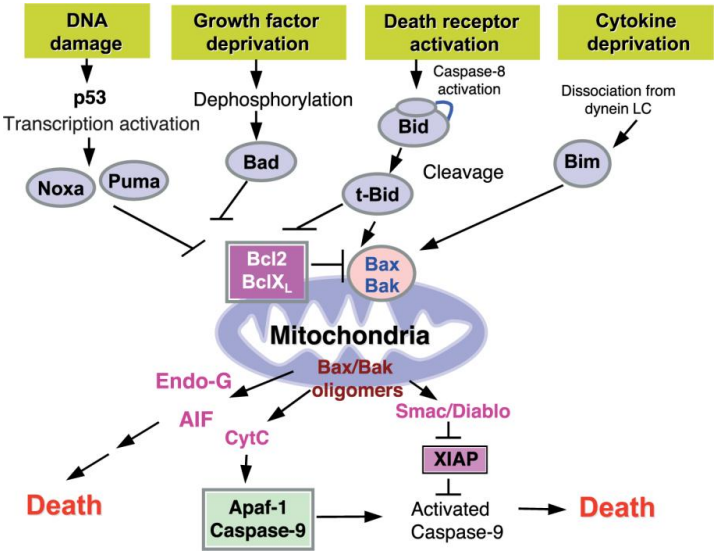


Figure 11. Schematic diagram demonstrating the putative molecular function and regulation of selected members of the Bcl-2 family during apoptosis.[57]

BH3-only family members are sensors for the various apoptotic stimuli and undergo post-translational modifications; these proteins also relay signals to the mitochondria following activation. At the mitochondria, they induce conformational changes and oligomerisation of the pro-apoptotic proteins BAX and BAK, which insert into the OMM (activator proteins). BH3-only proteins also antagonise the function of the anti-apoptotic proteins, such as Bcl-xL and Bcl-2, and induce the activation of BAX and BAK (sensitiser proteins) (Figure 11).[49,57]

BAX and BAK integrate the apoptotic signals and undergo oligomerisation to facilitate the permeabilisation of the OMM, allowing efflux of apoptogenic proteins (Figure 11).[57] BAX is

predominantly a cytosolic monomer in healthy cells, though it undergoes conformational changes during apoptosis and translocates to the OMM where it oligomerises.[57] BAK is present in the OMM in healthy and apoptotic cells and associates with the voltage dependent anion channel (VDAC); however, BAK also changes conformation and forms larger aggregates during apoptosis.[49]

The pro-apoptotic proteins BAX and BAK initiate either MPT (mitochondrial permeability transition)-dependent or MPT-independent **mechanisms for mitochondrial outer membrane permeabilisation** (MOMP).

In **MPT-dependent MOMP**, apoptotic signals open the putative **MPT pore**.[50] The classical MPT pore model (Figure 12A) is thought to be composed of 1) adenine nucleotide translocase (ANT), which interacts with the MPT pore regulator, cyclophilin D, in the inner mitochondrial membrane (IMM), and 2) VDAC, which binds to hexokinase-2 (HK2) in the OMM.[58] However, discussions regarding the exact protein composition of the MPT pore are ongoing (Figure 12B).[59] Experiments from VDAC-null mice and cells lacking VDAC confirmed the existence of a VDAC-independent model of Bcl-2 family member-mediated cell death.[60] ANT has been also excluded as an essential pore component, but it is possible that it serves some regulatory function.[61] Only the role of cyclophilin D has been clearly established as an important regulator of the MPT pore (Figure 12B).[62] He et al. proposed a new model of pore formation and suggested that MPT pores form from aggregations of misfolded integral membrane proteins that have been damaged by oxidation and other stresses. Chaperone-like proteins initially block conductance through these misfolded protein clusters; however, increased Ca^{2+} opens these regulated MPT pores.[63] Another candidate protein is the mitochondrial phosphate carrier protein (PiC), which binds cyclophilin D like ANT. Another model has been proposed for the MPT pore, which includes ANT and PiC forming dimers in the IMM.[59,64,65]

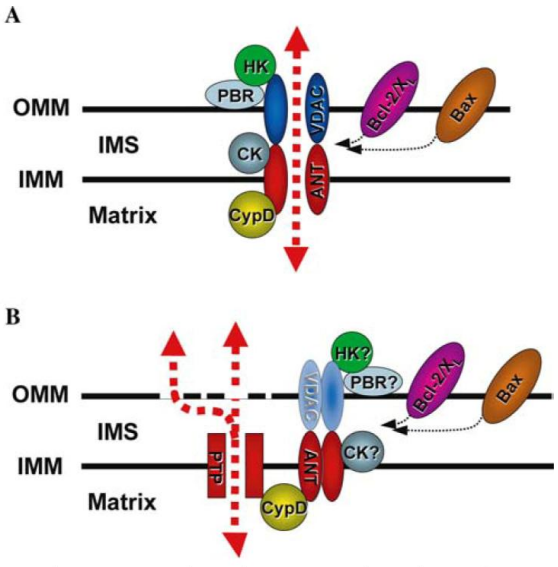


Figure 12. Proposed MPT pore complex architecture: (A) Classical view. The pore structure is formed by the VDAC-ANT-cyclophilin D complex, which is located at the contact sites between the IMM and OMM. HK2 and CK are regulatory kinases. PBR and Bcl-2 family members are regulatory components. (B) Current view. The elements comprising the pore are presently unidentified but are most likely regulated by the adjacent elements. VDAC is not an essential pore component in this model.[58]

Opening the pore is Ca^{2+}- and voltage-dependent and causes water to enter the matrix, which allows ions to equilibrate, thus dissipating the mitochondrial membrane potential (MMP). The matrix swells, and the OMM ruptures to release proteins and solutes smaller than 1500 Da from the mitochondrial intermembrane space (IMS).[58,65] The released proteins include cytochrome c and others.[49,50,57]

In **MPT-independent MOMP**, the BAX-like death factors cause the formation of pores in the OMM, which releases proteins from the IMS. Pore formation occurs either by BAX and/or BAX-related pro-apoptotic proteins forming large channels (Figure 13) or by associating with other proteins such as VDAC[53] or ANT.[50,66] The pores are large enough to allow the passage of cytocrome c, and BH3-only proteins induce the oligomerisation of BAX and associated proteins.

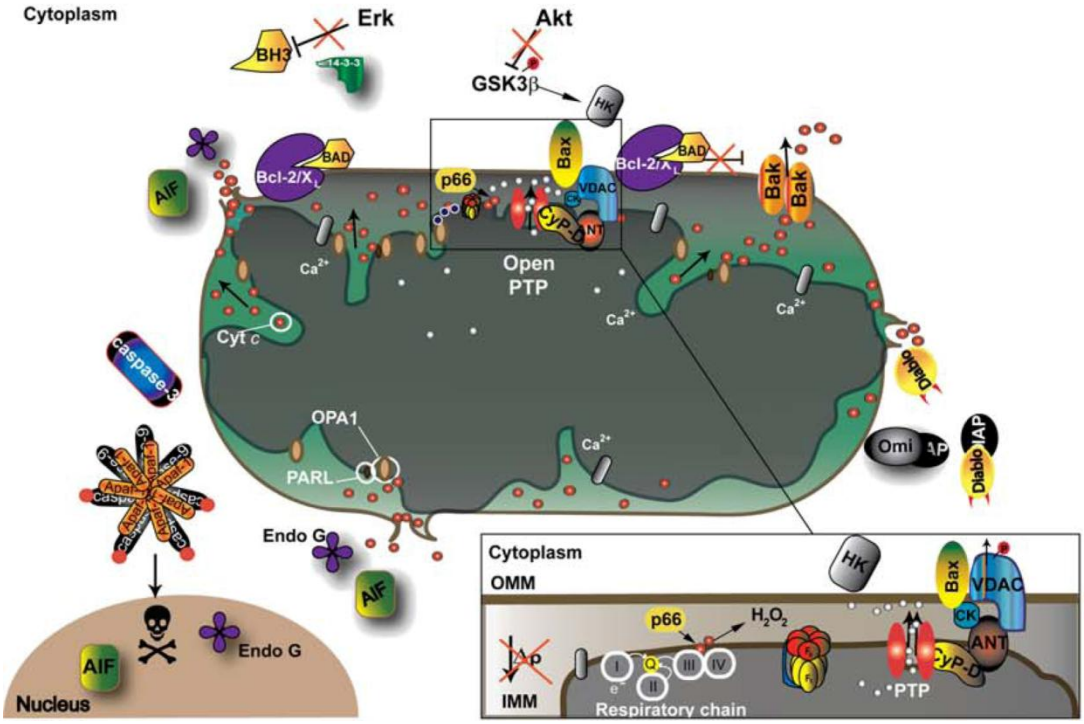


Figure 13. The mitochondria exposed to death stimuli and the MPT pore.[66]

In addition to the mitochondria as one of the key regulatory players during apoptosis, the ER is another organelle that is critical during apoptosis and takes part in complex interactions with the mitochondria. The ER is susceptible to ER stress, which is a critical initiator of apoptosis, and this process is known as the **ER stress apoptotic pathway**.[67] In the ER, quality control mechanisms ensure that only properly folded proteins can continue along the secretory pathway. Conditions such as disturbed glycosylation, perturbed Ca^{2+} homeostasis, nutrient deprivation, or abnormal protein synthesis can reduce the protein folding capacity of the ER, resulting in the accumulation and/or aggregation of unfolded proteins, a condition termed ER stress.[68] Pancreatic β-cells are susceptible to ER stress. In situations demanding high insulin secretion, an imbalance in protein homeostasis may occur and lead to ER stress, which is one of the molecular mechanisms that contributes to diabetes.[69]

To overcome the deleterious effects of ER stress, there are adaptive and protective strategies termed the unfolded protein response (UPR).[68] UPR involves 1) reduced protein synthesis to

prevent further aggregation and accumulation of unfolded proteins, 2) induction of ER-resident chaperones and folding catalysts, and 3) activation of ER-associated protein degradation to eliminate aggregates. However, if unresolved, ER stress is lethal because it induces apoptosis.[50]

ER stress leads to integrated transcriptional and translational responses through the activation of the ER transmembrane receptors IRE1 (inositol requiring ER-to-nucleus signal kinase 1), ATF6 (activating transcription factor 6) and PERK (PKR-like ER kinase). These ER stress transducers remain inactive when bound to the ER chaperone HSPA5 (heat shock 70 kDa protein 5, also known as GRP78 or BiP). When the chaperone dissociates from its luminal side to assist in protein folding, the transducers are activated, signalling ER-chaperone depletion (Figure 14).[68,70]

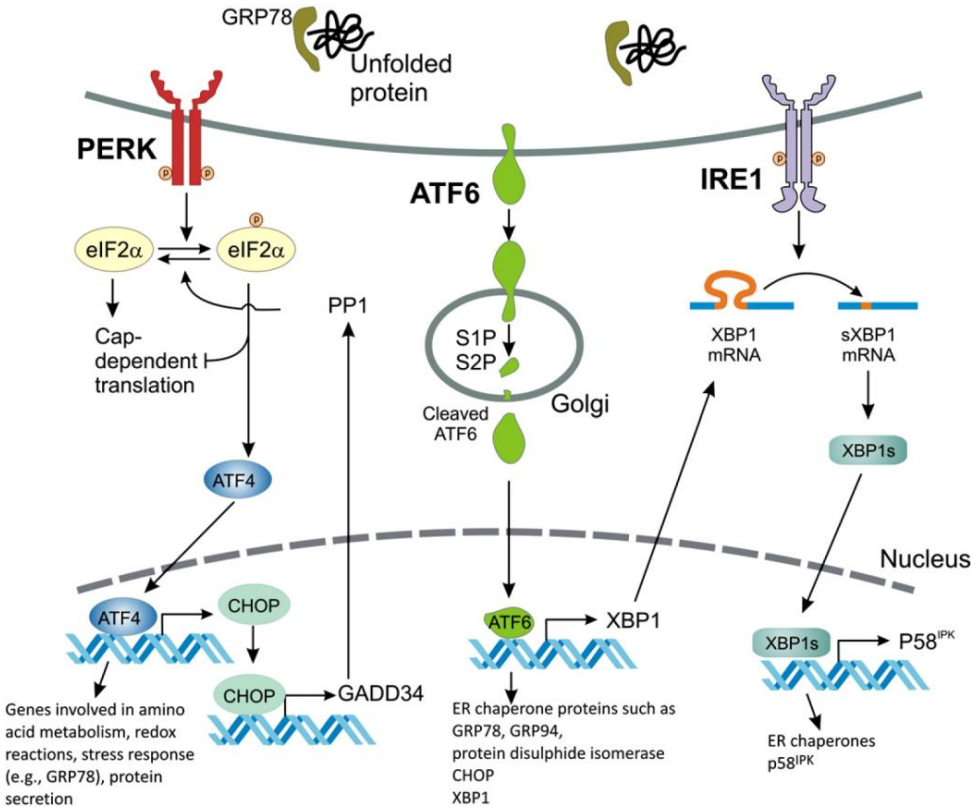


Figure 14. The UPR is mediated by three ER stress sensors: PERK, ATF6 and IRE1.[68]

Activation of transmembrane receptors is initially protective, but they are also involved in ER stress mediated apoptosis. PERK leads to the induction of CHOP (C/EBP homologous protein), which promotes apoptosis by shifting the balance of pro- and anti-apoptotic Bcl-2 family members in favour of the former.[68,70] In addition, IRE1 is important for the initiation of pro-apoptotic signals. IRE1, in a complex with TNF-receptor-associated factor 2 (TRAF2), phosphorylates and activates JNK (c-Jun NH_2-terminal kinase) in an ER stress-dependent manner. Subsequently, JNK phosphorylates Bcl-2, inhibiting its ability to regulate ER Ca^{2+} homeostasis in addition to impeding its ability to inhibit pro-apoptotic proteins. Increased cytosolic Ca^{2+} from the ER is taken up by the mitochondria, which initiates the apoptotic process. JNK also phosphorylates BIM, releasing it from its inhibitory complex; released BIM can exert its pro-apoptotic effects.[48,67,68] Finally, caspase-12 is localised to the cytosolic face of the ER, which places it in a position to respond to ER stress. Caspase-12 may be activated by calpain in response to Ca^{2+} or activated by IRE1.

Caspase-12 can directly trigger caspase-9 activation and apoptosis independent of the mitochondrial cytocrome c and APAF-1 pathway.[48,71]

As previously mentioned, β-cell apoptosis occurs at very low rates under normal conditions. However, there is a considerable decrease in β-cell mass and an increase in β-cell apoptosis in diabetes.

1.3. Pancreatic diseases

1.3.1. Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases that are characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. Deficient insulin action results from inadequate insulin secretion and/or a diminished tissue response to insulin.[72]

Several pathogenic processes are involved in the development of diabetes, and symptoms of marked hyperglycaemia include polyuria, polydipsia, weight loss, and occasionally polyphagia and blurred vision.[72]

Diabetes mellitus is one of the most common chronic diseases in nearly all countries, and its prevalence continues to increase, as changing lifestyles lead to reduced physical activity and increased obesity. In 2010, the number of diabetes cases in adults was 285 million (6.4% of the adult population), and this value is predicted to increase by approximately 54%, rising to nearly 439 million people (7.7% of the adult population) by 2030 (Table 2).[73] The increase in prevalence is predicted to be much greater in developing countries than in developed countries due to a shift toward a western lifestyle (high-energy diets and reduced physical activity).[74]

Table 2. Prevalence* of diabetes and estimated diabetes incidence by region in adults (aged 20-79 years) for the years 2010 and 2030.[73]

	2010			2030			2010/2030
	Total adult population (000s)	No. of adults with diabetes (000s)	Diabetes prevalence (%)	Total adult population (000s)	No. of adults with diabetes (000s)	Diabetes prevalence (%)	Increase in the no. of adults with diabetes (%)
Africa	379	12.1	3.8	643	23.9	4.7	98.1
EMME	344	26.6	9.3	533	51.7	10.8	93.9
Europe	646	55.4	6.9	659	66.5	8.1	20.0
N America	320	37.4	10.2	390	53.2	12.1	42.4
SACA	287	18.0	6.6	382	29.6	7.8	65.1
S Asia	838	58.7	7.6	1200	101.0	9.1	72.1
W Pacific	1531	76.7	4.7	1772	112.8	5.7	47.0
World	4345	284.8	6.4	5589	438.7	7.7	54.1

* Prevalence for each region is standardised to the world distribution of that year.

EMME = Eastern Mediterranean and Middle-East

SACA = South and Central America

1.3.1.1. Type 1 Diabetes Mellitus (T1DM)

T1DM, also known as insulin-dependent diabetes, accounts for only 5-10% of diabetes cases.[72] It results from a cell-mediated autoimmune assault against β-cells of the pancreas, and includes the invasion of islets by mononuclear cells in an inflammatory reaction termed "insulinitis". These processes induce progressive β-cell death. Apoptosis is the main mechanism of β-cell death, and it is initiated by direct contact with activated macrophages and T-cells, and exposure to soluble mediators secreted by these cells. These mediators include cytokines such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), and TNF-α; as additionally, nitric oxide (NO) and reactive oxygen and nitrogen species (ROS/RNS) are involved. These cytokines also induce their own expression in β-cells, further contributing to the inflammatory environment. Therefore, the interaction between immune cells and islets amplifies cytotoxicity and sensitises the β-cells to apoptosis. The mechanisms of apoptosis in T1DM include activation of stress-activated protein kinases JNK, MAPK, and ERK; triggering of ER stress; and disruption of the MMP, accompanied with the release of death signals from the mitochondria (Figure 16).[75–79]

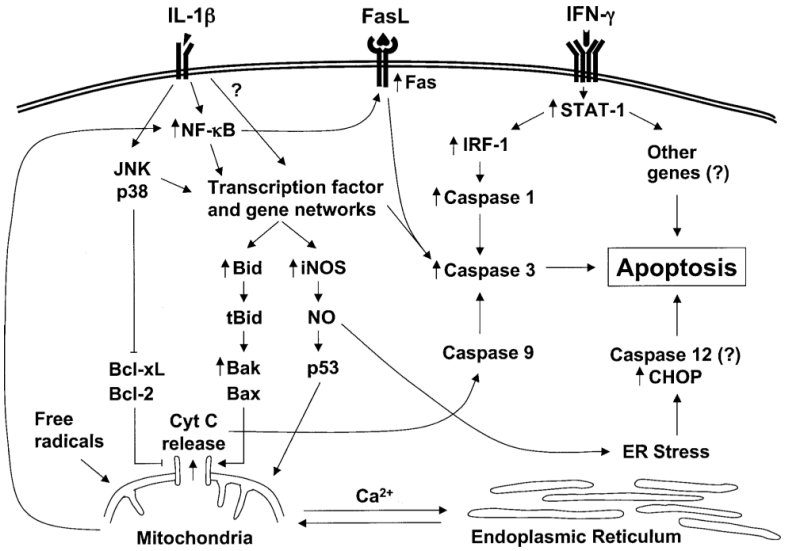


Figure 16. Model of the different pathways contributing to cytokine-induced β-cell apoptosis, including JNK, ER stress, and the liberation of pro-apoptotic proteins from the mitochondria.[75]

β-cell destruction is generally rapid in children and slow in adults.[72] β-cell mass is often reduced by 70-80% at the time of diagnosis.[75] T1DM has multiple genetic predispositions and is associated with environmental factors that are still poorly defined. Although patients are rarely obese when they present with T1DM, the presence of obesity is not incompatible with the diagnosis. T1DM patients are also prone to other autoimmune disorders.[72]

Despite the fact that apoptosis has a central role in the development of T1DM, proliferation processes could also be involved in this disorder. One study of an 89-year-old patient provided evidence of a compensatory β-cell proliferation mechanism in T1DM.[80]

1.3.1.2. Type 2 Diabetes Mellitus (T2DM)

T2DM, or non-insulin-dependent diabetes, accounts for 90-95% of diabetes cases. It is a metabolic disorder of fuel homeostasis characterised by hyperglycaemia and altered lipid metabolism. β-cells are unable to secrete adequate insulin in response to varying degrees of overnutrition, inactivity, obesity, and insulin resistance.[74]

T2DM involves a combination of genetic and environmental or lifestyle factors. The heritability of T2DM is high (estimated to be >50%). Genome-wide association studies have helped to identify more than 40 confirmed diabetes-associated loci. *TCF7L2* locus is associated with strongest susceptibility for developing T2DM and is associated with β-cell dysfunction. Additionally, a westernised lifestyle, which involves a high-energy diet and reduced physical activity, has been linked to obesity and T2DM pandemics.[74]

Several criteria may be independently used to diagnosis T2DM. These criteria include a glycated haemoglobin A_{1c} (HbA_{1c}) concentration of 6.5%, a 75-g oral glucose tolerance test with a 2-h value of 11.1 mmol/L or more, a random plasma glucose of 11.1 mmol/L with classic symptoms of diabetes, or a fasting plasma glucose of 7.0 mmol/L or more on more than one occasion.[72,81]

Prediabetes refers to an intermediate stage in which abnormalities in glucose metabolism are present, but the elevated glucose level is below the cut-off point for diagnosing T2DM.[82] Prediabetes includes individuals with impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), and these individuals are considered to be at risk for the development of diabetes and macrovascular disease.[81,83] Subjects with IFG have high fasting plasma glucose levels (5.6 to 6.9 mmol/L) and normal responses to glucose loads. In contrast, those with IGT have abnormal postprandial glucose excursion (2-h plasma glucose value of 7.8 to 11.0 mmol/L) but normal fasting plasma glucose concentrations.[72,82,83] Both IGT and IFG are insulin-resistant states, but they differ in the site of insulin resistance. Subjects with IGT have moderate-to-severe insulin resistance in muscle and impaired first- and second-phase insulin secretion. Conversely, individuals with IFG have moderate insulin resistance in the liver, impaired first-phase insulin secretion, and normal or near-normal muscle insulin sensitivity.[83] IFG and IGT are associated with obesity (especially abdominal or visceral obesity), dyslipidemia with high triglycerides and/or low HDL cholesterol, and hypertension. Although one third of these patients will eventually develop diabetes, dietary modifications and exercise can lower the risk of progression from IGT to T2DM. These lifestyle changes may also prevent the development of IGT in nondiabetic individuals at high risk. Pharmacological agents may also limit the progression of IGT to diabetes.[81]

The development of T2DM is a two-step process. In the first step, glucose tolerant individuals progress to IGT with insulin resistance as the primary determinant. Plasma insulin levels are elevated, and β-cell function is clearly impaired. In step two, IGT advances to T2DM because of a progressive decline in β-cell function.[84]

The primary pathogenic event that drives the development of T2DM is chronic fuel excess in genetically and epigenetically susceptible people. However, many chronically overnourished and overweight or obese individuals do not develop diabetes or develop it very late in life. These individuals are referred to as being resistant to T2DM. Susceptible overnourished individuals

develop T2DM because of the inability of β-cells to compensate for fuel excess. Furthermore, the liver produces increased endogenous glucose, and muscle takes up less glucose due to the development of peripheral insulin resistance. Impaired expansion of subcutaneous adipose tissue, hypoadiponectinaemia, inflammation of adipose tissue, reduced incretin responses in the gut, hyperglucagonaemia, increased renal reabsorptive capacity for glucose, and impaired appetite regulation by insulin due to neurotransmitter dysfunction can also occur. These symptoms are depicted in the ominous octet (Figure 17).[85]

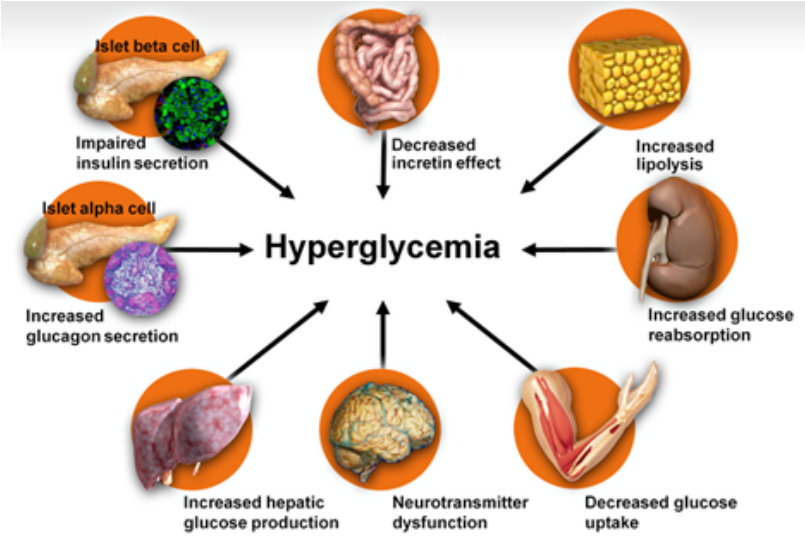


Figure 17. The ominous octet.[85]

In the development of T2DM, peripheral insulin resistance, pancreatic β-cell dysfunction, and decreased β-cell mass play important roles.[86]

Insulin resistance

Insulin resistance involves liver, muscle, and adipose tissue. In the liver, insulin resistance is manifested by an overproduction of glucose during the basal state despite the presence of fasting hyperinsulinemia. Additionally, there is impaired suppression of hepatic glucose production in response to insulin, often occurring after a meal. In muscle, insulin resistance is manifested by impaired glucose uptake after ingestion of a carbohydrate-rich meal, resulting in postprandial hyperglycaemia.[83] Finally, adipocytes are resistant to insulin's antilipolytic effect, leading to an elevation in the plasma free fatty acid (FFA) concentration. Excessive cytokines are also produced, which exacerbate insulin resistance.[85]

There is a substantial compensatory effort by the β-cells to maintain normal glycaemic levels in obesity and insulin resistance. Two types of adaptative responses occur. One response involves each β-cell secreting more insulin, and the second response involves increasing the β-cell mass. Nonetheless, the β-cell mass is the major factor determining the amount of insulin that can be secreted.[21,87]

β-cell dysfunction

Hyperglycaemia results not only from defects in the action of insulin (insulin resistance), but it also results from a defect in the appropriateness of insulin secretion by the pancreatic β-cell.[88] β-cell function is estimated to be approximately 50% lower compared to normal function at time of diagnosis, and it further deteriorates during the natural course of diabetes. This decline is the main reason for the loss of control occurring over the years in patients with T2DM.[88]

Multiple factors including genetic predisposition, insulin resistance, increased insulin secretory demand, glucotoxicity, lipotoxicity, impaired incretin release/action, amylin accumulation, and decreased β-cell mass play a causative role in progressive β-cell dysfunction.[84]

The mechanisms underlying β-cell failure are varied and complex. β-cell dysfunction generally occurs when compensation is required to deal with fuel excess. The mix of β-cell susceptibility factors determine the initial mechanism of damage, but once substantial hyperglycaemia has developed, glucotoxic and glucolipotoxic mechanisms occur in most patients, accelerating the rate of failure.[74]

β-cell dysfunction is initially characterised by an impaired first phase of insulin secretion during glucose stimulation, which may precede the onset of glucose intolerance in T2DM. Initiation of the insulin response depends on the transport of glucose and the coupling of glucose to the glucose sensor. Glucose transport in the β-cells of T2DM patients is greatly reduced, thus shifting the control point for insulin secretion from glucokinase to the glucose transport system. Later in the course of the disease, the second phase release of newly synthesised insulin is impaired. This secondary phenomenon, termed desensitisation or β-cell glucotoxicity, is the result of a paradoxical inhibitory effect of glucose on insulin release and may be attributed to the accumulation of glycogen within the β-cell as a result of sustained hyperglycaemia. Other β-cell function defects in T2DM include defective glucose potentiation in response to non-glucose insulin secretagogues, asynchronous insulin release, and a decreased conversion of proinsulin to insulin.[81]

β-cell mass in T2DM

Postpandrial hyperglycaemia and dyslipidemia are common features that occur before the development of insulin secretory defects in T2DM, and both alterations may be implicated in the pathogenesis of T2DM.[89] Moreover, most subjects with T2DM have increased plasma insulin levels (hyperinsulinemia), especially during the fasting state.[88] These conditions can modify β-cell mass. During the first stages of insulin resistance, β-cell mass is increased to compensate for the high levels of glucose. However, when β-cells are unable to compensate for increased insulin demand, there is a decrease in β-cell mass, which is characteristic of T2DM onset. The decrease in β-cell mass is due to an increase in β-cell apoptosis, which far outweighs the modest increases in β-cell replication and neogenesis. As T2DM progresses, the situation worsens. The incidence of β-cell replication decreases, and the β-cell population declines.[18]

Mild hyperglycaemia produced by glucose infusion in mice causes an increase in β-cell replication.[41] Another study demonstrated that β-cell neogenesis is also involved in the β-cell

mass dynamics during prolonged hyperglycaemia, and β-cell neogenesis contributes to the expansion of the β-cell mass after several days of glucose infusion in mice.[90]

It is important to note that glucose affects the survival of human and rodent islets differently. Glucose can stimulate proliferation in rodents, but its mitogenic effects, at least *in vitro*, seem to be time-limited in human islets. However, β-cell apoptosis is long-lasting.[91]

Moreover, there is almost no β-cell replication in humans, and new islet formation is the predominant pathway for increased β-cell mass in response to obesity. However, there was no difference in new islet formation in T2DM patients. Thus, the mechanism for decreased β-cell mass in T2DM patients is an increase in β-cell apoptosis.[92]

Chronic hyperglycaemia and chronic hyperlipidemia are two mechanisms that could trigger an increase in β-cell apoptosis.[93]

· Glucotoxicity

Glucose metabolism in pancreatic β-cells leads to ATP generation, closure of ATP-regulated K^+ channels, plasma membrane depolarisation, opening of voltage-dependent Ca^{2+} channels, and an increase in free cytosolic Ca^{2+} concentration that results in insulin release. In contrast, chronic exposure to a hyperglycaemic environment causes β-cell dysfunction and death, a phenomenon termed glucotoxicity.[94] Hyperglycaemia causes increased apoptosis in cultured human pancreatic islets and a dose-dependent reduction in gene expression of insulin, GLUT-2, and transcription factors involved in β-cell failure.[88] Hyperglycaemia also increases apoptosis in cultured rat islets and rat β-cells in a Ca^{2+}-dependent manner.[95]

Chronic hyperglycaemia can lead to chronic activation of a nutrient-sensing serine/threonine protein kinase, mammalian Target of Rapamycin (mTOR) in β-cells. Activated mTOR triggers serine/threonine phosphorylation of IRS-2, which subsequently results in IRS-2 ubiquitination, proteosomal degradation, and β-cell apoptosis. IRS-2 is critically important for maintaining β-cell mass by promoting β-cell survival.[96]

Chronic exposure to high glucose induces β-cell apoptosis by decreasing glucokinase (GCK) and its interactions with VDAC in the OMM, causing decreased BAD phosphorylation. Decreased binding of GCK with mitochondria promotes the binding of BAX with VDAC, and subsequently, BAX oligomerisation, cytocrome c release, apoptosis, and decreased cellular ATP production and insulin secretion occur.[94,97] Additionally, high glucose mediates a decrease in glucose-6-phosphate dehydrogenase activity, leading to increased ROS and apoptosis.[98]

Hyperglycaemia can also be harmful to β-cells due to inflammation and oxidative stress. Increased glucose concentration alone has been suggested to induce apoptosis in human β-cells *in vitro* through the induction of IL-1β, which leads to nuclear factor-kappa B (NF-κB) activation and up-regulation of Fas receptors. This mechanism ultimately leads to DNA fragmentation, and the mitochondrial pathway appears to not be involved.[91,99] This result implies that IL-1β plays a role in inducing β-cell apoptosis in T2DM and in T1DM. However, these findings were not reproduced by others.[100] Conversely, high glucose concentrations have been shown to increase ROS in human islets as a consequence of chronically increased glucose metabolism in β-cells. β-

cells have relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase; thus, they are more sensitive to ROS assault when exposed to oxidative stress.[88,101] Elevated ROS leads to an accumulation of mitochondrial DNA mutations and the disruption of mitochondrial membrane integrity, resulting in the induction of the intrinsic mitochondrial cell death pathway.[93]

Another player in hyperglycaemia-induced apoptosis is ER-stress. High glucose levels greatly increase the demand of β-cell insulin secretion and lead to increased proinsulin biosynthesis to replenish β-cell insulin stores. The increase in proinsulin biosynthesis causes an increased flux in protein through the ER. Flux of β-cells through the ER is higher compared to other cell types even under physiologic conditions, and any further increase in flux is expected to tilt the balance in favour of ER-stress-induced apoptosis.[102] Moreover, hyperglycaemia could also lead to long-term increases in cytosolic Ca^{2+}, which could be pro-apoptotic.[102,103]

· Lipotoxicity

Elevated plasma free fatty acid (FFA) concentration may contribute to progressive loss of insulin action, β-cell function, and β-cell mass.[87,104] Lipotoxicity is related to high FFA availability in T2DM and the formation of excessive amounts of products, such as ceramide, acyl-CoA, and diacylglycerol.[88] Although FFAs are critical for normal insulin release, chronic exposure to FFAs is associated with marked impairments in glucose-stimulated insulin secretion, decreases in insulin biosynthesis and increases in apoptosis.[87,88] The increase in FFA concentration is a result of increased fat deposits in obesity and is normally associated with T2DM. Conversely, ectopic fat accumulation can also occur in the endocrine pancreas, and harmful cross-talk between the adipocytes and the β-cells might be facilitated by paracrine effects.[88]

Saturated fatty acids such as palmitate are highly toxic to β-cells, whereas the mono-unsaturated fatty acids such as oleate are protective against both palmitate and glucose-induced apoptosis.[105] This difference in the pro-apoptotic effects of FFAs is thought to be due to a greater ability of unsaturated FFAs to form intracellular triacylglycerides (TAG).[106] Similarly, VLDL and LDL are pro-apoptotic, whereas HDL protects β-cells against pro-apoptotic effects via activation of AKT and inhibition of caspase-3 cleavage.[107]

Some of the mechanisms involved in FFA-induced β-cell apoptosis have been described. FFA, through the production of intracellular long chain acyl-CoA, can activate a novel class of protein kinase C isoforms (nPKC) independent of [Ca^{2+}], leading to serine/threonine phosphorylation of IRS molecules that would promote IRS-2 degradation in β-cells and lead to β-cell apoptosis.[18] The toxic effects of saturated FFAs may also be related to ceramide formation. Ceramide induces cytocrome c release, which is prevented by monounsaturated FFAs, such as palmitoleic and oleic, most likely via up-regulation of Bcl-2.[105,108,109]

FFA-induced β-cell toxicity might also occur at the ER level, where FFA esterification occurs. High FFA loads exceeding the β-cell's esterification capacity impair ER functions and trigger the ER stress response. Moreover, FFAs might also impair ER Ca^{2+} handling.[70,75] Palmitate, but not oleate, exposure induces ER stress in INS-1 cells, activating PERK, ATF4, XBP-1, and CHOP; these proteins mediate ER stress-induced apoptosis.[110] However, both palmitate and oleate

induced ER stress in another study.[104] Furthermore, oleate only induced the activation of ATF6, whereas palmitate also induced PERK and IRE1.[70] ATF signalling might protect β-cells because HSP5 overexpression protects β-cells against palmitate.[111] Palmitate also induces rapid degradation of carboxyperptidase E, which results in ER stress and apoptosis.[112]

Finally, at least part of the detrimental effects of palmitate on β-cells is caused by changes in the levels of specific miRNAs. Prolonged exposure of rodent β-cells and islets to palmitate causes an increase in miR34a and miR146, and this change in expression has also been observed in islets of diabetic *db/db* mice. miR34a is known to favour apoptosis by inhibiting the expression of Bcl-2, whereas the mechanism through which miR146 affect β-cell survival is unclear.[113]

An *in vivo* mouse study demonstrated that the beneficial effects of hepatocyte growth factor (HGF) overexpression in β-cells, which include glucose homeostasis, β-cell proliferation, and β-cell expansion under basal conditions, disappear in obesity and insulin resistance induced by high-fat feeding. *In vitro*, HGF decreases FFA oxidation, enhances ceramide accumulation, and exacerbates apoptosis in rodent and human β-cells in lipotoxic environments. Because circulating HGF levels are increased in obese subjects, HGF might participate in β-cell failure, leading to T2DM in obesity.[114]

FFAs, mainly linoleic acid and palmitic acid, inhibit glucose-induced β-cell proliferation, without interfering with cyclin D2 induction or nuclear localisation by glucose, but expression of INK4 family cell cycle inhibitors p16 and p18 increases.[115]

This lipotoxic effect can also act synergistically with glucose to produce even greater deleterious effects, commonly referred to as glucolipotoxicity.[87] Under elevated glucose conditions, long-chain fatty acyl-CoA derived from elevated FFAs accumulates due to the inhibitory effect of glucose on lipid detoxification by β-oxidation and enhanced lipid synthesis.[89,106]

· Hyperinsulinemia and others

The protective effects of **insulin** on β-cell mass are lost at high concentrations; therefore, the hyperinsulinemia commonly found in T2DM may have additional deleterious effects on β-cell apoptosis and glucose homeostasis.[116] In fact, prolonged exposure of β-cells to high levels of insulin induces apoptosis *in vitro*.[117]

In obesity-linked diabetes, certain adipocyte-derived products are elevated in circulating blood. Adipocyte-released **cytokines**, such as TNF-α or IL-6, can induce β-cell apoptosis through induction of signalling pathways that activate the transcription factor NF-κB (reviewed in [118]). Cytokine-induced activation of NF-κB leads to the loss of differentiated β-cell functions by down-regulation of PDX-1, up-regulation of inducible nitric oxide synthase (iNOS), excessive NO production, up-regulation of chemokines such as monocyte chemoattractant protein-1 (MCP-1), and down-regulation of the Ca^{2+} pump sarcoendoplasmic reticulum Ca^{2+} ATPase type 2b (SERCA-2b), which leads to ER Ca^{2+} depletion and ER stress.[104]

Finally, **leptin** is a protein hormone released by adipocytes and is an important factor regulating body weight and glucose homeostasis. At physiological concentrations, leptin decreases rat β-cell apoptosis by increasing Bcl-2.[119] However, chronic exposure to high concentrations of leptin

induces apoptosis in human β-cells, decreasing β-cell production of the IL-1 receptor agonist and inducing IL-1β release. Finally, caspase-3 is activated, which induces apoptosis.[120] Chronically high leptin concentration also activate JNK and induce caspase-1, the enzyme that converts pro-IL-1β to IL-1β.[121]

1.3.2. Pancreatic cancer

Pancreatic cancer is one of the most aggressive cancers in developed countries, and despite recent diagnostic and therapeutic advances, it retains a poor prognosis.[122] It accounts for the 4th most cancer-related deaths in the United States, despite being only the 10th most common type of cancer. Furthermore, the 5-year survival rate is only 5%, which is the lowest of all malignancies, as estimated between 1996-2004.[123] The majority of patients with pancreatic cancer present with metastatic disease, and only 15-20% of tumours are surgically resectable.[124]

Pancreatic cancers are classified by a variety of malignancies, including the preponderant form, pancreatic ductal adenocarcinoma (80-90% of the cases). Additionally, islet tumours, rare acinar cell tumours, mucinous neoplasms, lymphomas, and sarcomas are classified as pancreatic cancers.[125] Pancreatic adenocarcinoma arises from epithelial cells in the pancreatic ducts, though they can also develop from resident stem cells.[126] Development of the adenocarcinoma is thought to be preceded by proliferative intraductal changes, such as pancreatic intraepithelial neoplasia.[127] This cancer is characterised by invasiveness, rapid progression, and profound resistance to treatment.[5] Moreover, features characteristic of the malignancy include large mural nodules, marked dilation of the main pancreatic duct, and symptoms of pain, weight loss, and pancreatitis.[123]

Pancreatic cancer arises from genetic dysregulation (including chromosomal abnormalities, point mutations, and epigenetic silencing) that accumulates over time due to demographic (advancing age or male gender) and/or environmental (smoking and obesity) factors.[122,126] Moreover, it is estimated that 10% of pancreatic cancers are due to an inherited predisposition.[5]

Pancreatic carcinogenesis progresses via the accumulation of genetic alterations resulting in a gain of cell growth and proliferation and increased dissemination and metastatic potential. Gain or loss of gene function may result from the up-regulation of oncogenes, down-regulation of tumour-suppressor genes, or by the dysregulation of genomic maintenance/DNA repair genes and house-keeping genes. Additionally, genes that regulate the apoptosis/cell death/immortalisation cascade and growth factors, cytokines, and cell adhesion molecules play a role in carcinogenesis.[128] The dysregulation of oncogenes and tumour-supressor genes may also be due to aberrant miRNA expression. In fact, aberrant expression of several miRNAs has been observed in pancreatic tumour tissues and cell lines, and these miRNAs could be used as biomarkers for pancreatic cancer diagnosis and treatment (reviewed in [129]).

The tumour-supressor gene p16^{INK4a} is the most frequently inactivated gene in pancreatic cancer and its inactivation causes inappropriate phosphorylation of pRb, thereby facilitating cell cycle progression through the G1/S transition. In addition, inactivation of p53, a tumour-suppressor, occurs in 50-75% of pancreatic cancers, and its functional loss allows the cell cycle to progress in

the presence of underlying DNA damage.[126] Conversely, mutations in the oncogene K-ras are present in 90% of pancreatic adenocarcinomas, and these mutations occur early in these cancers. Mutated K-ras leads to constitutive activation of epidermal growth-factor family signalling, which includes MAPK, PI3K, and NF-κB. This signalling leads to cellular proliferation, survival, and invasion.[5,126,130] In addition, overexpression of cyclin D1 has been observed in pancreatic cancer, and it causes constitutive phosphorylation of pRb, leading to dysregulated and increased E2F activity.[131]

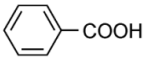
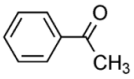
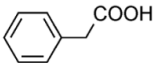
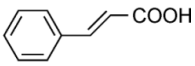
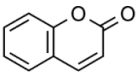
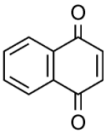
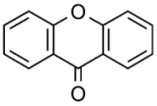
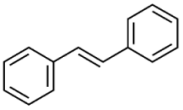
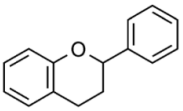
It has been assumed that inflammation promotes tumorigenesis, which may occur, at least in part, via the production of growth factors, cytokines, and ROS. These molecules induce cell proliferation, disrupt cell differentiation, and select for oncogenic mutations. Increased cell turnover and ROS might also lead to increased telomere attrition and dysfunction, setting the stage for potentially oncogenic genomic instability.[5] Moreover, high levels of ROS have been proposed to play a role in cancer metastasis.[132]

2. Phenolic compounds

Phytochemicals are plant secondary metabolites. As they are dietary components, they are not essential for short-term well-being but have the capacity to impact human health.[133] Polyphenols form one of the major groups of phytochemicals.[134]

Polyphenols are composed of a wide range of plant substances that possess at least one aromatic ring bearing one or more hydroxyl substituents.[135] They are widely dispersed throughout the plant kingdom and are widespread constituents of fruits, vegetables, cereals, olives, dry legumes, chocolate, and beverages (tea, coffee, and wine).[136] Polyphenols are divided into several classes according to the number of phenol rings that they contain and the structural elements that bind these rings to one another (Table 3).[136]

Table 3. Basic structural skeletons of phenolic and polyphenolic compounds.[134]

Skeleton	Classification	Basic structure
C_6–C_1	Phenolic acids	
C_6–C_2	Acetophenones	
C_6–C_2	phenylacetic acid	
C_6–C_3	Hydroxycinnamic acids	
C_6–C_3	Coumarins	
C_6–C_4	Naphthoquinones	
C_6–C_1–C_6	Xanthones	
C_6–C_2–C_6	Stilbenes	
C_6–C_3–C_6	Flavonoids	

2.1. Flavonoids

2.1.1. Structure and classification of flavonoids

Flavonoids compose the largest group of phenolic compounds (more than 4000 flavonoids have been identified) and are found in the epidermis of leaves, in flowers, and in the skin of fruits.[134,136,137]

Their structure is based on the flavonoid nucleus, which consists of three phenolic rings referred to as the A, B, and C rings (Figure 18). The benzene ring A is condensed with a six-member ring (C), which carries a phenyl benzene ring (B) as a substituent on the 2-position.[137]

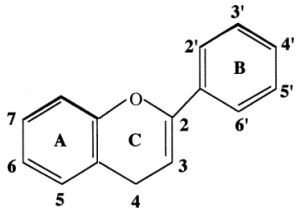
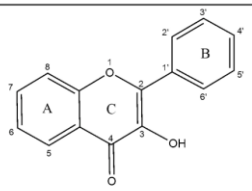
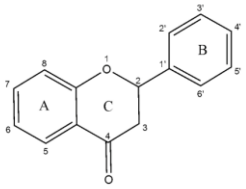
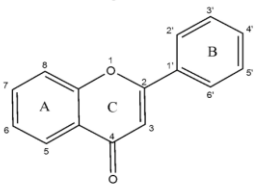
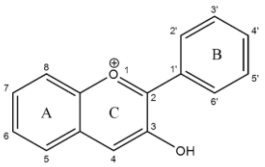
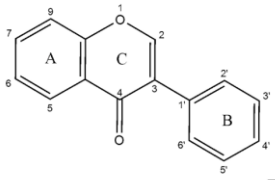
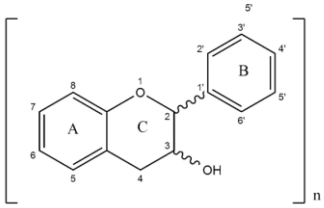


Figure 18. Flavonoid nucleus.[137]

Flavonoids are divided into 6 subclasses depending on the oxidation state of the C ring and on the connection of the B ring to the C ring (Table 4).[136] Flavan-3-ols (also known as flavanols) and anthocyanidins have a heterocyclic pyran as their C ring. In contrast, flavonols, flavones, flavanones, and isoflavones have a pyrone as their C ring.[137]

Table 4. Classification and representative structures of the main flavonoids.[138]

Flavonoids	Structure
Flavonols (for example Quercetin)	
Flavanones (for example Naringenin)	
Flavones (for example Apigenin)	
Anthocyanidins (for example Pelargonidin)	
Isoflavones (for example Genistein)	
Flavanol monomer, dimers, trimers,etc. (for example EGCG)	

The basic flavonoid skeleton can have numerous substituents, including glycosylation, hydrogenation, hydroxylation, malonylation, methylation, and sulfation.[137] Hydroxyl groups are usually present at the 4'-, 5- and 7-positions, and sulphate groups and glycosides increase the water solubility of flavonoids. In contrast, methyl and isopentyl groups make flavonoids lipophilic.[134,139] Moreover, the degree of polymerisation increases the diversity of flavonoid compounds. Flavonoid molecules that are not attached to sugar moieties are referred to as being in the aglycone form, whereas flavonoid molecules attached to sugar moieties are called flavonoid glycosides.[137]

2.1.2. Structure and classification of flavan-3-ols

Flavan-3-ols are the most structurally complex subclass of flavonoids. They range from simple monomers, (+)-catechin and its isomer (-)-epicatechin, to complex structures that include the oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins (Figure 19). The monomeric forms can be hydroxylated at the 5' position of the B ring to form gallocatechins, and monomers can also undergo esterification with gallic acid at the 3 hydroxyl position of the C ring.[134,140]

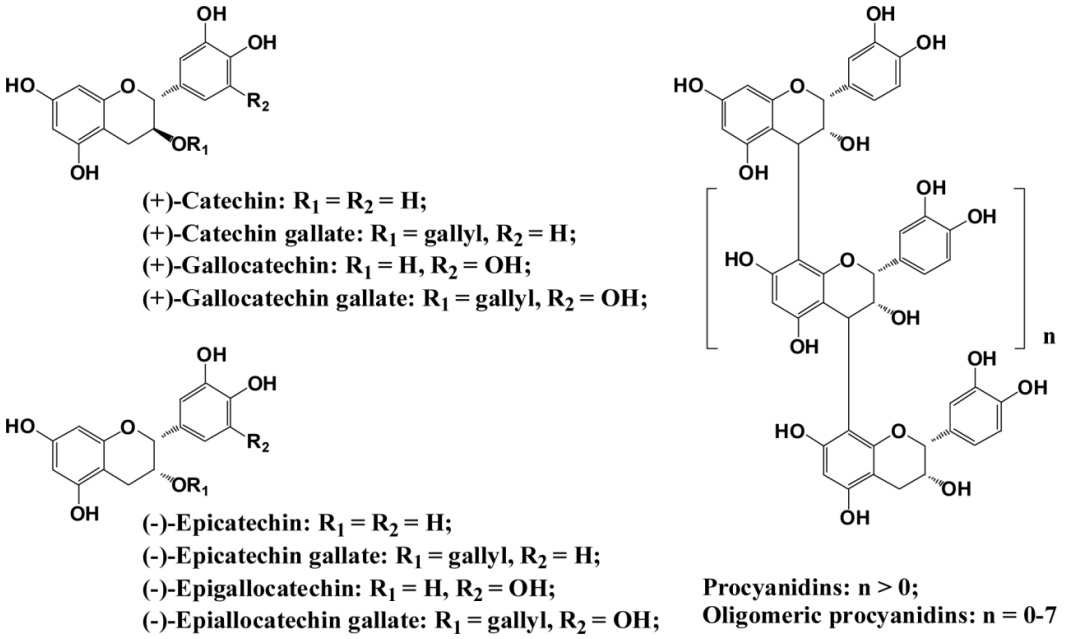


Figure 19. Flavan-3-ols and procyanidins.[141]

Fifteen subclasses of proanthocyanidins have been identified, but procyanidins are the most prominent in human foods of plant origin. Procyanidins consist exclusively of (epi)catechin units and are the most abundant type of proanthocyanidins in nature.[133] Prodelphinidins and propelargonidins contain (epi)gallocatechin and (epi)afzelechin units, respectively, and are usually mixed with procyanidins.[142] The flavan-3-ol subunits may carry acyl (mainly gallic acid) or glycosyl substituents.[143] In these tannins, the monomeric units are linked by single C4→C6 or C4→C8 bonds (B-type proanthocyanidins) or by C4→C8 linkages and C2→O→C7 bonds (A-type proanthocyanidins). Procyanidin C1 is a trimer.[141]

2.1.3. Sources and intake

Catechins are found in many fruits, including cherries and apricots (250 mg/kg), but the richest sources of catechins are green tea (800 mg/L), chocolate (600 mg/L), and red wine (300 mg/L).[136] Catechin and epicatechin are the main flavanols in fruit, whereas gallocatechin (GC), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) are found in certain seeds of legumes, in grapes, and in tea.[144] In contrast to other classes of flavonoids, flavanols are not glycosylated in foods.[144]

Proanthocyanidin content is difficult to determine due to the wide range of structures.[136] They are major components in the human diet because of their widespread presence in fruits, berries, nuts, beans, some spices, cocoa-based products, wine, and beer.[145] They are also responsible for the astringent character of fruit and beverages and for the bitterness of chocolate.[143]

In 1976, Kuhnau calculated that dietary flavonoid intake in the United States was approximately 1 g/day.[146] More recent studies have estimated that the polyphenol intake is approximately 1 g/day in Finland and France.[147,148] When only flavonoid intake was evaluated, the value falls to approximatey 370 mg/day.[149] The exact proanthocyanidin content in food is difficult to asses because of the numerous proanthocyanidin structures, degrees of polymerisation, and the various analytical methods used. Thus, the average human intake of procyanidins is not well defined. The average proanthocyanidin intake in the United States is estimated to be approximately 53.6 mg/day (57.7 mg/day including monomers), with apples, chocolate and grapes as major contributors.[145] However, regular wine drinkers and fruit eaters will easily have a higher daily intake of flavan-3-ols[143], varying from 10 mg to 500 mg/day[145].

2.1.4. Absorption and metabolism

Once flavonoids have been ingested, most of the glycosides resist acid hydrolysis in the stomach and arrive intact in the intestine. Studies with rodents demonstrated that absorption at the gastric level is possible for some flavonoids, such as quercetin and anthocyanins, but is not possible for their glycosides (reviewed in [136]). Moreover, aglycones can be absorbed by the small intestine. However, most of the flavonoids (except catechins) do not exist in plants as aglycones; instead, they exist as glycoside conjugates, esters, and polymers that cannot be absorbed in their natural form.[139] Therefore, absorption of these molecules is associated with hydrolysis by lactase phloridizin hydrolase (LPH), which releases the aglycone. Hydrolysis occurs at the brush-border of the small intestine epithelial cells. The released aglycone may then enter the epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cell membrane.[150] Alternatively, polar glucosides may be transported into epithelial cells. Transport may involve the active sodium-dependent glucose transporter SGLT1[151] and hydrolysis by cytosolic β-glucosidase (CBG) within the epithelia.[152] These two possible routes are named “LPH/diffusion” and “transport/CBG”.[134] However, recent studies have indicated that some catechins can inhibit the glucose transporter SGLT1.[153] It has been estimated that 5 to 10% of consumed flavonoids are absorbed by the small intestine.[154]

The remaining 90 to 95% of flavonoids[154] are not absorbed by the small intestine, and their conjugates excreted by the intestine and bile reach the colon, where they can be metabolised by the gut microflora. Chemical transformations include removal of glucuronides and sulphates, breaking of the glycoside bonds formed between polyphenol aglycones and sugars, and fission and reduction of the aromatic rings, these transformations lead to the production of phenolic acids and hydroxycinnamates, which can be absorbed by colonic cells.[139]

Prior to absorption into the blood stream, aglycones undergo metabolic processes to form sulphate, glucuronide, and/or methylated metabolites via the respective actions of sulfotransferases (SULT), uridine-5'-diphosphate glucuronosyltransferases (UGTs), and catechol-O-methyltransferases (COMT). There is efflux of some of the metabolites back into the lumen of the small intestine, which is thought to involve members of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters.[134]

After absorption, flavonoids are bound to proteins, mainly albumin,[155] and transported to the liver via the portal vein. In the liver, metabolites can be subjected to phase II metabolism and are further converted by hydroxylation, methylation, and reduction reactions.[134,137]

The concentration of polyphenols in the body following consumption is highly variable depending on the nature of the polyphenol and the food source. The plasma concentrations of intact flavonoids rarely exceed 1 μM, and the plasma metabolite concentrations are approximately 10 μM. To maintain a high concentration of polyphenols in plasma, repeated ingestion over time is required. In fact, the maximum concentration of polyphenols is most often achieved between 1 and 2.5 h after ingestion, with the exception of polyphenols that require degradation prior to absorption (5 to 12 h).[136,154]

Once in the circulation, flavonoids can reach organs and tissues. Flavonoids have been detected by HPLC analysis in a wide range of tissues in mice and rats, including brain, endothelial cells, heart, kidney, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone, and skin.[144]

The conjugation of flavonoids is a metabolic detoxification process that increases their hydrophilicity in order to facilitate their biliary and urinary elimination. Normally, the conjugation of polyphenols is highly efficient because their aglycones are generally either absent in blood or present at low concentrations after consumption.[139] Extensively conjugated metabolites are more likely to be eliminated in the bile, whereas small conjugates are preferentially excreted in urine.[136] The urinary recovery is 0.5-6% of the intake for some tea catechins,[156] 2-10% for red wine catechin,[157] and up to 30% for cocoa epicatechin.[158]

Numerous studies in animals and humans have shown that polymeric proanthocyanidins are not absorbed (Figure 20). The majority of them pass unaltered through the small intestine, and they are then metabolised by the colonic microflora to yield a number of simple phenolic acids.[159] However, procyanidin dimers and trimers have been detected in rat urine[160] and plasma[161] following administration of grape seed procyanidins. Additionally, oligomers (up to pentamer size) were detected in rat plasma following administration of a procyanidin extract from apples.[162] Some *in vitro* assays that mimic gastrointestinal conditions demonstrated the degradation of

procyanidin oligomers to yield bioavailable monomers.[163] However, *in vivo* studies rejected the notion that procyanidins contribute to the pool of circulating flavanols via their breakdown into monomers in rats[160,164] and humans.[165] The detection of dimeric procyanidins in human plasma has been reported in some studies.[166–168]

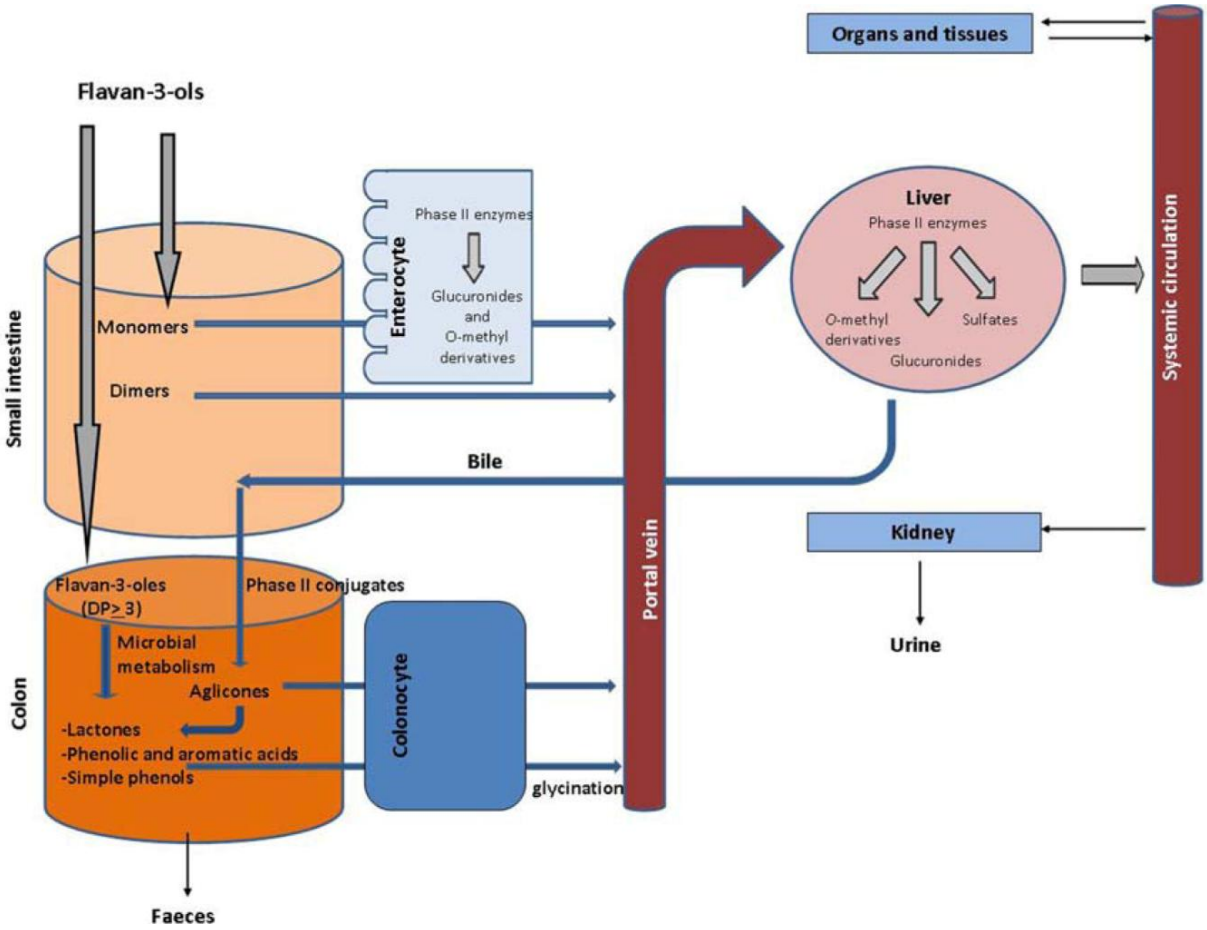


Figure 20. Schematic diagram of organs, reactions and agents involved in the bioavailability of flavan-3-ols, as an example of flavonoid absorption and metabolism.[142]

2.1.5. Beneficial effects

Flavan-3-ols and, concretely, procyanidins exert a protective effect against cardiovascular diseases (reviewed in [143]), and they can act as antioxidant,[169,170] anti-inflammatory,[171–173] antigenotoxic,[174] and anti-carcinogenic[175] molecules. Additionally, they can improve lipid homeostasis.[176] These effects are due to the fact that procyanidins can scavenge free radicals, complex with metal ions, and interact with proteins[177] critical for intracellular signalling cascades.[178] These properties of procyanidins lead to the modulation of protein function or to modifications in gene expression.[179]

The beneficial effects of procyanidins under glucose homeostasis-disrupted conditions have also been investigated. In a situation of T1DM, in which little or no insulin is present, procyanidins seem to have insulin-mimetic effects, likely targeting the liver and peripheral tissues. However, this effect is not reproduced under physiological conditions. In situations of insulin resistance,

procyanidins prevent damage in fructose-fed animals by limiting hyperglycaemia. Nevertheless, the use of high-fat diet or genetically obese animal models have produced controversial results (reviewed in [180]).

In a study conducted by the Nutrigenomics group, the administration of a procyanidin extract from grape seeds reduced plasma insulin levels in rats that were fed a cafeteria diet. Procyanidins may act on adipocytes to stimulate glucose uptake,[181] but they may also act on the pancreas to improve β-cell function.[182] However, it is unclear if procyanidins could act on the pancreas to modulate β-cell mass.

2.2. Flavonoid effects on proliferation and apoptosis

2.2.1. Effects of flavonoids on β-cell apoptosis

The effects of procyanidins on β-cell apoptosis have not been studied. However, studies of some catechins and other flavonoids in β-cells and pancreatic islets have reported anti-apoptotic or pro-apoptotic effects depending on the compound, the concentration, and the situation in which they were tested. Moreover, the mechanism by which they exert their effects on apoptosis also depends on the treatment.

Concerning *in vitro* studies, genistein reduced NaF-induced apoptosis in rat and human pancreatic islets and in the β-cell line RINm5F at low doses, whereas high doses enhanced apoptosis.[183] EGCG also reduced apoptosis induced by hypoxia-reperfusion.[184]

Oxidative stress is important for the progression of diabetes, and some flavonoids have been reported to possess antioxidant activity in β-cells by increasing the activity of antioxidant enzymes. Accordingly, puerarin[185] and anthocyanins[186] reduced apoptosis that was induced in rodent islets and β-cells by H_2O_2. Furthermore, catechin 7-O-β-D glucopyranoside reduced streptozotocin (STZ)-induced cell damage,[187] and kaempferol protected HIT-T15 cells from 2-deoxy-D-ribose, which produces ROS.[188]

Cytokine-induced cell death in β-cell lines and in rodent and human islets plays an important role in diabetes, and it can be prevented by EGCG,[189–191] silymarin,[192] kazinol U,[193] genistein,[194] sulfuretin,[195] quercetin, naringenin,[196] and by flavonoid-rich extracts.[197,198] The main proposed mechanism of cytoprotection is the inhibition of NF-κB activity. However, the suppression of the ERK and JNK signal transducer and activator of transcription (STAT) pathways, the activation of the PI3K pathway, and the induction of the mitochondrial pathway via the reduction in the translocation of BAX to the mitochondria, the loss of MMP, and release of cytochrome c may also play substantial roles.

Glucolipotoxicity is another mechanism of apoptosis in T2DM. The flavonol kaempferol promotes viability, inhibits apoptosis and reduces caspase-3 activity in INS-1E cells and human islets chronically exposed to palmitate. These actions by kaempferol prevent the lipotoxicity-induced down-regulation of anti-apoptotic proteins AKT and Bcl-2.[199] Glyceollins, which are found in soybeans, also decrease palmitate-induced apoptosis in MIN6 cells by attenuating ER stress.[200] Similarly, gallic acid protects RINm5F cells from glucolipotoxicity, reducing apoptosis that is

induced by high glucose, palmitate or a combination of both.[201] Finally, genistein-treated human β-cells inhibited glucose-induced apoptosis.[202]

In the absence of damage, flavonoids normally have no effect on β-cell or islet viability. However, resveratrol (a stilbene) demonstrated pro-apoptotic and antiproliferative effects in the insulinoma cell line INS-1E at concentrations higher than 50 μM; these effects coincided with activation of caspases and inhibition of AKT phosphorylation.[203]

In vivo flavonoid studies are scarce. However, quercetin[204] and anthocyanins have been shown to protect pancreatic tissue from STZ-induced apoptosis; protection though to be mediated by the regulation of caspase-3, BAX, and Bcl-2 proteins.[205] Conversely, the treatment of STZ-induced diabetic rats with EGCG further impaired β-cell loss, suggesting that EGCG acts as a pro-oxidant.[206]

2.2.2. Effects of flavonoids on β-cell proliferation

There are few studies concerning the effects of flavonoids on pancreatic β-cell proliferation, and the effects of procyanidins in this process have not been reported.[138]

In vitro, under physiological conditions, genistein inhibits the proliferation of cultured islet cells.[207] In contrast, the same isoflavone enhances the proliferation of INS-1 cells and human islets by activating the ERK signalling pathway and increasing the expression of cyclin D1.[208] Moreover, an anthocyanidin-rich blueberry extract increased the proliferation of β TC-tet cells.[209] Additionally, genistein enhanced the proliferation of rat islets after alloxan injury,[210] and it rescued the glucose-induced reduction in proliferation of human β-cells.[202] Furthermore, chrysin, quercetin, catechin, and caffeic acid protect β-cells from oxidative-stress-induced inhibition of proliferation.[211]

In vivo, quercetin increased the number of pancreatic islet cells in both STZ-diabetic rats and non-diabetic rats.[212] In another study, quercetin was proposed to improve pancreatic functions in STZ-induced diabetic mice by enabling cell proliferation via the inhibition of $p21^{CIP1}$ expression.[213] Genistein increased β-cell proliferation, survival, and mass in STZ-induced diabetic mice[208] and in mice with nongenetic T2DM, which was induced with a high fat diet and a low dose of STZ.[214]

Taking into account all of these studies analysing the effects of flavonoids on proliferation and apoptosis in β-cells, flavonoids generally exert anti-apoptotic and proliferative effects.

2.2.3. Effects of flavonoids on proliferation and apoptosis in other cell types

Additional studies have investigated the effects of flavonoids on apoptosis and proliferation processes in other cell types. For example, genistein[215] and quercetin[216] can inhibit cell proliferation in adipocytes[215] and osteoblasts[216].

The effects of procyanidins, which have not been characterised in β-cells, have been studied in other cell types. A grape seed procyanidin extract (GSPE) inhibited adipogenesis and proliferation in the 3T3-L1 cell line,[217] and oligomeric procyanidins from apple inhibited cell

migration and proliferation in human umbilical vein endothelial cells (HUVEC).[218] Similarly, polyphenolic fractions of grape extracts exerted antiproliferative effects in mouse hepatoma Hepa-1c1c7 cells, being the fractions that contained higher molecular weight molecules which had the greatest influence on cell cycle progression and apoptosis.[219] Oligomeric procyanidins from areca nuts induced apoptosis in isolated mice lymphocytes, whereas monomers and oligomers up to tetramers were inactive.[220] The treatment of chick cardiomyocytes with high doses of GSPE (500 mg/L) increased apoptotic cell death by increasing NO production and by depleting intracellular glutathione.[221] Conversely, the catechin monomer and the procyanidin B4 dimer induced the expression of cellular antioxidant enzymes in a dose-dependent manner in cardiac H9C2 cells, which lead to a reduction in ROS accumulation and apoptosis.[222]

Procyanidins may also serve as potential therapeutic tools for protecting multiple target organs from assaults by drugs, environmental agents, and chemicals (summarized in Tables 5 and 6).[223]

Table 5. Summary of published *in vitro* procyanidin effects against toxic assaults.

Cell type	Toxic assault	Treatment	Effect	Reference
Isolated rat hepatocytes	Ethanol (10 mL/L) or Carbon tetrachloride (10 mM)	Grape procyanidins (50 and 100 mg/L)	Decrease in apoptosis and stimulation of cell growth	224
Isolated human oral keratinocytes	Smokeless tobacco extract (300 mg/mL)	GSPE (100 mg/L)	Decrease in apoptosis, lipid peroxidation and cytochrome c reduction	225
Chang epithelial cells	Idarubicin (30 nM) or 4-hydroxyperoxy-cyclophosphamide (1 mg/L)	GSPE (25 mg/L)	Decrease in apoptosis via the up-regulation of Bcl-2 and down-regulation of p53 and c-myc	226
Rat pheochromocytoma cells (PC12)	4-hydroxynonenal (20 μM)	Cocoa procyanidin extract (5-10 mg/L) and dimeric procyanidin B2 (10-20 μM)	Decrease in apoptosis via inhibition of the JKN pathway and ROS accumulation	227
PC12 cells	H_2O_2 (200 μM)	Cocoa procyanidin extract (1-5 mg/L) and dimeric procyanidin B2 (1-5 μM)	Decrease in apoptosis by inhibiting the down-regulation of Bcl-xL and Bcl-2 through blocking the JNK and p38 MAPK pathways	228
Caco-2 cells	Acrylamide (5 mM)	Cocoa procyanidin extract (10 mg/L) and dimeric procyanidin B2 (10 μM)	Inhibition of apoptosis by blocking the JNK pathway and preventing oxidative stress	229
HUVEC cells	Advanced glycation end products (AGE) (200 mg/L)	Dimeric procyanidin B2 and resveratrol (10 μM)	Decrease in apoptosis via inhibition of the mitochondrial apoptosis pathway	230
Human aortic smooth muscle cells	AGE (200 mg/L)	Dimeric procyanidin B2 (2.5-10 μM)	Inhibition of AGE-induced proliferation and migration	231
Renal tubular LLC-PK_1 cells	3-morpho-linosydnonimine (800 μM)	Wen-Pi-Tang extract rich in catechin and fractions (5-50 mg/L)	Increase in viability via inhibition of peroxynitrite formation	232

Table 6. Summary of published *in vivo* procyanidin effects against toxic assaults.

Organ	Toxic assault	Treatment	Effect	Reference
Rat kidney	Mercuric chloride (2.4 mg/kg of bw)	Procyanidins (450 mg/kg bw)	Reduction in ROS generation, enhancement of antioxidant enzyme expression and a decrease in apoptosis	233
Rat testes	Nickel sulfate (1.25-5 mg/kg)	GSPE (50 and 100 mg/kg)	Enhancement of sperm motility and offsetting of apoptosis (down-regulation of BAX) and oxidative stress	234
Mouse liver	Acetaminophen (400-500 mg/kg)	GSPE (100 mg/kg bw)	Inhibition of apoptotic liver cell death via inhibition of Bcl-xL phosphorylation	235
Mouse lung	Amidarone (AMI) (50 mg/kg)	GSPE (100 mg/kg bw)	Counteraction of AMI-induced DNA fragmentation	236
Mouse liver and brain	12-O-tetradecan-oylphorbol-13-acetate (0.1 mg)	GSPE (25-100 mg/kg bw)	Decreased DNA fragmentation, ROS production and lipid peroxidation	237
Mouse spleen	Dimethylnitrosa-mine (10 mg/kg)	GSPE (100 mg/kg)	Inhibition of DNA fragmentation and apoptosis	238

Studies of flavonoid effects on proliferation and apoptosis are mainly conducted in cancer cell lines, where these processes are markedly dysregulated. Flavonoids are consumed in the everyday diet and can be useful as chemopreventive or chemotherapeutic agents.[175] Procyanidins are also considered to be anti-carcinogenic and anti-mutagenic. Their chemopreventive activities include reduced proliferation, increased apoptosis and cell cycle arrest, and their main molecular targets are NF-κB, MAPK, PI3K/AKT, caspases, cytokines, angiogenic molecules, and cell cycle regulatory proteins (Figure 21).[175,239]

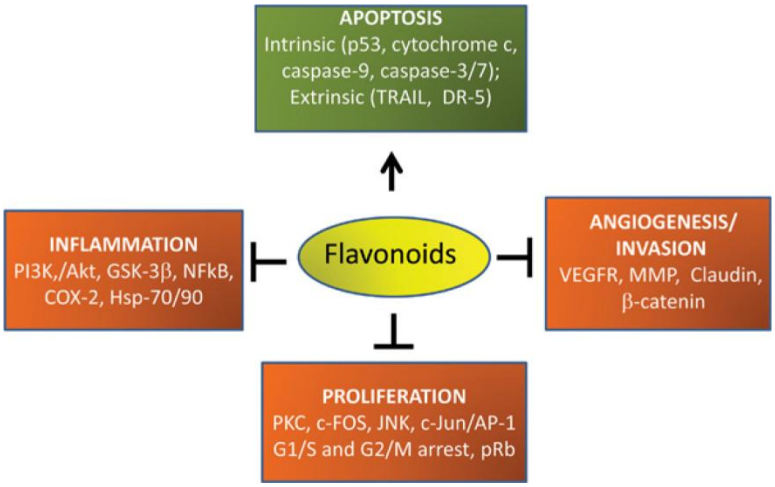


Figure 21. Molecular targets of flavonoids in cancer prevention. Flavonoids repress molecular targets that stimulate proliferation, inflammation, invasion/metastasis, and angiogenesis. They also induce pro-apoptotic pathways.[239]

An inverse association between dietary proanthocyanidin intake and the risk of gastric,[240] colorectal,[241] lung,[242] and pancreatic[243,244] cancer has been reported. Moreover, procyanidins derived from grape seed, cocoa, apple, Japanese quince fruit, and hop inhibit growth and apoptosis in colon cancer cells.[245–250] Grape seed procyanidins have also been reported to arrest the cell cycle and to induce apoptosis in human prostate cancer cells,[251] oral cancer cells,[252] and pancreatic adenocarcinoma cells.[253]

The procyanidin extract fractions containing the highest percentage of galloylation and degree of polymerization have the strongest antiproliferative activity and pro-apoptotic effects in breast cancer cells,[140] prostate carcinoma cells,[254] colon cancer cells,[248,255,256] and other human cancer cells.[257]

Gallic acid has also been identified as a major active constituent in GSPE, and it inhibits growth and induces apoptosis in prostate carcinoma cells.[258] This phenolic acid inhibits proliferation and increases apoptosis in lung cancer,[259] prostate cancer,[260] and pancreatic adenocarcinoma cells.[261] This effect has also been observed in leukaemia cells treated with other dietary phenolic acids.[262]

In conclusion, the procyanidin effects on proliferation and apoptosis have been widely studied in several cell types, though mainly in cancer cells. They exert pro-apoptotic and antiproliferative effects. However, to our knowledge, they have not been studied in pancreatic β-cells.

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II. HYPOTHESIS AND OBJECTIVES

HIPÒTESI I OBJECTIUS

Phytochemicals are plant secondary metabolites that are not essential, but they may improve human health. Polyphenols form one of the largest groups of phytochemicals, and flavonoids are the largest group of phenolic compounds. Flavonoids are present in a wide range of fruits and vegetables. Among flavonoids, procyanidins are one of the most abundant subclasses, and they are oligomeric forms of flavan-3-ols. The Nutrigenomics research group is focused on studying the beneficial effects of procyanidins from grape seed extracts. Previous studies have reported that procyanidins provide several benefits via their anti-inflammatory and antioxidant properties, and these molecules are involved in lipid and glucose homeostasis.

In a previous study conducted by our research group, the effects of a grape seed procyanidin extract (GSPE) were evaluated in an insulin resistance model induced by feeding female rats with a cafeteria diet. There was a reduction in the HOMA-IR index and in plasma insulin levels, suggesting improved insulin resistance in the peripheral tissues. Moreover, these results indicated that procyanidins could potentially target the pancreas, the main organ responsible for nutrient homeostasis, and improving β-cell functionallity or β-cell mass. In fact, this doctoral thesis has been conducted in parallel with another doctoral thesis (Castell-Auví, A. The Effects of Grape Seed Procyanidin Extract on Insulin Synthesis and Secretion. 2012), which focused on the effects of GSPE on β-cell functionality. These studies demonstrated that procyanidins act on the pancreas to modulate insulin synthesis, secretion and degradation.

Type 2 diabetes mellitus is characterised by hyperglycaemia and altered lipid metabolism, which is accompanied by peripheral insulin resistance, pancreatic β-cell dysfunction, and decreased β-cell mass. Although this condition involves genetic factors, it is also tightly linked to obesity. Both obesity and type 2 diabetes are becoming worldwide epidemics. During the first stages of insulin resistance, β-cell mass increases to compensate for hyperglycaemia. However, when β-cell mass is unable to compensate for the increased insulin demand, there is a decrease in β-cell mass due to an increase in β-cell apoptosis.

Additionally, pancreatic adenocarcinoma is one of the most aggressive cancers and is highly resistant to treatment. This cancer progresses through the accumulation of genetic alterations, which results in increased cell growth and proliferation and decreased apoptosis.

Thus, understanding these natural compounds and their beneficial effects on proliferation and apoptosis of pancreatic cells is of great importance. These two processes are tightly linked and are often altered in these diseases.

Procyanidin effects on proliferation and apoptosis have been widely studied in several cell types. In cancerous cell lines, procyanidins decrease proliferation and increase apoptosis, acting as anti-carcinogenic agents. In other cell types, they act as a therapeutic tool and protect the cell from environmentally- or chemically-induced damage, decreasing apoptosis and stimulating cell growth. However, there is little information concerning the effectsof procyanidins on the pancreas.

Therefore, taking all of the data presented above into consideration, our **hypothesis** was the following:

> **Procyanidins can modulate proliferation and apoptosis processes in pancreatic cells.**

The main **objectives** proposed to prove this hypothesis were:

1. To ascertain whether GSPE modulates β-cell proliferation and apoptosis.

2. To assess whether the regulation of these processes by GSPE is dependent on the condition of the experiment. To test this hypothesis, healthy rats and different animal obesity models will be used.

3. To investigate the mechanisms by which GSPE modulates β-cell proliferation and apoptosis.

4. To evaluate whether GSPE modulates proliferation and apoptosis in pancreatic adenocarcinoma cells.

5. To identify the extract components that are responsible for the proliferation and apoptosis effects.

The work presented in this thesis was performed in the Nutrigenomics group at the University Rovira i Virgili, together with 3 month-stay at the Laboratoire of Biologie et Pathologie du Pancréas Endocrine in the University Paris-Diderot, to which I received a grant from the Generalitat de Catalunya. Funding for the first 3 months was provided by the Universitat Rovira i Virgili. After the initial 3 months, I received the FI fellowship from the Generalitat de Catalunya, which funded the rest of my thesis work.

Els fitoquímics són metabòlits secundaris de les plantes, els quals, tot i no ser compostos essencials, poden aportar una millora de la salut. Els polifenols formen part d'un dels grups més grans de fitoquímics i, a la vegada, els flavonoids són el grup de compostos fenòlics més nombrós, presents en un gran ventall de fruites i vegetals. Entre els flavonoids, una de les subclasses més abundants són les procianidines, que són formes oligomèriques de flavan-3-ols. El grup de recerca de Nutrigenòmica es centra, en part, en l'estudi dels efectes beneficiosos de les procianidines de pinyol de raïm, de les quals s'ha trobat que tenen efectes beneficiosos, incloent propietats antiinflamatòries i antioxidants, i que poden participar en l'homeòstasi dels lípids i la glucosa.

En un estudi previ realitzat en el nostre grup de recerca, es van avaluar els efectes d'un extracte de procianidines de pinyol de raïm (GSPE) en un model de resistència a la insulina induït per l'alimentació de rates femelles amb una dieta de cafeteria. Es va veure que GSPE reduïa l'índex HOMA-IR i els nivell d'insulina plasmàtica, suggerint una millora de la resistència a la insulina en teixits perifèrics. A més a més, aquests resultats semblaven indicar que les procianidines podrien estar afectant el pàncrees, el principal òrgan responsable de l'homeòstasi dels nutrients, ja sigui modificant la funcionalitat o la massa de les cèl·lules β pancreàtiques. De fet, aquesta tesi doctoral s'ha dut a terme en paral·lel amb una altra (Castell-Auví, A. The Effects of Grape Seed Procyanidin Extract on Insulin Synthesis and Secretion. 2012), centrada en l'estudi de l'efecte del GSPE en la funcionalitat de les cèl·lules β, en la qual es va concloure que les procianidines actuen en el pàncrees modulant la síntesi, secreció i degradació de la insulina.

Els individus amb diabetis del tipus 2 presenten hiperglucèmia i un metabolisme lipídic alterat, juntament amb resistència a la insulina, disfunció de les cèl·lules β i disminució de la massa β. Tot i que determinats factors genètics hi estan implicats, la diabetis del tipus 2 està estretament lligada a l'obesitat, i ambdós patologies estan assolint proporcions d'epidèmia a nivell mundial. En els primers estadis de la resistència a la insulina, la massa β s'incrementa per compensar la hiperglucèmia. Tot i així, quan les cèl·lules β ja no són capaces de compensar l'augment de la demanda d'insulina, la massa β es veu reduïda degut a un augment de l'apoptosi.

A més a més, considerant el pàncrees, l'adenocarcinoma pancreàtic és un dels càncers més agressius, caracteritzat per una elevada resistència al tractament. L'acumulació d'alteracions genètiques resulta en un augment del creixement cel·lular i de la proliferació i en una inhibició de l'apoptosi.

D'aquesta manera, l'obtenció d'informació sobre els compostos naturals amb efectes beneficiosos sobre la proliferació i l'apoptosi en les cèl·lules pancreàtiques, processos estretament lligats i alterats en les malalties mencionades anteriorment, és de gran interès.

Els efectes de les procianidines sobre la proliferació i l'apoptosi han estat molt estudiats en diferents tipus cel·lulars. En línies cel·lulars de càncer, les procianidines baixen els nivells de proliferació i incrementen l'apoptosi, actuant com a anticarcinogèniques. En altres tipus cel·lulars, les procianidines actuen com a eina terapèutica, protegint les cèl·lules del dany induït per factors ambientals o químics, disminuint l'apoptosi i estimulant el creixement cel·lular. Tot i així, existeix poca informació relativa als efectes de les procianidines en el pàncrees.

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Per tant, tenint en compte tota la informació presentada anteriorment, la nostra **hipòtesi** era:

> **Les procianidines poden modular la proliferació i l'apoptosi en les cèl·lules pancreàtiques.**

Els principals **objectius** que es van proposar per provar aquesta hipòtesi van ser:

1. Comprovar si el GSPE modula la proliferació i l'apoptosi en les cèl·lules β pancreàtiques.

2. Avaluar si la modulació d'aquests processos pel GSPE és depenent de les condicions experimentals, usant rates sanes o diferents models animals d'obesitat.

3. Investigar els mecanismes pels quals el GSPE modula la proliferació i l'apoptosi en les cèl·lules β pancreàtiques.

4. Avaluar si el GSPE modula la proliferació i l'apoptosi en cèl·lules d'adenocarcinoma pancreàtic.

5. Identificar els components de l'extracte responsables dels efectes del GSPE observats sobre la proliferació i l'apoptosi.

El treball presentat en aquesta tesi doctoral es va realitzar en el grup de Nutrigenòmica de la Universitat Rovira i Virgili, juntament amb una estada de 3 mesos al laboratori de Biologie et Pathologie du Pancréas Endocrine a la Universitat Paris-Diderot, per la qual vaig rebre una beca de viatge de la Generalitat de Catalunya. Durant els primers tres mesos de tesi, el finançament va provenir de la Universitat Rovira i Virgili. Després, vaig rebre una beca FI de la Generalitat de Catalunya que va cobrir la resta de la tesi doctoral.

III. RESULTS

1. Grape seed procyanidin extract modulates proliferation and apoptosis of pancreatic beta-cells

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In an attempt to determine the effects of GSPE on proliferation and apoptosis in pancreatic β-cells, the INS-1E cell line was used as an *in vitro* model. In *in vitro* studies of β-cells, the use of primary cells is preferable. However, this requires large quantities of isolated pancreatic islets, and a mixed population of other islet cell-types is generally obtained.[1] Alternatively, rodent β-cell lines have proven to be useful until clonal human β-cells became available.[1] In fact, only recently has a suitable human β-cell line been developed, named EndoC-βH1.[2] The INS-1 cell line was isolated from a radiation-induced rat insulinoma, but these cells are heterogeneous and not stable due to their nonclonal nature. In contrast, INS-1E cells, derived from the INS-1 cell line, are stable. These cells also represent a valuable β-cell model based on their insulin content and their secretory response to glucose.[1]

To determine conditions allowing GSPE to modulate proliferation and apoptosis in INS-1E cells, the effects of this extract were tested under normal culture conditions as well as conditions mimicking a pro-diabetic state. This pro-diabetic state was achieved by treating the cells with high concentrations of glucose, insulin and palmitate. In addition, the components of the extract responsible for the proliferation effects were identified. These components were identified by treating INS-1E cells with pure monomeric catechins, and dimers and trimers of procyanidins; fractions of the extract enriched for monomers, dimers or procyanidin oligomers were also used in these studies.

Finally, the effects observed *in vitro* were investigated *in vivo*, using the cafeteria experiment, which had previously demonstrated that GSPE causes a reduction in plasma levels of insulin.

(1) Merglen, A. et al. Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology.* **2004**, *145* (2), 667–678.

(2) Ravassard, P. et al. A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. *J. Clin. Invest.* **2011**, *121* (9), 3589–3597.

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Grape seed procyanidin extract modulates proliferation and apoptosis of pancreatic beta-cells

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ABSTRACT

Grape seed procyanidin extract (GSPE) modulates glucose homeostasis and insulinemia in several animal models. Under pathological conditions, insulin levels are dependent on pancreatic beta-cell functionality, as well as on the beta-cell mass expansion or apoptosis in the pancreas. In this study, we analysed the effects of GSPE on modulating apoptosis and proliferation in beta-cells. We tested the effects of GSPE in the INS-1E pancreatic beta-cell line, either under basal or altered conditions with high glucose, insulin or palmitate levels. GSPE enhanced the pro-apoptotic effect of high glucose and showed clear antiproliferative effects under high glucose, insulin and palmitate conditions. These antiproliferative effects are likely due to high molecular weight compounds contained in the extract. GSPE also modulated pro- and anti-apoptotic markers in the pancreas of rats fed a cafeteria diet, with the effect depending on the dose of GSPE and duration of treatment. Thus, GSPE is able to modulate apoptosis and proliferation of beta-cells under altered, but not basal, conditions.

1. Introduction

Procyanidins are the most abundant polyphenols found in foods such as grapes and red wine. They provide several benefits; namely anti-inflammatory and antioxidant properties and protection against cardiovascular disease (Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005). Furthermore, they have been shown to participate in glucose homeostasis although their effects are not fully understood (Pinent, Cedó, Montagut, Blay, & Ardévol, 2011). Type 2 diabetes mellitus is a disorder characterised by hyperglycemia, impaired insulin action in peripheral tissues, deficiency in the β-cell insulin-secretory response to glucose and altered lipid metabolism. Given the high prevalence of the disease, obtaining knowledge about natural compounds with potential beneficial effects on glucose homeostasis is of great interest.

In a previous study, we analysed the effect of procyanidins in an insulin resistance model induced by feeding female rats a cafeteria diet, and found a reduction in the Homeostatic model assessment for insulin resistance (HOMA-IR) index suggesting improved insulin resistance (Montagut et al., 2010). In that experiment, rats fed with 13 weeks on cafeteria diet were supplemented for either 10 or 30 days with grape seed procyanidin extract (GSPE). We found that GSPE treatment reduced plasma insulin levels (Montagut et al., 2010), suggesting that procyanidins might target the pancreas, the main organ responsible for insulin production.

We have previously shown that grape seed procyanidins can modulate insulin secretion in isolated Wistar rat islets and pancreatic beta-cell lines (Castell-Auví et al., 2012). However, changes in beta-cell insulin production may also be due to variations in the number of insulin-producing cells. Under insulin resistance conditions, the beta-cell mass increases in response to heightened insulin demand (Ackermann & Gannon, 2007), and an imbalance between beta-cell proliferation and apoptosis accompanies the development of conditions leading to type 2 diabetes mellitus (Butler et al., 2003; Wajchenberg, 2007). Procyanidins interfere with apoptotic processes, but their effects differ depending on the cell type. It has been suggested that grape seed procyanidins act as chemopreventives, inducing apoptosis and inhibiting cell proliferation in cancerous cell lines (Lin, Chen, Liu, & Nieh, 2012; Lizarraga et al., 2007). Other procyanidins and the corresponding dimers can serve pro-apoptotic roles in cancerous cell lines (Actis-Goretta, Romanczyk, Rodriguez, Kwik-Uribe, & Keen, 2008; Chung, Miranda, Stevens, & Maier, 2009) and anti-apoptotic roles in other cell lines. Grape procyanidins inhibited ethanol- and carbon tetrachloride-induced damage in rat hepatocytes (Zhong, Cong, & Zhang, 2007) and protected against cardiac cell apoptosis via the induction of endogenous antioxidant enzymes (Du, Guo, & Lou, 2007). Taken together, these results show that procyanidins modulate apoptosis and proliferation in several cell types; however, there is little information concerning their effects on beta-cells.

Considering that GSPE modulates apoptosis and proliferation in several cell types and given the importance of these processes in

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the development of insulin resistance and diabetes, this study was conducted to determine the effects of procyanidins on the apoptosis and proliferation of beta-cells using the INS-1E cell line. Furthermore, we studied the effects of GSPE on markers of these processes in the pancreas of rats with cafeteria-induced obesity.

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%). Fractions II, VI and X were obtained by size-exclusion chromatography of GSPE, for which 11 major polymeric fractions were identified. Fraction II mainly contains monomeric structures, fraction VI contains procyanidin dimers, and fraction X contains oligomeric structures. These fractions were vacuum dried and kept at −20 °C for subsequent biological studies. The monomeric procyanidins catechin, epicatechin and epicatechin gallate (ECG) and the dimeric procyanidin B2 were purchased from Sigma–Aldrich (Madrid, Spain). Procyanidin C1 was purchased from Transmit (Giessen, Germany). A stock solution of sodium palmitate (4 mM) was prepared by dissolving sodium palmitate (Sigma–Aldrich, Madrid, Spain) in 12.5% (w/v) fatty acid-free BSA (Sigma–Aldrich, Madrid, Spain).

2.2. Cell culture and treatment

Rat insulinoma INS-1E cells were kindly provided by Prof. Pierre Maechler, University of Geneva (Merglen et al., 2004), and were cultured in RPMI 1640 supplemented with 5% foetal 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. Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium).

The effects of treating cells with 25 mg/L GSPE for 24 h were determined in different conditions. The first was a basal condition, for which cells were maintained in culture medium. Second was a high glucose treatment, for which cells were incubated with 25 mM glucose and GSPE for 24 h. In proliferation assays, cells were previously depleted of serum (and maintained in 0.1% BSA) overnight (o/n). For the third condition, high insulin treatment, cells were incubated for 24 h in conditions of serum depletion (0.1% BSA) and low glucose (5 mM), then treated for 24 h with 20 or 200 nM insulin (Novo Nordisk Pharma SA, Madrid, Spain) and GSPE under the same conditions of depletion. An analogous experiment was carried out using GSPE fractions and monomeric procyanidins. The fourth condition was high palmitate treatment, for which cells were incubated with 0.2 or 0.4 mM palmitate for 24 h in serum-depleted medium with GSPE, and then for an additional 24 h with palmitate only.

2.3. Animal experimental procedures

The animals were treated as previously described (Montagut et al., 2010). Briefly, Wistar female rats were divided in two groups, a control group fed a standard diet (Panlab A03) and a cafeteria group fed a cafeteria diet in addition to standard chow and water. The latter group was fed a cafeteria diet for 13 weeks to induce obesity. Afterwards, it was divided into three subgroups: (A) a cafeteria control group of rats treated with vehicle (sweetened condensed milk), (B) a cafeteria + GSPE 25 group of rats treated with 25 mg of GSPE/kg of body weight (bw) per day, and (C) a cafeteria + GSPE 50 group of rats treated with 50 mg of GSPE/kg of bw per day. After 10 days of GSPE treatment, the first six animals from each group were sacrificed (deemed "short treatment"), and after 30 days, the remaining six (long treatment). After 3 h of fasting, the animals were sacrificed by beheading, and their pancreases were excised, frozen immediately in liquid nitrogen, and stored at −80 °C until analysis. All the procedures were approved by the Experimental Animals Ethics Committee of the Universitat Rovira i Virgili.

2.4. Apoptosis assay and caspase 3/7 activity

INS-1E cells cultured in 96-well plates were treated as previously described. At the end of the treatment, cells were lysed and 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 activities of Caspase-3 and Caspase-7 were measured by luminescence, using the Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to the manufacturer's instructions.

2.5. Proliferation assay

INS-1E cells cultured in 96-well plates were treated as previously described. On the last day of the treatment, bromodeoxyuridine (BrdU) was added to each well and incubated o/n. At the end of the treatment, proliferation (quantified as incorporation of BrdU during DNA synthesis) was measured using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.6. Quantitative Real Time PCR

Total RNA from the pancreas was extracted using TRYzol LS Reagent (Invitrogen, Carlsbod, CA) following the manufacturer's instructions. Total RNA from INS-1E cells was isolated using RNeasy Mini Kit (Qiagen, Barcelona, Spain). cDNA from all the experiments was generated with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and was subjected to quantitative Real Time PCR amplification using the TaqMan Master Mix (Applied Biosystems, Madrid, Spain). Specific TaqMan probes (Applied Biosystems, Madrid, Spain) were used for each gene: Rn99999125_m1 for *Bcl2*, Rn01480160_g1 for *Bax*, Rn01492401_m1 for *Ccnd2* (Cyclin D2) and Rn01451446_m1 for *Mki67*. *Actb* was used as the reference gene in INS-1E cells (Rn00667869_m1) and *Ppia* as the reference in animal experiments (Rn00690933_m1). Reactions were run on a quantitative RT-PCR 7300 System (Applied Biosystems, Madrid, Spain) according to the manufacturer's instructions. The relative mRNA expression levels were calculated using the ΔΔCt method.

2.7. Western blot

The protein levels of Bax, Bcl-2 and Cleaved Caspase-3 were quantified by Western Blot as previously described (Castell-Auví et al., 2011) using rabbit anti-β-actin antibody (Sigma–Aldrich, Madrid, Spain) as a reference. Primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). For pancreas lysates, 100 μg of protein was loaded to the gel and the following antibody dilutions used: 1:1000 for Bax, 1:1500 for Bcl-2, 1:750 for Cleaved Caspase-3 and 1:750 for β-actin. For INS-1E lysates, 20–25 μg of protein was loaded and the following antibody dilutions used: 1:1500 for Bax and 1:750 for β-actin. After incubation with peroxidise-conjugated monoclonal anti-rabbit secondary antibody (Sigma–Aldrich, Madrid, Spain) at a 1:10,000 dilution, immunoreactive proteins were visualised with the ECL Plus Wes-

tern Blotting Detection System (GE Healthcare, Buckinghamshire, UK) using an Alpha Innotech system and software 6.0.2v (San Leonardo, CA). Densitometric analysis of the immunoblots was performed using ImageJ 1.44p software, with all proteins quantified relative to the loading control.

2.8. 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 (version 19, SPSS, Inc., and IBM).

3. Results

3.1. GSPE enhances the pro-apoptotic effects of glucose but not those of palmitate in INS-1E beta-cell line

Pancreatic damage under conditions of insulin resistance leads to diabetes and has been attributed to several physiological triggers, such as high glucose concentrations (glucotoxicity), and elevated fatty acids (lipotoxicity). To determine whether GSPE can modulate the beta-cell apoptosis in a diseased state, we simulated these conditions in the beta-cell line INS-1E. High glucose concentrations have been shown to induce apoptosis in pancreatic beta-cells (Efanova et al., 1998; Joe et al., 2008; Kim et al., 2005). In agreement with this finding, we observed significantly increased apoptosis after 24 h of treatment with 25 mM glucose compared to the optimal glucose concentration for INS-1E cell growth (11 mM), as well as down-regulation of gene expression of the anti-apoptotic marker *Bcl2*, increased Bax protein expression and increased Caspase 3/7 activity (Table 1). A 24 h-GSPE treatment simultaneous to the high glucose treatment significantly increased the apoptosis and the activity of the Caspase 3/7, compared to the high glucose and vehicle-treated cells (Fig. 1). This increase in apoptosis was accompanied by down-regulated *Bcl2* gene expression, although the protein expression of the apoptotic marker Bax was also down-regulated by GSPE (Fig. 1). Remarkably, GSPE alone under basal glucose conditions (11 mM) neither induces apoptosis nor modifies any of the apoptosis-related parameters (last column in Table 1).

Table 1
Effects of 24-h treatment with high glucose (25 mM) or GSPE (25 mg/L) compared to the basal condition (11 mM glucose, vehicle-treated). Data are normalised with respect to the basal condition and is shown as the mean ± SEM.

	Basal	High glucose	Basal + GSPE
Apoptosis	1.00 ± 0.0	1.26 ± 0.1*	1.00 ± 0.1
Bcl2 mRNA	0.99 ± 0.0	0.72 ± 0.0*	0.94 ± 0.1
Caspase 3/7 activity	1.00 ± 0.1	1.58 ± 0.1*	1.1 ± 0.1
Bax protein	1.00 ± 0.1	1.49 ± 0.1*	1.07 ± 0.0

* $p \leqslant 0.05$ versus basal condition.

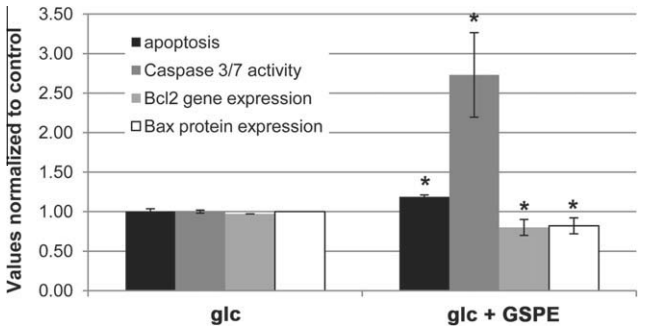

Fig. 1. After 24 h of treatment with 25 mM glucose (glc) and 25 mg/L GSPE, INS-1E cells were assessed for apoptosis (black columns), Caspase 3/7 activity (dark grey columns), *Bcl2* gene expression (light grey columns) and Bax protein expression (white columns) as described in materials and methods. Data are shown as the mean ± SEM. *$p \leqslant 0.05$.

To assay the effects of GSPE with respect to lipotoxicity, we treated cells with the saturated fatty acid palmitate, which has been previously shown to induce apoptosis in pancreatic beta-cells (El-Assaad et al., 2003; Karaskov et al., 2006; Kim et al., 2005). A 48 h treatment with 0.4 mM palmitate increased apoptosis in INS-1E cells (1.84 ± 0.3 versus 1.00 ± 0.0, $p \leqslant 0.05$ palmitate versus control); GSPE added during the first 24 h did not modify the effects of palmitate on apoptosis (1.81 ± 0.2 versus 1.84 ± 0.3, GSPE + palmitate versus palmitate alone).

3.2. GSPE reverts the INS-1E beta-cell proliferation induced by several stimuli

Because insulin resistance is linked to a compensatory increase in beta-cell mass, we next analysed the effects of GSPE on beta-cell proliferation in the INS-1E cell line. In this way, we tested the effects of GSPE under several conditions that affect cell proliferation and that mimic the environment induced upon disruption of glucose homeostasis. Beta-cell proliferation is increased by insulin, a well-known mitogenic stimulus. We first tested the effects of GSPE on proliferation induced by 20 nM insulin for 24 h. Fig. 2A shows that GSPE reduced the insulin-induced proliferative effects on cells and normalised the levels of *Mki67*, which was up-regulated by insulin. GSPE also normalised the gene expression of Cyclin D2, although in this case the effect of insulin was to down-regulate it. When a higher dose (200 nM) of insulin was used to elicit a stronger proliferative effect, we observed that GSPE also reduced the insulin-proliferative effects (Fig. 2A).

We then measured the effects of glucose and GSPE on proliferation under high glucose conditions and found that glucose slightly, but significantly, increased proliferation, measured by BrU incorporation to the cells, and up-regulated the proliferation gene markers Cyclin D2 and Mki67 (Fig. 2B). Twenty five milligram per litre of GSPE concomitant with the high glucose negated the effects of glucose on proliferation and *Mki67* gene expression, and tended to revert Cyclin D2 mRNA towards its basal level (Fig. 2B).

We next tested a condition in which proliferation is inhibited, as might occur in advanced insulin resistance, during which pancreas beta-cell mass starts to decrease. Using palmitate as the antiproliferative agent, we found that 48 h treatment with 0.2 mM palmitate reduced the beta-cell proliferation rate and that GSPE potentiated this reduction (Fig. 2C). In this case, Cyclin D2 gene expression increased in the presence of palmitate and showed no correlation with the presence of GSPE (Fig 2C).

Finally, the effects of 24 h treatment with 25 mg/L GSPE on proliferation were tested under basal conditions in the absence of mitogenic and/or antimitogenic stimuli. We found that GSPE influenced neither BrU incorporation (0.96 ± 0.3 versus 1.00 ± 0.2) nor Cyclin D2 gene expression (0.95 ± 0.1 versus 1.01 ± 0.1).

3.3. The antiproliferative effects of GSPE are due to its high molecular weight forms

Because the studies were performed on whole extracts containing mixtures of different flavonoids, we analysed which component of the mixture was responsible for the measured effects. The proliferation assay with mitogenic insulin was chosen for such study because it had been shown to demonstrate significant changes. Rather than inhibit proliferation, the purified monomers examined herein significantly enhanced it (Table 2). Purified forms of dimer B2 and trimer C1 were also tested. At a dose of 5 mg/L, the former slightly increased the proliferation; the latter showed no

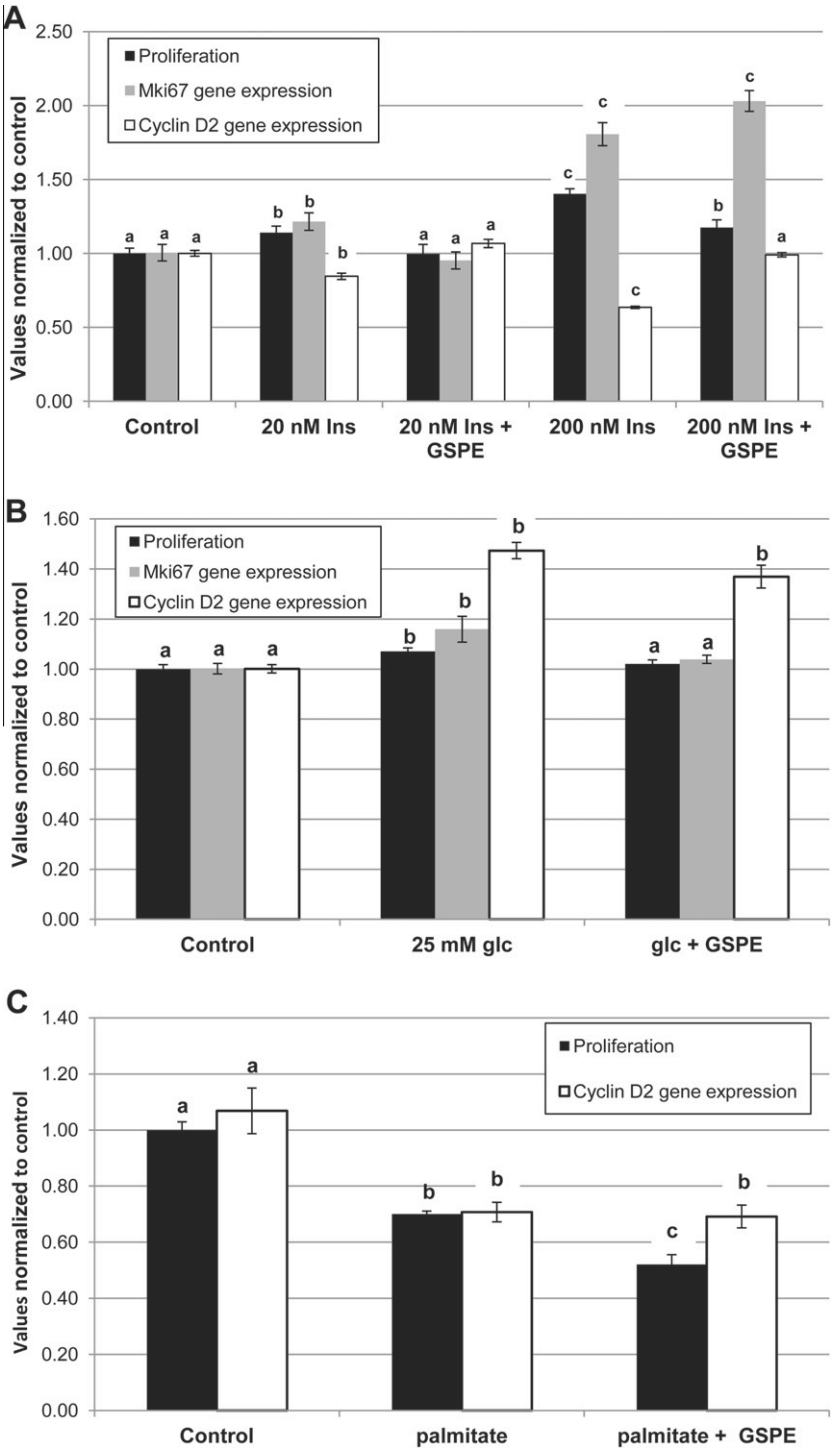

Fig. 2. (A) Treatment of INS-1E cells with 25 mg/L of GSPE and either 20 or 200 nM insulin for 24 h. At the end of the treatment, proliferation (black columns) and mRNA expression of Mki67 (grey columns) and Cyclin D2 (white columns) were tested. B) INS-1E cells were treated with 25 mM glucose (glc) and 25 mg/L of GSPE for 24 h. At the end of the treatment, proliferation (black columns) and mRNA expression of Mki67 (grey columns) and Cyclin D2 (white columns) were assessed. C) INS-1E cells were incubated with 0.2 mM palmitate in serum-free media for 24 h with 25 mg/L of GSPE, followed by a 24 h treatment with palmitate only. At the end of the treatment, proliferation (black) and Cyclin D2 gene expression (white) were quantified. Different letters indicate significantly different groups ($p \leqslant 0.05$).

significant effect at a low concentration, but inhibited proliferation at a higher (25 mg/L) concentration (Table 2). Next, GSPE was chromatographically separated into fractions representing different degrees of polymerisation, and selected fractions were tested. Fraction II (enriched in monomers) and fraction VI (enriched in dimers) showed slight, but significant, inhibition of proliferation. The fraction containing higher molecular weight forms (fraction X) demostrated the strongest antiproliferative effects (Table 2).

3.4. GSPE treatment modulates apoptosis markers in pancreas of cafeteria-diet fed rats

In a former study, we had shown that GSPE treatment (10 and 30 days) reduced the fasting insulinemia of cafeteria-fed rats. This experiment was carried out at two different doses of GSPE (25 and 50 mg/kg bw). In the present study, we monitored apoptosis markers to determine whether GSPE-induced insulin reduction could be

Table 2
Effects of purified compounds and fractions on proliferation induced with 200 nM insulin. Data are normalised with respect to insulin condition and is shown as the mean ± SEM.

	BrdU incorporation vs insulin	
200 nM insulin	1.00 ± 0.0	
Dose	5 mg/L	25 mg/L
Catechin	1.10 ± 0.0**	
Epicatechin	1.10 ± 0.0**	
Epicatechin gallate	1.06 ± 0.0**	
Procyanidin B2	1.08 ± 0.0*	
Procyanidin C1	0.93 ± 0.0	0.96 ± 0.0**
Fraction II	0.90 ± 0.0**	0.85 ± 0.0***
Fraction IV	0.91 ± 0.0*	0.94 ± 0.0***
Fraction X	0.80 ± 0.0***	0.77 ± 0.0***

* $p \leqslant 0.05$.
** $p \leqslant 0.01$.
*** $p \leqslant 0.001$ versus insulin.

a consequence of pancreatic damage. We selected the apoptosis-involved protein Bax and the anti-apoptotic protein Bcl-2 as markers, and analysed their expression both at gene and protein levels. As reported in Table 3, GSPE significantly modify apoptosis markers in the pancreas of cafeteria-fed rats, with the induced changes depending on both on the dose and treatment period.

To better define the effects of GSPE on modulating apoptosis, we examined the markers that change during the progression of the cafeteria diet. As summarised in Table 3, Bcl-2 gene and protein expression are down-regulated after a longer (30 days) exposure to the cafeteria diet; the ratio Bcl-2/Bax at gene and protein levels is similarly down-regulated by the cafeteria diet longer treatment. GSPE impedes the down-regulation of Bcl-2 protein (but not gene) expression, with increased and normalised levels observed after 10 and 30 days of treatment, respectively. Fifty milligram per kilogram bw GSPE also counteracts the decrease in the ratio Bcl-2/Bax protein after 10 days, although no GSPE effects are measured for Bcl-2/Bax gene expression level with any dose or treatment duration compared to the cafeteria-fed animals.

4. Discussion

In an attempt to describe and understand the effects of GSPE on pancreatic beta-cells, we first tested the effects of GSPE on proliferation and apoptosis *in vitro* using the INS-1E beta-cell line. Our results show that GSPE does not modulate any of these processes under basal conditions. Though not conclusive, these results suggest GSPE is innocuous under healthy conditions. Because the GSPE effects largely depend on metabolic environments (Pinent et al., 2011), we also simulated altered conditions that can be found in insulin resistance and diabetes, i.e., high concentrations of glucose, insulin or fatty acid. In the case of glucose, the major regulator of pancreatic beta-cell function, long term exposure to a hyperglycaemic environment leads to beta-cell dysfunction (Ling et al., 1996) and apoptosis (Efanova et al., 1998; Joe et al., 2008; Kim et al., 2005). Our results show that GSPE enhances glucose-induced apoptosis. Exposure of islet cells to high glucose concentrations down-regulates glucokinase (Gck) protein expression and decreases the interactions of Gck with voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane (Kim et al., 2005). We have previously demonstrated down-regulation in Gck gene expression under high glucose conditions (Castell-Auví et al., 2012). In this high glucose environment, GSPE is also able to stimulate glucose uptake in INS-1E cells (Castell-Auví et al., 2012). The fatty acid palmitate had previously been shown to induce apoptosis in beta-cells (El-Assaad et al., 2003; Karaskov et al., 2006). In the present study, we found that this phenotype is not modified by GSPE treatment. The precise mechanisms involved in the toxic actions of free fatty acids remain to be defined, but likely involve the interplay of a number of factors (Maestre et al., 2003) and differ from those induced by glucose. Palmitate-induced apoptosis is suggested to be due to LC-CoA and/or metabolites thereof (El-Assaad et al., 2003), to increase endoplasmatic reticulum stress (Karaskov et al., 2006), and by causing mitochondrial dysfunction (Lee et al., 2009). From these results, it seems likely that GSPE pro-apoptotic effects in pancreatic beta-cells are mediated by enhancing glucose uptake under high glucose conditions, leading first to down-regulation of Gck and translocation of Bax protein to the mitochondrial membrane, then to its oligomerisation and, finally, to apoptosis.

In this study, we also show that GSPE modulates apoptosis markers in pancreas *in vivo*, with the exact effects depending on the dose and period of treatment. The changes attributed to the cafeteria treatment show signs of inducing apoptosis (Castell-Auví et al., 2011), similar to that observed in mice fed with high-fat diet (Owyang et al., 2010). Depending on the dose and time, GSPE modifies some of the markers that suggest increased apoptosis in the cafeteria-fed population, but the changes do not point to an increased apoptosis as observed *in vitro*. In fact, *in vitro* we found that GSPE only increases apoptosis when it is already activated by high glucose conditions. As previously published (Montagut et al., 2010), these cafeteria-fed animals were still normoglycemic and despite signs of apoptosis, the pancreases were still functional (Castell-Auví et al., 2011). Therefore, the GSPE-enhanced glucose uptake hypothesised to occur in beta-cells in a high glucose environment would not occur in this animal model.

Table 3
Effects of GSPE on Bax and Bcl-2 gene and protein expression in cafeteria-fed rats. Data are shown as the mean ± SEM, with different letters indicating significantly different groups ($p \leqslant 0.05$).

	Control	Cafeteria	25 mg/kg GSPE	50 mg/kg GSPE
Short term (10 days)				
Bcl2 mRNA	1.05 ± 0.1 a	0.92 ± 0.0 a	0.69 ± 0.1 b	0.83 ± 0.1 ab
Bax mRNA	1.00 ± 0.0 a	0.86 ± 0.2 ab	0.67 ± 0.1 b	0.61 ± 0.0 b
Ratio *Bcl2/Bax* (mRNA)	1.33 ± 0.1 a	1.31 ± 0.3 ab	1.04 ± 0.1 b	1.44 ± 0.3 ab
Bcl-2 protein	1.00 ± 0.1 a	1.14 ± 0.1 a	1.96 ± 0.3 b	3.12 ± 1.3 ab
Bax protein	1.00 ± 0.0 a	1.65 ± 0.1 b	1.57 ± 0.3 ab	1.79 ± 0.2 b
Ratio Bcl-2/Bax (protein)	1.15 ± 0.4 ab	0.34 ± 0.1 a	0.38 ± 0.1 a	0.67 ± 0.1 b
Long term (30 days)				
Bcl2 mRNA	1.00 ± 0.0 a	0.72 ± 0.1 b	0.73 ± 0.1 ab	0.66 ± 0.1 b
Bax mRNA	1.07 ± 0.2 a	0.86 ± 0.1 a	0.75 ± 0.1 a	1.13 ± 0.2 a
Ratio *Bcl2/Bax* (mRNA)	1.12 ± 0.0 a	0.88 ± 0.1 b	1.03 ± 0.3 ab	0.65 ± 0.1 b
Bcl-2 protein	1.00 ± 0.2 a	0.40 ± 0.1 b	0.88 ± 0.2 ab	0.87 ± 0.2 a
Bax protein	1.00 ± 0.2 a	1.50 ± 0.1 ab	2.52 ± 0.5 b	1.97 ± 0.6 ab
Ratio Bcl-2/Bax (protein)	1.12 ± 0.2 a	0.46 ± 0.2 b	0.32 ± 0.1 b	0.49 ± 0.3 ab

We also show antiproliferative effects of GSPE in pancreatic beta-cells in this study. The antiproliferative effects of procyanidins implicate these molecules as preventative agents toward cancer, and thus procyanidins have been extensively studied in cancerous cell lines (Singh, Shankar, & Srivastava, 2011). Very recently, a pancreatic carcinoma cell line has also been shown to be sensitive to the antiproliferative effects of grape seed procyanidins (Chung et al., 2011). Similarly, tea catechins inhibit proliferation in pancreatic carcinoma cell lines (Kürbitz et al., 2011; Vu et al., 2010). However, the effects of procyanidins in beta-cells were not described. We found that a non-toxic dose of GSPE is able to reduce beta-cell proliferation when it is stimulated by a proliferative agent, such as glucose or insulin. GSPE also boosts inhibition of proliferation induced by the fatty acid palmitate. Regulation of cell division in beta-cell regeneration is complex, and mitogens influence D-type Cyclin expression levels involving multiple signalling pathways (Lee & Nielsen, 2009). It has been suggested that glucose induces beta-cell proliferation via increased Cyclin D2 (Alonso et al., 2007). Subsequently, it was postulated that the presence of Cyclin D2 may be necessary but not sufficient for beta-cell replication and that other components of the cell cycle machinery may be responsible for mitogen-induced replication in beta-cells (Salpeter et al., 2011). Glucose controls Cyclin D2 at mRNA level (Salpeter et al., 2011). Consistent with that finding, we have demonstrated increased Cyclin D2 mRNA expression after a treatment with high glucose concentrations in INS-1E cells. We also found that in cells with both high and basal glucose levels, GSPE does not modulate Cyclin D2 gene expression. This result suggests that the antiproliferative effects of GSPE might be downstream of Cyclin D2 modulation by glucose, perhaps at the regulatory level of Cyclin-dependent kinases and their inhibitors. The effect of procyanidins in enhancing the antiproliferative effect of FFAs could also be mediated by the induction of cell-cycle inhibitors. Pascoe et al. (2012) postulated that the cell-cycle inhibitors p16 and p18 block the cell cycle downstream of D-type Cyclins in beta-cell treated with FFAs (Pascoe et al., 2012). Indeed, grape seed procyanidin-induced cell-cycle arrest is accompanied by increased Cdk inhibitors in several cell types (Agarwal, Sharma, & Agarwal, 2000; Lin et al., 2012; Pinent et al., 2005).

The antiproliferative effects of GSPE could be of interest because procyanidins have been shown to increase peripherial insulin sensitivity (Pinent et al., 2004) during some treatments, thus promoting glucose uptake by peripheral tissues while preventing the increase in pancreatic mass that leads to hyperinsulinemia. In the present *in vivo* study, the proliferation markers analysed were unchanged and showed no correlation with cafeteria treatment (Castell-Auví et al., 2011). According to the literature, high fat diets increase functional beta-cell mass in rodents. We are currently assessing the effects on beta-cell mass using other methods and/or markers of proliferation. Regardless, the GSPE treatment was carried out after 13 weeks on a high fat diet, a duration after which beta-cell mass had likely already increased. Therefore, the antiproliferative effects of GSPE in beta-cells might not be attributable to reduced insulinemia in the previous *in vivo* cafeteria model.

The GSPE antiproliferative effects found *in vitro* might be relevant if GSPE treatments had been performed at the beginning of insulin resistance induction. One of the caveats in working with natural extracts *in vitro* is that the molecules in the extract might be modified via metabolism before reaching the organ (Kroon et al., 2004). Therefore, we focused on which of the extract compounds could be the responsible for the antiproliferative effects. We found that the non-galloylated monomers (catechin and epicatechin) enhanced proliferation in pancreatic beta-cells, while EGC did not. Increased proliferation by the non-galloylated monomers has also been demonstrated in other cell types, including the colon cells LoVo (Tan et al., 2000) and the fibroblast cells of type I GD patients (Lee, Kim, & Heo, 2011). When testing a GSPE fraction enriched in monomers, we found a slight inhibition of proliferation, indicating that a combination of monomers present in the extract better reflect the effect of the whole extract when compared to a single pure molecule. Similarly, the dimer procyanidin B2 enhanced proliferation while the GSPE fraction enriched in dimeric forms reduces it. Finally, polymerised structures seem to demonstrate enhanced antiproliferative effects, as the purified trimer C1 inhibited proliferation, and the fraction enriched in higher-order polymers demonstrated the most potent antiproliferative effects. This is in agreement with studies conducted on a colon carcinoma cell line (HT29), from which it was concluded that grape oligomers are more efficient than monomers as antiproliferative agents (Lizarraga et al., 2007).

Together, these results indicate that a possible GSPE-antiproliferative effect in beta-cells *in vivo* cannot be ruled out, because structures found in plasma (e.g., monomers, dimers and trimers) show effects *in vitro*. However, the fact that the most antiproliferative compounds are the higher molecular weight forms necessitates caution when interpreting *in vitro* data.

In conclusion, we demonstrate in the current study that GSPE modulates apoptosis and proliferation of INS-1E pancreatic beta-cells. GSPE pro-apoptotic effects might be due in part to increases in glucose uptake in a high glucose environment. Additionally, GSPE possesses clear antiproliferative effects, with higher molecular weight compounds giving rise to the largest effects.

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2. Grape seed procyanidin extract improves insulin production but enhances Bax protein expression in cafeteria-treated male rats

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To better understand the effects of GSPE on pancreatic proliferation and apoptosis *in vivo*, the extract was administrated to male rats that were fed a cafeteria diet.

Pharmacokinetic and pharmacodynamic parameters in females are different from the parameters in males.[1] Furthermore, female rats have been reported to be more sensitive to cafeteria-induced obesity than males,[2,3] suggesting that female rats may be more resistant to treatment. Thus, the results obtained in male rats were compared to those observed in a previous study of female rats.

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Grape seed procyanidin extract improves insulin production but enhances Bax protein expression in cafeteria-treated male rats

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ABSTRACT

In a previous study, the administration of a grape seed procyanidin extract (GSPE) in female Wistar rats improved insulin resistance, reduced insulin production, and modulated apoptosis biomarkers in the pancreas. Considering that pharmacokinetic and pharmacodynamic parameters in females are different from these parameters in males, the aim of the present study was to evaluate the effects of GSPE in male Wistar cafeteria-induced obese rats.

The results have confirmed that the cafeteria model is a robust model mimicking a pre-diabetic state, as these rats display insulin resistance, increased insulin synthesis and secretion, and increased apoptosis in the pancreas. In addition, GSPE-treatment (25 mg/kg of GSPE for 21 days) in male rats improves insulin resistance and counteracts the cafeteria-induced effects on insulin synthesis. However, the administration of the extract enhances the cafeteria-induced increase in Bax protein levels, suggesting increased apoptosis. This result contradicts previous results from cafeteria-fed female rats. In female rats, GSPE seemed to counteract the increased apoptosis induced by the cafeteria diet.

KEYWORDS

cafeteria diet; male rats; procyanidins; insulin; pancreas; apoptosis.

INTRODUCTION

Procyanidins are the second most abundant natural phenolic after lignin, and they are widely distributed in fruits, berries, beans, nuts, cocoa, and wine.[1] They are potent antioxidants that possess biological properties that may protect against cardiovascular diseases.[1] They participate in glucose homeostasis[2] and modulate insulin synthesis, secretion and degradation.[3] Moreover, changes in β-cell insulin production may also be due to variations in the number of insulin-producing cells. β-cell mass adapts to increased metabolic demands caused, for example, by obesity, pregnancy or insulin resistance. However, when β-cells are unable to compensate for increased insulin demand, there is a decrease in β-cell mass characteristic of the onset of type 2 diabetes mellitus (T2DM).[4] Procyanidins modulate apoptotic and proliferation processes, mainly reported in cancerous cell lines.[5] Moreover, they protect cells from diverse drug- or chemical-induced toxic assaults by decreasing apoptosis and inducing cell growth.[5] However, there is little information available regarding their effects on β-cells. Other studies by our research group have reported that procyanidins modulate proliferation and apoptosis of the pancreatic β-cell line INS-1E under altered conditions.[6] Procyanidins also alter the protein and/or gene expression of factors involved in apoptosis in Zucker Fatty rats[7].

Obesity has become a worldwide problem, leading to an explosion of obesity-related health issues.[8] Obese individuals develop resistance to the cellular actions of insulin, a key etiological factor for T2DM, which is also becoming an epidemic.[9] T2DM is characterised by peripheral insulin resistance as well as pancreatic β-cell dysfunction and decreased β-cell mass.[10] It involves a combination of genetic and environmental or lifestyle factors. These lifestyle changes, involving high-energy diets and reduced physical activity, are linked to the pandemics of obesity and T2DM.[11] Given the high prevalence of the disease, obtaining knowledge about natural compounds with potential beneficial effects on glucose homeostasis is of great interest.

Several animal models have been used to study obesity, including both genetic and diet-induced obesity models. However, the cafeteria diet is a more robust model to reproduce the diet in Western society.[12] In a previous study, we analysed the effects of procyanidins in an insulin resistance model induced by cafeteria diet administration in female Wistar rats. We also considered the effects of the cafeteria diet on insulin production and apoptosis in the pancreas. The study showed that the cafeteria diet increased insulin production as well as activated apoptosis biomarkers.[13] Furthermore, procyanidin administration caused a reduction in the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) index, suggesting improved insulin resistance.[14] Moreover, in the pancreas, procyanidins caused a decrease in insulin production[15] and modulated pro- and anti-apoptosis markers.[6]

The pharmacokinetics and pharmacodynamics in females are different from the same parameters in males because of the female's unique anatomy and physiology.[16,17] Thus, the aim of the present study was to evaluate the effects of GSPE in male Wistar cafeteria-induced obese rats and to compare the results on insulin synthesis, apoptosis and proliferation in the pancreas with those observed in the previous study of female rats.

MATERIALS AND METHODS

GSPE

The procyanidin extract was derived from grape seed and contained the following: catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (15.68 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g), and hexameric procyanidins (0.38 μmol/g).[18]

Animal experimental procedures

Wistar male rats weighting between 250-330 g were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22 ºC with a 12 h light/dark cycle. After 1 week in quarantine, the animals were divided in two groups, a diet-control group (7 animals) fed a standard diet (Panlab A03) and a cafeteria group (21 animals) fed a cafeteria diet (bacon, biscuits with pâté, biscuits with cheese, muffins, carrots and milk with sugar) in addition to standard chow and water. Every day at 9 a.m., food was withdrawn, and it was replaced at 6 p.m. Obesity was induced in the animals on the cafeteria diet for 52 days. Afterwards, the diet-control group and 7 animals from the cafeteria-fed group were sacrificed, as a reference for the state of the animals before the beginning of treatment. The rest of the cafeteria-fed rats were divided in two subgroups (7 animals/group). These two groups were the (i) cafeteria group: rats treated with a vehicle (gum arabic 5% w/v) and the (ii) GSPE-treated group: rats treated with 25 mg of GSPE/kg of body weight (bw) per day. The treatment was administrated every evening for 21 days before the replacement of the food. Three days before the end of the treatment and after 8 h of fasting, blood was collected from the tails of the rats to measure glucose and insulin levels. At the end of the treatment regimen and after 3 h of fasting, the animals were anesthetised using sodium pentobarbital (50 mg/kg of bw, Sigma-Aldrich, St. Louis, MO) and sacrificed by abdominal aorta exsanguination. The pancreas was isolated from all of the animals, frozen immediately in liquid nitrogen and stored at −80 °C until analysis. All of the procedures were approved by the Experimental Animals Ethics Committee of the Universitat Rovira i Virgili.

Plasmatic and pancreatic measurements

Insulin plasma levels were assayed using an ELISA method following the manufacturer's instructions (Mercodia, Uppsala, Sweden). Glucose plasma levels were determined using an enzymatic colorimetric kit (QCA, Amposta, Spain).

The HOMA-IR and HOMA-β were calculated using the fasting values of glucose and insulin and the following formulas:

$$HOMA-IR = \frac{\text{insulin } (\mu U/mL) \times \text{glucose } (mM)}{22.5}$$

$$\text{HOMA} - \beta = \frac{20 \times \text{insulin}\ (\mu\text{U/mL})}{\text{glucose}\ (\text{mM}) - 3.5}$$

Triglycerides (TAG) and non-esterified fatty acids (NEFAs) from the pancreas were extracted by homogenising the tissue with PBS containing 0.1% triton X-100 (Sigma-Aldrich, St. Louis, MO), and their concentrations were determined using enzymatic colorimetric kits (QCA, Amposta, Spain for TAG and Wako chemicals GmbH, Neuss, Germany for NEFAs).

The pancreas was homogenised with six volumes of PBS containing 50 mM EDTA at pH 7.4 and centrifuged at 3000 g for 5 min at 4 ºC. Reactive oxygen species (ROS) in the supernatants were quantified using 20 μM DCFH-DA (2',7'-dichlorofluorescin diacetate) (Sigma-Aldrich, St. Louis, MO), and the fluorescence was measured after 50 minutes at 37 ºC at λ_{ex} = 485 nm and λ_{em} = 530 nm. The values were normalised to the protein content, which was analysed by the Bradford method.[19]

Quantitative Real Time PCR

Total RNA from the pancreas was extracted using the RNeasy Mini Kit (Qiagen, Barcelona, Spain). cDNA was generated with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and was subjected to quantitative Real Time PCR amplification using the TaqMan Master Mix (Applied Biosystems, Madrid, Spain). Specific TaqMan probes (Applied Biosystems, Madrid, Spain) were used for each gene: Rn99999125_m1 for *Bcl2*, Rn01480160_g1 for *Bax*, Rn01492401_m1 for *Ccnd2* (Cyclin D2), Rn01774648_g1 for *Ins*, and Rn00565544_m1 for *Cpe*. *Actb* was used as the reference gene (Rn00667869_m1). Reactions were run on a quantitative RT-PCR 7300 System (Applied Biosystems, Madrid, Spain) according to the manufacturer's instructions. The relative mRNA expression levels were calculated using the ΔΔCt method.

Western Blot

The protein levels of Bax and Bcl-2 were quantified by Western Blot as previously described.[13] Primary antibodies were purchased from Cell Signalling Technology (Beverly, MA). 25 μg of protein was loaded onto the gel, and the antibody dilution was 1:1500 for Bax and Bcl-2. After incubation with peroxidise-conjugated monoclonal anti-rabbit secondary antibody (Sigma-Aldrich, Madrid, Spain) at a 1:10000 dilution, immunoreactive proteins were visualised with the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Chemiluminescence and densitometric analysis of the immunoblots was performed using ImageJ 1.44p software, and all proteins were quantified relative to the loading control.

Calculations and Statistical Analysis

The results are expressed as the mean ± SEM. Effects were assessed using Student's *t*-test. All calculations were performed with SPSS software v19.

RESULTS

Cafeteria diet increases insulin production in the pancreas and GSPE-treatment counteracts this diet

We first examined the effects of the cafeteria diet on pancreatic insulin production after 52 days of diet administration. Insulin and glucose plasma levels were quantified at day 49, and the cafeteria diet-fed rats showed significantly higher plasma insulin levels and no changes in glucose levels (Table 1). The HOMA-IR index indicated that the cafeteria-fed animals had peripheral insulin resistance, and their HOMA-β index tended to increase (Table 1). Therefore, there was a tendency to increase pancreatic functionality response to glucose in order to counteract peripheral insulin resistance. The increased plasma insulin levels agree with an increase in the insulin gene expression, as well as with increased gene expression of carboxypeptidase E (Cpe) (Table 2).

After induction of obesity via the cafeteria diet, rats were treated with 25 mg/kg of bw GSPE for 21 days, concomitantly with cafeteria diet administration. The animals treated with the procyanidin extract had lower insulinemia and decreased HOMA-IR and HOMA-β indexes (Table 1), counteracting the effects observed in the cafeteria-fed rats. Moreover, insulin gene expression tended to decrease in these rats, and decreased expression of Cpe was also observed (Table 3).

Effects of cafeteria diet and GSPE on apoptosis biomarkers

To examine the effects of the cafeteria diet and GSPE on apoptosis and proliferation in the pancreas, several markers were analysed at the gene and protein level.

The cafeteria-fed rats showed a decrease in the anti-apoptotic marker Bcl-2 at both the gene (Table 2) and protein levels (Figure 1A). For the pro-apoptotic marker Bax, the mRNA levels of this gene were not significantly altered (Table 2), but we did observe an increase in the protein levels of Bax in the cafeteria diet-fed group (Figure 1A). Therefore, the ratio of Bcl-2/Bax was reduced both at the gene and protein level, suggesting an increase in apoptosis in the pancreas (Table 2 and Figure 1A).

The administration of GSPE had no effect on Bcl-2 and Bax at gene expression compared to the cafeteria diet (Table 3) and on Bcl-2 protein expression (Figure 1B). In contrast, Bax protein levels were increased by the GSPE treatment, enhancing the effects observed in the cafeteria-fed rats (Figure 1B). The ratio Bcl-2/Bax was significantly reduced at protein level (Figure 1B) compared to the cafeteria-fed animals.

Finally, we also analysed Cyclin D2, a proliferation marker, but no changes were observed in the cafeteria-fed animals or in the GSPE-treated rats (Table 2).

GSPE treatment avoids the increase of TAG in the pancreas induced by cafeteria diet

Pancreas malfunction is in part due to the accrual of TAG in its cells. To measure it, we examined the TAG content in this tissue, and found that TAG triplicated its levels in the pancreas of cafeteria-fed rats compared to the standard diet-fed rats (33.15 ± 2.7 versus 11.42 ± 0.3 μg TAG/mg tissue, $p \leq 0.01$). After the 21 days of treatment, the TAG contents in the pancreas increased, due to cafeteria diet, but GSPE avoided this (Figure 2A).

In contrast, the content of NEFAs were not modified neither in the cafeteria group compared to the standard diet-fed rats (3.37 ± 0.9 versus 4.40 ± 0.4 μg NEFA/mg tissue), nor in the GSPE-treated rats compared to the vehicle-treated rats (2.43 ± 0.3 versus 10.29 ± 4.6 μg NEFA/mg tissue).

ROS content in the pancreas was also analysed, and no significant differences were observed in either the cafeteria-fed rats or in the GSPE-treated animals (Figure 3).

DISCUSSION

This study was designed to examine the effects of the cafeteria diet on insulin production in male Wistar rats by evaluating pancreas functionality, apoptosis, and proliferation. We also evaluated the effects of procyanidins on these processes. Procyanidins have been shown to have positive effects on glucose metabolism under conditions of slightly disrupted homeostasis.[2]

We had previously shown that 17 weeks of a cafeteria diet led to insulin resistance, high plasma insulin levels, and increased insulin synthesis and secretion in female Wistar rats.[13] It has been reported that female rats are more sensitive to cafeteria-induced obesity than males.[20,21] However, with respect to insulin resistance, we have now shown that 52 days of cafeteria diet (nearly 7 and a half weeks) administrated to male Wistar rats confirms the effects observed in the previous study of female rats. In the males, we observed high insulin plasma levels and elevated HOMA-IR index, indicating peripheral insulin resistance in these animals. Additionally, we saw an elevated HOMA-β index, which indicates an increase in pancreatic functionality in terms of glucose response to counteract peripheral insulin resistance.[22] We also found an increase in the expression of the insulin gene in the cafeteria-fed rats; an increase in the gene expression of Cpe was also demonstrated. Cpe is the enzyme thought to be involved in the cleavage of proinsulin, which results in insulin and C-peptide molecules.[23] Therefore, the pancreas of cafeteria diet-fed rats is still functional, and it tries to counteract peripheral insulin resistance despite the increased lipid accumulation in the pancreas.

Previously, we demonstrated that GSPE acts peripherally on adipose tissue to improve glycemia, which leads to lower insulinemia in female Wistar cafeteria-fed rats.[14] In that experiment, 25 mg of GSPE/kg of bw was administered to the rats for 30 days; this treatment resulted in decreased insulin production. This result can be explained at least in part through GSPE's lipid-lowering effect, and GSPE reduces the triglyceride content in the pancreas, likely via decreased fatty acid synthesis and increased β-oxidation.[15] In this study, male Wistar rats were fed a cafeteria diet (25

mg/kg of GSPE for 21 days), which decreased insulin production, decreased plasma insulin levels, decreased pancreatic insulin, and decreased Cpe gene expression. Moreover, the triglyceride content was also reduced in the pancreas of GSPE-treated rats.

Increased deposits of fat are associated with obesity and lead to an increase in free fatty acids (FFA). The induction of apoptosis in the *in vivo* high-fat diet model and *in vitro* models of FFA-induced apoptosis are important evidence for β-cell lipotoxicity.[24] In mice, administration of a high-fat diet for 12 weeks leads to increased β-cell mass, despite showing an increase in β-cell apoptosis.[25] In our study, we found a decrease in the Bcl-2/Bax ratio both at the gene and protein levels, which also suggests an increase in apoptosis in the pancreas of cafeteria-fed rats. Cpe degradation mediates β-cell endoplasmic reticulum stress and death in response to palmitate.[26,27] However, the levels of this gene are increased by the cafeteria diet, suggesting that the apoptosis observed in the cafeteria-fed rats is not mediated by lipotoxicity. In fact, the levels of NEFA in the pancreas are not altered in the cafeteria-fed rats when compared to the standard diet-fed rats.

In rats fed high fat diets, no changes in Ki67 (a proliferation marker) expression were observed.[25] We also found no changes in the expression of the Cyclin D2 gene, suggesting that at the time of the analysis, β-cell mass had likely already increased. The results from the apoptosis markers and Cyclin D2 are in accordance with the data obtained in the previous study, which evaluated the cafeteria diet on Wistar female rats.[13] The data suggests that the effects of the cafeteria diet on insulin synthesis, secretion, and apoptosis are not influenced by gender or treatment duration.

As above mentioned, GSPE reduced the accumulation of triglycerides in the pancreas. However, the apoptosis markers were not modulated to counteract the effects of the cafeteria diet. Instead, GSPE-treated rats showed an increase in Bax protein levels and a decreased ratio of Bcl-2/Bax, suggesting an enhancement in the apoptosis observed in cafeteria-fed rats. Therefore, the lipid-lowering effects do not involve a reduction in apoptosis in the pancreas. Actually, *in vitro* GSPE does not modulate fatty acid-induced apoptosis.[6] In fact, GSPE increases glucose uptake in β-cells under high-glucose conditions and impairs mitochondrial and cellular membrane potentials.[3] Moreover, GSPE was reported to enhance the pro-apoptotic effects of high glucose *in vitro*.[6] Therefore, the enhanced apoptosis observed in the GSPE-treated rats could be due to increased glucose uptake in the β-cells that potentiate glucotoxicity. ROS is one of the players in glucose-induced apoptosis in β-cells; ROS is increased as a consequence of chronically increased glucose metabolism. β-cells have relatively low expression of antioxidant enzymes and are more sensitive to ROS attack when they are exposed to oxidative stress.[28] However, the levels of ROS in the pancreas were not modified by the cafeteria diet or by GSPE treatment, suggesting that glucose toxicity could be mediating by another mechanism.

The apoptosis marker results conflict with those in the previous study of female Wistar rats, which showed that 25 and 50 mg/kg of GSPE seemed to counteract the deleterious effects of the cafeteria diet by inhibiting the down-regulation of Bcl-2 protein expression after 10 and 30 days of treatment.[6] In addition, 50 mg/kg bw of GSPE also counteracted the decrease in the Bcl-2/Bax ratio at the protein level after 10 days of administration. However, no GSPE effects were observed with respect to the ratio of Bcl-2/Bax gene expression at any dose or treatment duration.[6] We found no changes in the expression of Bcl-2, either at the gene or at the protein

level. Bax was modulated by GSPE, but this modulation was not identified in the previous study.[6] Therefore, the modulation of apoptosis biomarkers by GSPE in cafeteria-fed rats is clearly dependent on the dose and treatment period; these effects may also be dependent on gender.

In conclusion, the present cafeteria model study has confirmed that this is a suitable model to mimic the pre-diabetic state. This model induces an insulin resistance state, shows increased insulin synthesis and secretion, and exhibits increased apoptosis in the pancreas. Moreover, GSPE-treatment in male rats treated with 25 mg/kg of GSPE for 21 days improves the insulin resistance state and counteracts the cafeteria-induced effects on insulin synthesis. However, procyanidins enhance the elevated levels of Bax, a pro-apoptotic protein observed in the cafeteria-fed rats, potentially suggesting an increase in apoptosis. This result indicates that the effects of GSPE on apoptosis markers are dose-, time- and/or gender-dependent.

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TABLES

Table 1. Effects of the cafeteria diet and GSPE treatment on plasmatic glucose and insulin levels, HOMA-IR and HOMA-β index. ** p ≤ 0.01, *** p ≤ 0.001 and # p ≤ 0.1 for cafeteria diet versus standard diet. ‡ p ≤ 0.05 and † p ≤ 0.1 for cafeteria + GSPE versus cafeteria + vehicle.

	Standard diet	Cafeteria diet	Cafeteria + vehicle	Cafeteria + GSPE
Glucose (mM)	4.13 ± 0.2	4.49 ± 0.3	4.76 ± 0.2	4.88 ± 0.2
Insulin (ng/ml)	1.23 ± 0.1	2.47 ± 0.2 ***	2.78 ± 0.2	1.80 ± 0.3 ‡
Insulin/Glucose	6.02 ± 0.6	12.40 ± 1.6 **	13.25 ± 0.7	8.39 ± 1.2 ‡
HOMA-IR	4.97 ± 0.5	10.75 ± 1.0 ***	14.66 ± 2.8	8.37 ± 1.2 †
HOMA-β	609.45 ± 93.4	1508.98 ± 388.9 #	1084.1 ± 13.7	750.58 ± 121.2 ‡

Table 2. Effects of cafeteria diet on gene expression in the pancreas. * p ≤ 0.05 and # p ≤ 0.1 versus standard diet.

	Standard diet	Cafeteria diet
Ins2	1.22 ± 0.3	5.37 ± 1.4 *
Cpe	1.16 ± 0.3	3.71 ± 0.8 *
Bcl-2	1.15 ± 0.3	0.28 ± 0.1 *
Bax	1.08 ± 0.2	1.64 ± 0.4
Bcl-2/Bax	1.27 ± 0.5	0.24 ± 0.1 #
Ccnd2	1.31 ± 0.4	1.12 ± 0.3

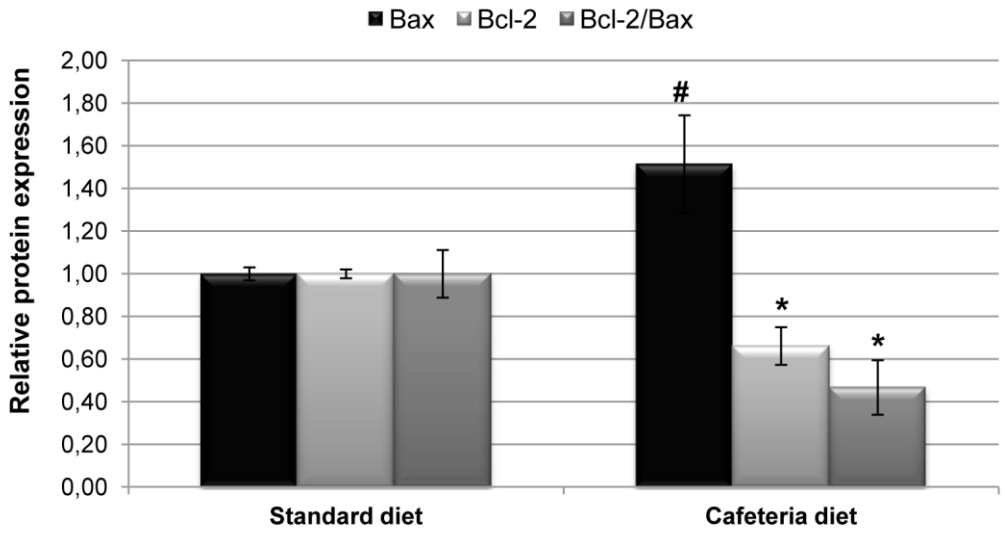
Table 3. Effects of GSPE treatment of cafeteria-fed rats on gene expression in the pancreas. ** p ≤ 0.01 and # p ≤ 0.1 versus vehicle-treated group.

	Cafeteria + vehicle	Cafeteria + GSPE
Ins2	1.16 ± 0.3	0.55 ± 0.1 #
Cpe	1.04 ± 0.2	0.2 ± 0.1 **
Bcl-2	1.12 ± 0.2	0.96 ± 0.3
Bax	1.13 ± 0.3	1.18 ± 0.2
Bcl-2/Bax	1.67 ± 0.4	0.95 ± 0.4
Ccnd2	1.15 ± 0.3	1.80 ± 0.3

FIGURES

Figure 1. Protein expression of the apoptosis markers Bcl-2 and Bax and the calculated ratio of Bcl-2/Bax in: (A) standard diet-fed rats and cafeteria diet-fed rats, and (B) in GSPE-treated rats and vehicle-treated rats assessed by Western Blot. Data are shown as the mean ± SEM. * $p \leq 0.05$ and # $p \leq 0.1$.

A



B

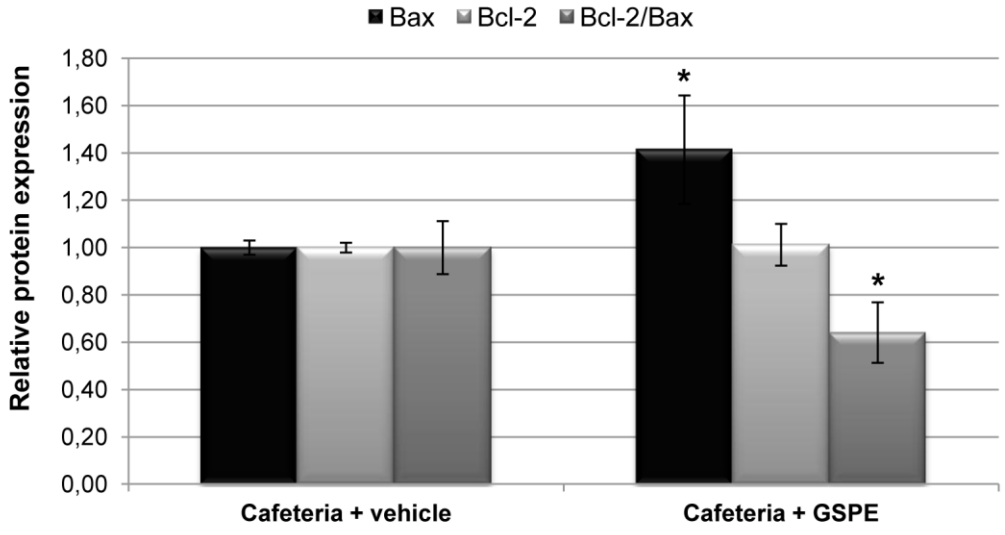


Figure 2. TAG content in the pancreas of cafeteria-fed rats treated with GSPE or vehicle, expressed as µg/mg of pancreatic tissue. Data are shown as the mean ± SEM. * $p \leq 0.05$.

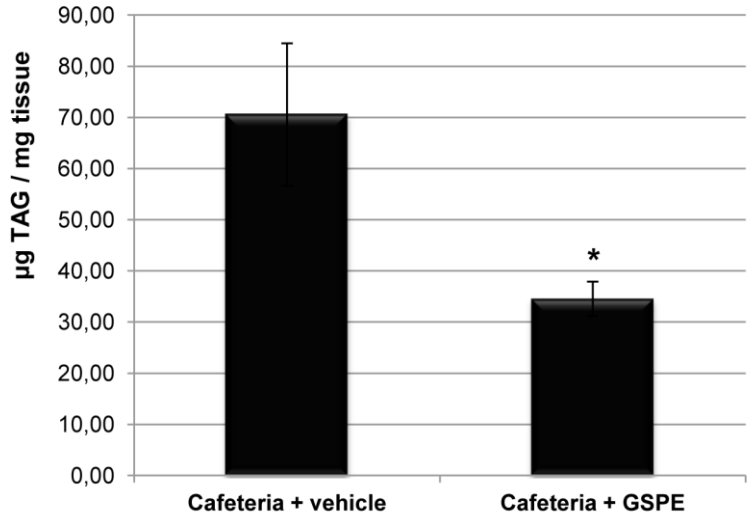
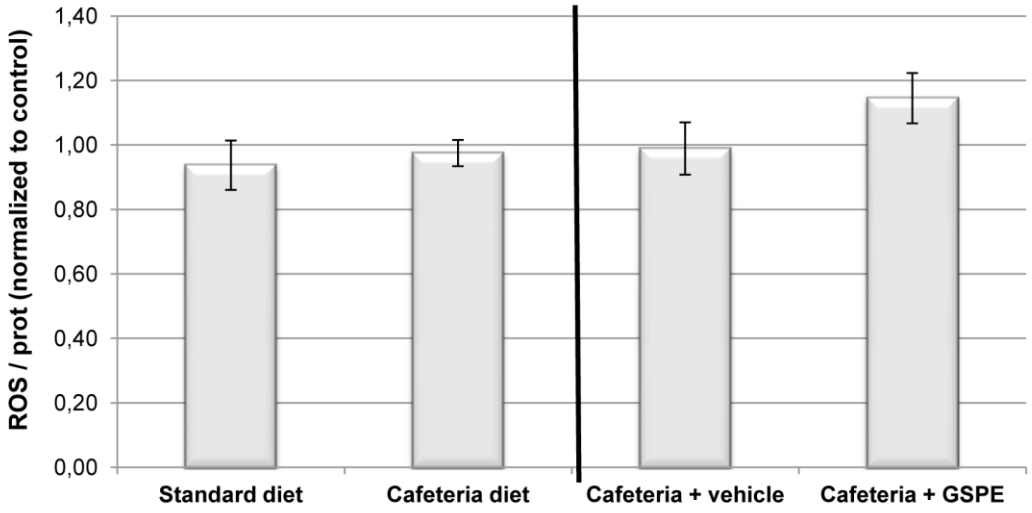


Figure 3. ROS content in the pancreas, expressed as fluorescence arbitrary units/(mg/mL) of protein and normalised to the respective control group. Data are shown as the mean ± SEM.



3. Pancreatic islet proteome profile in Zucker fatty rats chronically treated with a grape seed procyanidin extract

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Several animal models have been used to study obesity, both genetically- or diet-induced obesity. Concerning diet-induced obesity, the cafeteria model has been used in the previous studies to determine the effects of GSPE on pancreatic proliferation and apoptosis. In the present study, a genetically-induced obese model, Zucker Fatty rats, was chosen. Zucker Fatty rats have a mutation in the leptin receptor, which is the molecular base of their characteristic phenotype. These rats develop severe obesity associated with hyperphagia, defective thermogenesis and preferential deposition of energy in adipose tissue. They are extensively used as an obesity experimental model to study metabolic syndrome-related disorders, such as insulin resistance. In fact, Zucker Fatty rats present dyslipidemia, mild glucose intolerance, hyperinsulinemia, hypertrophy of the islets of Langerhans, and an increase in the number of islets (reviewed in [1]).

To better understand the mechanisms by which procyanidins modulate β-cell mass, a proteomics study was conducted in these rats. An isobaric tag for relative and absolute quantitation (iTRAQ) analysis was conducted. iTRAQ is a robust and sensitive technique, widely employed, and has a proven value in discovery-based proteomics. It allows for simultaneous protein identification and relative quantification, which is obtained at the MS/MS level from peptide fragments and low mass reporter ions of different samples.[2]

Finally, the differentially expressed proteins identified by iTRAQ were categorised according to Gene Ontology using DAVID, which extracts biological meaning from the protein list.[3]

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[#] The work presented in the following paper has been conducted with my colleague Anna Castell Auví. Only the results related to GSPE effects on proliferation and apoptosis belong to this thesis. The rest of the data belong to the thesis of my colleague.

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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 procyanidin extract (GSPE) has been reported to modify glucose metabolism and β-cell functionality through its lipid-lowering effects in a diet-induced obesity model. The objective of the present study was to evaluate the effects of chronically administrated GSPE on the proteomic profile of pancreatic islets from Zucker fatty (ZF) rats. An isobaric tag for relative and absolute quantitation (iTRAQ) experiment was conducted and 31 proteins were found to be differentially expressed in ZF rats treated with GSPE compared to untreated ZF rats. Of these 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 modulates factors involved in apoptosis, but these molecular changes are not sufficient to counteract the genetic background of the Zucker model at a physiological level.

1. 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 (Kahn, Hull, & Utzschneider, 2006; Qatanani & Lazar, 2007). T2DM is a metabolic disorder characterised by hyperglycemia, altered lipid metabolism and impaired insulin action in peripheral tissues (Baggio & Drucker, 2006; Nolan, Damm, & Prentki, 2006). T2DM is also associated with a deficient β-cell insulin-secretory response to glucose (Baggio & Drucker, 2006; Nolan et al., 2006) and involves a combination of genetic and environmental or lifestyle factors (Baggio & Drucker, 2006; DeFronzo & Abdul-Ghani, 2011). While genetic background is responsible for insulin resistance and β-cell failure, weight gain and physical inactivity exacerbate these inherited metabolic abnormalities (DeFronzo & Abdul-Ghani, 2011).

Procyanidins are the most abundant phenolic compounds in the human diet and they are found widely in fruits, berries, beans, nuts, cocoa-based products and wine (Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005). Procyanidins are known to exhibit protective effects against cardiovascular diseases, as they have antioxidant and anti-inflammatory properties and can help to prevent atherosclerosis (Rasmussen et al., 2005). However, there is little information about procyanidins' effects on the endocrine pancreas, which is a key organ of nutrient metabolism (Pinent et al., 2008). The effects of some doses of grape seed procyanidin extract (GSPE) on healthy animals have been recently described (Castell-Auví et al., 2012a). Moreover, in a previous study, GSPE was described to modify glucose metabolism by modulating plasma insulin levels and acting on peripheral tissues. The modifications were observed under conditions of obesity and mild insulin resistance induced by cafeteria diet (Montagut et al., 2010). 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 (Castell-Auví et al., 2012b).

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 utilising 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 characterised by insulin resistance and glucose intolerance. These rats are genetically obese

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due to a mutation in the leptin receptor gene (Augstein & Salzsieder, 2009; Berthiaume, Mika, & Zinker, 2003). Under the influence of obesity and insulin resistance, β-cells are exposed to elevated glucose, insulin and lipid levels. β-cells adapt physiologically to these conditions through increased β-cell mass and enhanced β-cell function (Augstein & Salzsieder, 2009; Jetton et al., 2005). An isobaric tag for relative and absolute quantitation (iTRAQ) experiment was completed and the proteins differentially expressed were identified. These were then clustered, categorised according to Gene Ontology (GO) terms, and visualised into network context in order to understand the proteome profile of isolated pancreatic islets from Zucker fatty rats chronically treated with GSPE.

2. Materials and methods

2.1. 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 (5–13 units, 35.7%), as well as phenolic acids (4.2%).

2.2. 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 22 °C 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 (Pajuelo et al., 2011). Briefly, the lean control group (ZL) and 10 randomly divided Zucker fatty rats (ZF) were treated with a vehicle (sweetened condensed milk diluted 1:6 with tap water). The other 10 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 a.m., food was withdrawn and at 4 p.m. the treatment (or vehicle) 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 anesthetised 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.

2.3. Islet isolation

Islets were obtained by collagenase digestion, as described previously (Castell-Auví et al., 2012a). Briefly, the rats were anesthetised and the pancreas was infused with 7 mL of ice-cold collagenase P (Roche Diagnostics, Mannheim, Germany) solution (1 mg/mL) before removal and incubation at 37 °C 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.

2.4. 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 (Bradford, 1976). 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 labelled with a different 8-plex iTRAQ reagent, as described in the iTRAQ protocol (Applied Biosystems, Foster City, CA). Finally, all of the labelled 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 analysed 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 × 5 mm, 3 μm, C18 trap column from LC Packings PepMap (Dionex Company, Amsterdam, The Netherlands) at a flow rate of 40 μL/min, utilising 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 × 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 nanoeletrospray 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 2.5–3.0 kV. Nitrogen was used as collision gas. The spray positions and other source parameters were optimised with a tryptic digest of a protein standard mixture (LC Packings, Amsterdam, The Netherlands).

2.5. 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) (www.matrixscience.com) and 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; 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 the 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) (Huang, Sherman, & Lempicki, 2009). Additionally, the data were clustered hierarchically using the Cluster 3.0 software (de Hoon, Imoto, Nolan, & Miyano, 2004) (version 1.5) and the results were visualised with Java TreeView software (Saldanha, 2004) (version 1.1.6r2). The list of significantly regulated proteins by GSPE was further analysed using the network building tool, Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc.), which uses the Ingenuity Pathways Knowledge Base. Hypothetical networks of proteins from the iTRAQ 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 = $-\log_{10}(P\text{-value})$. 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 (Ingenuity Systems, Inc., n.d.).

2.6. 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, presented as arbitrary units (AU), were normalised with the protein content of the islets, as analysed by Bradford method (Bradford, 1976).

2.7. 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.

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 $\leqslant$ 0.05).

2.8. 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.

3. Results

3.1. 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 (Pajuelo et al., 2011).

In order to analyse the proteomic profile of the pancreatic islets after the treatment with GSPE, an iTRAQ experiment was conducted. The information from the iTRAQ experiment was analysed using the ProteinPilot search algorithm against the SwissProt database. A total of 84 proteins were identified. With respect to the effect of the genetic background, of 21 proteins that were differentially expressed in ZL vs. ZF, 18 were associated with a *P*-value $\leqslant$ 0.05 and 3 were associated with a *P*-value $\leqslant$ 0.1. Additionally, four of these differentially expressed proteins were up-regulated, while the other 17 were down-regulated (Table 2). With respect to 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 $\leqslant$ 0.05, and 13 were associated with a *P*-value $\leqslant$ 0.1; furthermore, 10 were up-regulated, while 21 were down-regulated (Table 2).

The proteins modified by GSPE were analysed using DAVID according to the different categories in the Gene Ontology (GO) classification. In the cellular component category, the highest proportion of differentially expressed proteins was cytosolic. 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 (Fig. 1A–E).

3.2. 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 visualised with Java TreeView software (Fig. 2A). The values for the ZF group were assigned as 1 for all of the proteins. Hierarchical clustering revealed that the 36 proteins modified in ZL vs. ZF and/or ZF + GSPE vs. ZF were clustered in four expression patterns (Fig. 2B). Two clusters exhibited a profile in which ZF + GSPE counteracted the effect of ZF: Down–Up in which the expression of five proteins was down-regulated in ZF versus ZL and the effect was counteracted by GSPE; and Up–Down, in which the expression of 12 proteins was up-regulated 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

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	Haemoglobin:haemoglobin 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	Haemoglobin:haemoglobin 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 Rab1A	2	0.52**	1.10
Q9Z2L0	Vdac1	Voltage-dependent anion-selective channel protein 1	3	0.78#	0.80#

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

Table 3
Islets gene expression.

Gene Name	Commercial reference	ZL	ZF	ZF + GSPE
Ins2	Rn01774648-g1	**1.0 ± 0.1a**	**3.2 ± 0.2b**	**1.8 ± 0.4a**
Pdx1	Rn00755591-m1	1.0 ± 0.1ab	1.3 ± 0.1b	0.9 ± 0.2a
Pparg	Rn00440945-m1	1.1 ± 0.3a	2.4 ± 0.3b	1.9 ± 0.2ab
Gck	Rn00561265-m1	1.0 ± 0.0ab	1.4 ± 0.1b	1.0 ± 0.1a
Ucp2	Rn01754856-m1	1.1 ± 0.2a	2.1 ± 0.2b	1.6 ± 0.2ab
Cpe	Rn00565544_m1	**1.0 ± 0.1a**	**3.8 ± 0.2b**	**1.8 ± 0.4a**
Vdac1	Rn00821325_g1	**1.0 ± 0.1a**	**1.6 ± 0.0b**	**0.7 ± 0.1c**
Bcl-2	Rn99999125_m1	1.2 ± 0.3ab	1.3 ± 0.1a	0.9 ± 0.1b
Bax	Rn01480160_g1	0.9 ± 0.2a	1.1 ± 0.0a	1.0 ± 0.2a
Ratio Bcl-2/Bax		0.9 ± 0.1a	1.3 ± 0.1b	1.0 ± 0.1a
Ddit3	Rn01458526_m1	1.4 ± 0.5a	1.4 ± 0.3a	1.7 ± 0.4a
Ccnd2	Rn01492401_m1	1.0 ± 0.0a	1.4 ± 0.1b	0.8 ± 0.1a
Mki67	Rn01451446_m1	1.0 ± 0.1a	2.2 ± 0.3b	1.6 ± 0.2b
Gcg	Rn00562293_m1	**1.1 ± 0.2a**	**0.7 ± 0.1ab**	**0.5 ± 0.1b**
Ppy	Rn00561768_m1	1.0 ± 0.1a	0.7 ± 0.2ab	0.5 ± 0.1b

Different letters indicate the statistically significant differences between treatments (P-value $\leqslant 0.05$).
The genes in bold type were detected in the proteomics study.

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 eight proteins was not modified in ZF versus ZL but was down-regulated in ZF + GSPE versus ZF; and Up, in which the expression of 11 proteins was mainly not modified in ZF but was up-regulated 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.

3.3. 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 (Fig. 3A and B). Proteins in red are up-regulated and proteins in green are down-regulated. Solid lines represent direct relationships, dotted lines represent indirect interactions and different shapes represent different functional types of proteins (see legend (Fig. 3)). Network A, with a score of 48 (P-value = 10^{-48}), showed 35 proteins and included the following top functions: free radical scavenging, cell function and maintenance and cellular compromise. 24 of these 35 proteins were differentially expressed in the GSPE-treated rats' proteome and most of them were enclosed in the biological processes obtained in the GO analysis using DAVID (Fig. 1). Network B had a score of 25 (P-Value = 10^{-25}) and included the following top functions: drug metabolism, lipid metabolism and molecular transport. It showed 34 proteins, 11 of which were detected in ZF + GSPE rats proteome as differentially expressed. These 11 proteins were located in the periphery of the network.

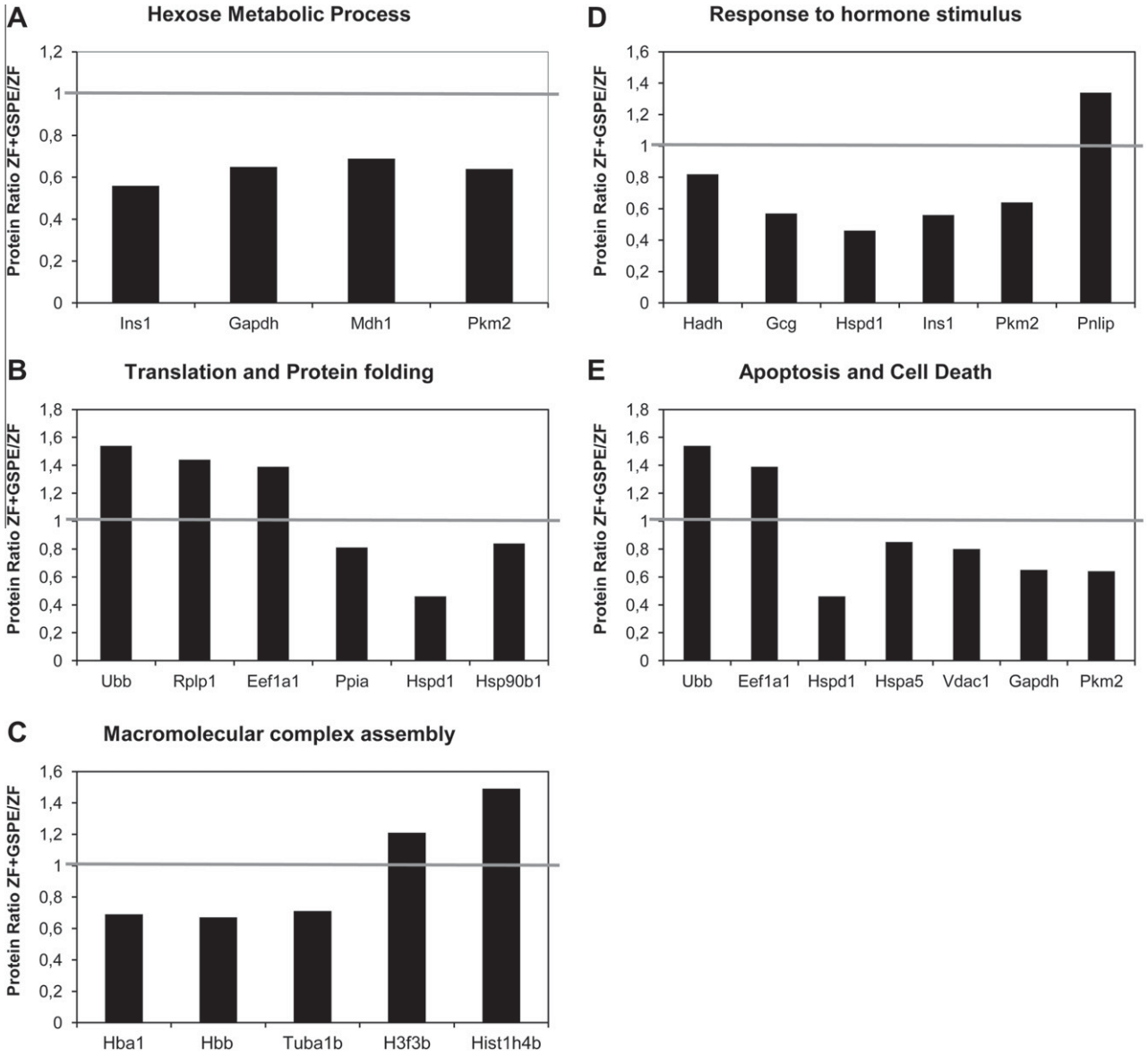

Fig. 1. Biological processes of proteins whose expression was significantly modulated by GSPE treatment assessed using the DAVID database. The proteins involved in (A) hexose metabolic process, (B) translation and protein folding, (C) macromolecular complex assembly, (D) response to hormone stimulus,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

3.4. 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. The results indicate that apoptosis was increased in the islets from ZF and ZF + GSPE rats compared to the islets from ZL rats (0.05 ± 0.0 AU/μg protein in ZL, 0.16 ± 0.2 in ZF and 0.16 ± 0.0 in ZF + GSPE). 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).

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, glucagon and Vdac1 followed the same profile that was obtained in the proteomic experiment. Glucagon protein levels were significantly down-regulated 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 down-regulated in ZF + GSPE compared to ZF. Insulin gene expression was up-regulated in ZF compared to ZL. GSPE counteracted this effect. In the same way, Vdac1 protein levels were up-regulated in ZF compared to ZL and GSPE counteracted this effect. It was found that the mRNA levels of this gene followed the same profile. In addition, the effect of GSPE down-regulating Cpe protein levels was also observed at gene expression level. The same gene expression profile of insulin was also observed in the expression of the transcription factor that regulates insulin gene expression, i.e., Pdx1; in one of the regulators of insulin secretion, i.e., glucokinase; and in a protein that can alter the insulin secretion process, i.e., Ucp2 (Table 3).

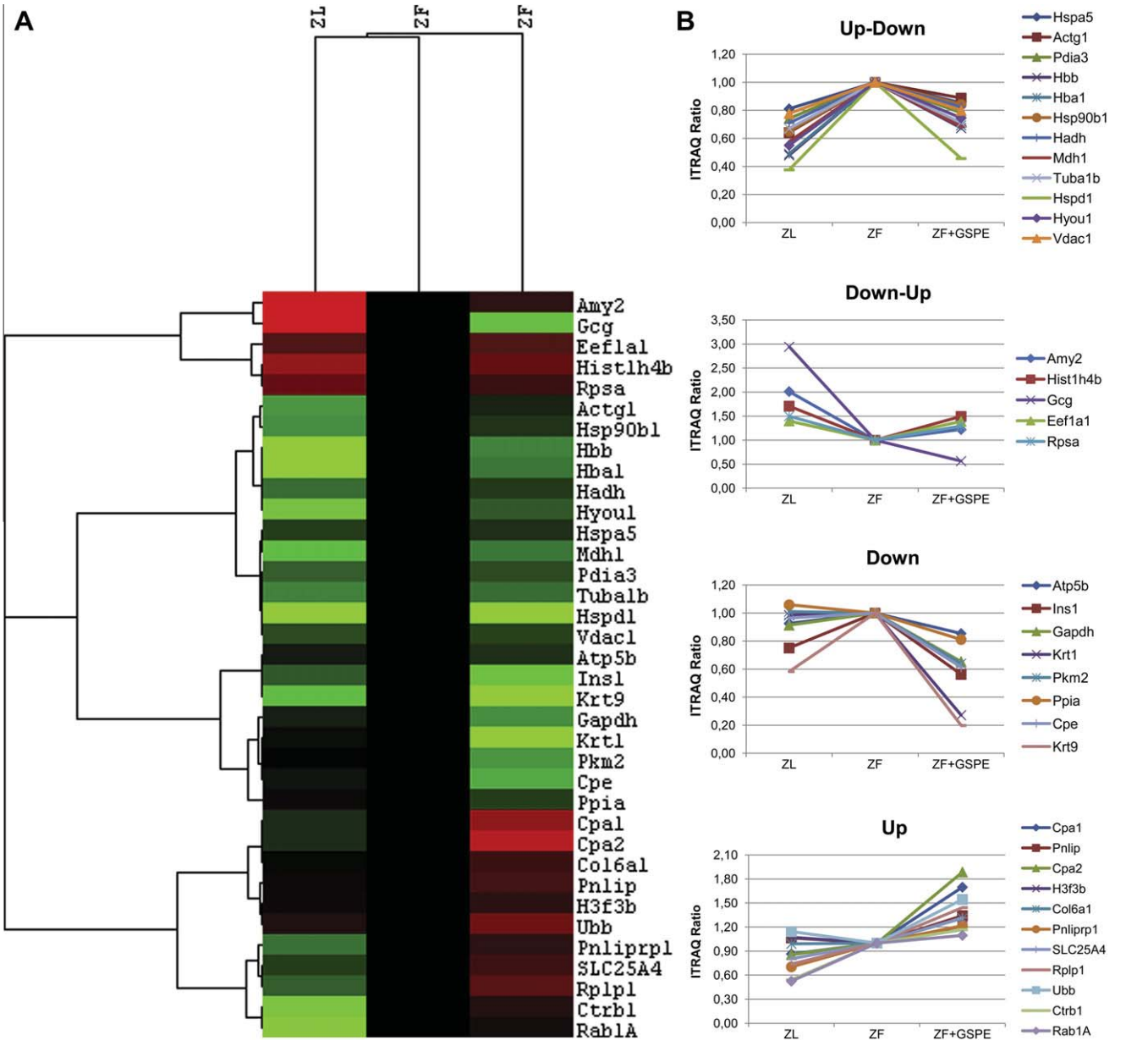

Fig. 2. (A) Hierarchical clustering for differentially expressed proteins performed using the Cluster 3.0 programme (de Hoon et al., 2004). Red gradients represent up-regulated proteins, and green gradients represent down-regulated proteins compared to ZF rats (which ratio is 1, represented in black). (B) Six different clusters were derived from the analysis and are represented in different graphics.

4. 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 (Pinent et al., 2008). Since obesity is associated with insulin resistance, ZF rats are a useful model in which to analyse the effects of natural compounds in pancreatic islets under conditions of insulin resistance. Therefore, the aim of the present study was to analyse the effect of GSPE on the proteomic profile of isolated pancreatic islets of Zucker fatty rats. For this purpose, a proteome analysis was carried out and 31 proteins differentially expressed in islets isolated from ZF + GSPE rats compared to ZF rats were obtained. 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, the effect of the genetic obese background was also analysed by comparing ZF and ZL rats. A similar proteome analysis had previously been performed (Han et al., 2011). Cluster 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.

The 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

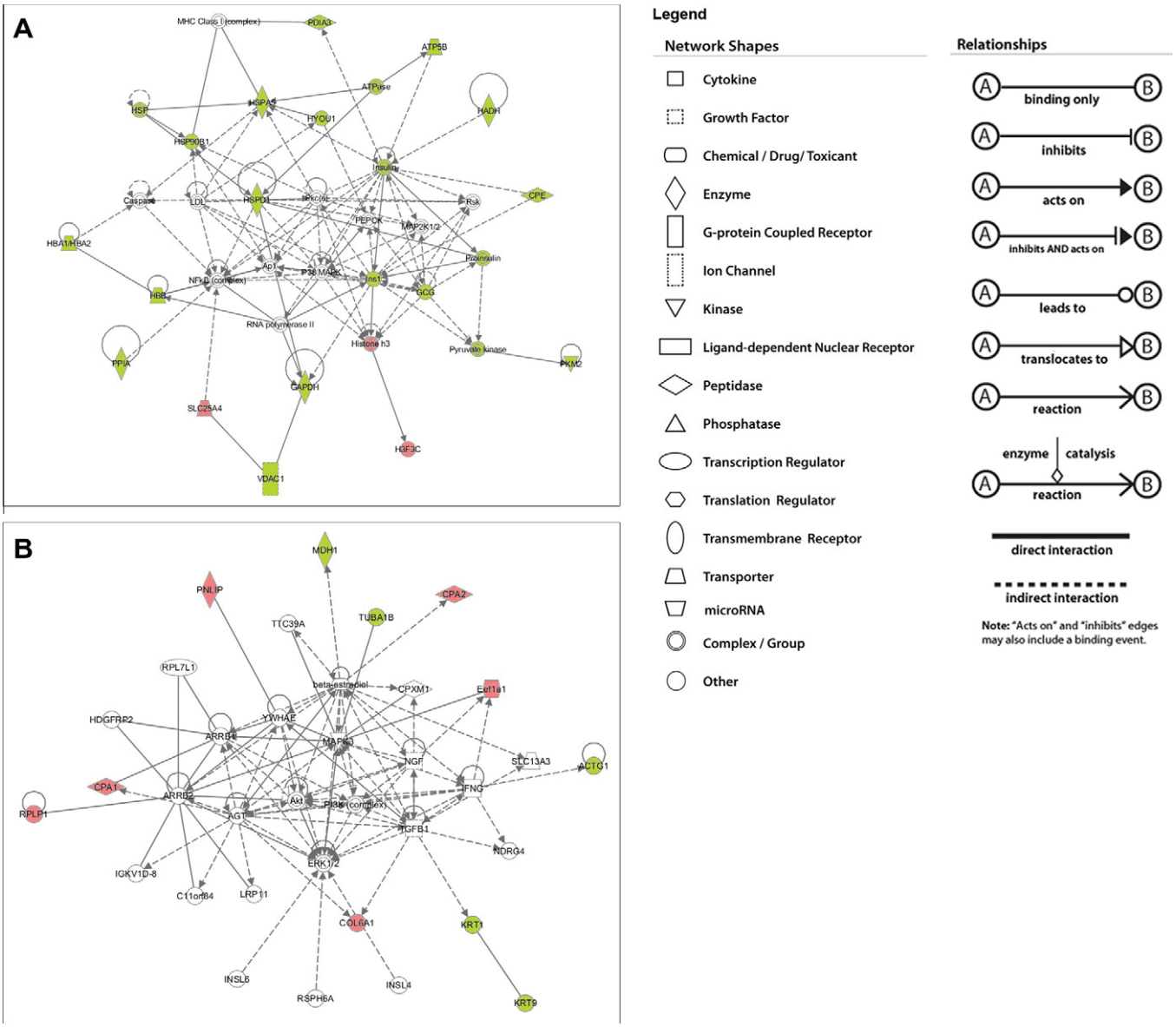

Fig. 3. Hypothetical networks associated with the proteins differentially expressed following treatment of GSPE in Zucker Fatty rats generated by Ingenuity Pathway Analysis software. In the analysis, two networks were generated, (A) and (B). Proteins are represented as nodes with different shapes that represent different functional types of proteins (see legend). Up-regulated proteins are shown in red and down-regulated 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 (see legend).

decreased the cell viability of human colon cancer HT-29 cells (Chung, Miranda, Stevens, & Maier, 2009), and dimeric procyanidins produced significant cytotoxicity in numerous human cancer cell lines (Actis-Goretta, Romanczyk, Rodriguez, Kwik-Uribe, & Keen, 2008). 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 (Zhong, Cong, & Zhang, 2007) and they protected against cardiac cell apoptosis via the induction of endogenous antioxidant enzymes (Du, Guo, & Lou, 2007). Previous experiments showed that GSPE modulates apoptosis markers in the pancreas (submitted results); therefore, this subcategory was analysed in more detail. Apoptosis and apoptosis markers in the pancreatic islets were checked. 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 (Borradaile et al., 2006; Han et al., 2011); this treatment also decreased the levels of chaperones involved in endoplasmatic reticulum (ER) homeostasis and the unfolded protein response (UPR) following ER stress (Hspd1 (Parcellier, Gurbuxani, Schmitt, Solary, & Garrido, 2003), Hspa5 (Laybutt et al., 2007), and Hsp90b1 (Bando, Katayama, Aleshin, Manabe, & Tohyama, 2004)). Procyanidins also decreased at gene and protein level Cpe, which positively controls β-cell survival via effects on ER stress (Johnson, 2009) and Vdac1, the mitochondrial element of the cell-death pathway (Ahmed, Muhammed, Kessler, & Salehi, 2010). All of these proteins were mapped and connected in the Network A and 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. This activates the UPR, which mitigates stress. ER stress seems to be one of the molecular mechanisms of beta-cell dysfunction contributing to diabetes (Fonseca, Urano, Burcin, & Gromada, 2010). Analysis of differentially expressed proteins in Zucker diabetic rats compared to ZL rats showed a reduction of Hspd1/Hspe1 chaperone complex (Han et al., 2011). GSPE decreased protein levels of the chaperones Hspd1, Hsp90b1, and Hspa5, proteins that promote protein folding and degradation (Kriegenburg, Ellgaard, & Hartmann-Petersen, 2011); 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 (Ahmed et al., 2010; D'Hertog et al., 2010; Maris et al., 2010). GSPE treatment also increased Polyubiquitin-B (Ubb), a molecule that targets misfolded proteins for degradation (Kriegenburg et al., 2011). Taken together these results could point 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. 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 the 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 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, hexose metabolic process and response to hormone stimulus that were also identified as being modified at the protein level by GSPE treatment. Although it was found that these biological processes were targets of procyanidins in the clustering analysis, changes due to the fa/fa genotype were not detected. 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 the previous studies that describe procyanidins as modulators of glucose homeostasis (Montagut et al., 2010; Pinent, Cedó, Montagut, Blay, & Ardévol, 2012) and insulin metabolism (Castell-Auví et al., 2012a; Montagut et al., 2010). The reduced insulin protein and gene expression in the islets confirm GSPE as repressor of insulin production, as was previously suggested (Castell-Auví et al., 2012a). The down-regulation of insulin production is also supported by the action of GSPE as a repressor of Pdx1 (Docherty et al., 2005) gene expression, as was observed in previous studies (Castell-Auví et al., 2012a) (and submitted results), and as a repressor of Cpe gene expression and protein levels (Hutton, 1994). 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 (Boyer, 1997). Reduced expression of a mitochondrial metabolic enzyme, malate dehydrogenase, can also contribute to decreased insulin secretion (Jitrapakdee, Wutthisathapornchai, Wallace, & MacDonald, 2010). One of the key regulators of insulin secretion is glucokinase (Matschinsky, 2002). Although glucokinase protein levels were not modified by GSPE treatment, gene expression analysis showed that procyanidins inhibited glucokinase mRNA levels. Therefore, the present data suggests that GSPE was able to affect insulin secretion. GSPE limits insulin synthesis and secretion, as it was found in other assayed animal models, i.e., healthy animals (Castell-Auví et al., 2012a) and cafeteria-fed animals (Castell-Auví et al., 2012b).

5. 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.

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4. Procyanidins Modulate MicroRNA Expression in Pancreatic Islets

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Once the effects of GSPE on proliferation and apoptosis in the pancreas were analysed in insulin resistance and pre-diabetes states using cafeteria- and genetically-induced obesity rats, the effects of the extract on proliferation and apoptosis in healthy rats were analysed.

The results presented here concerning the immunohistochemistry study analysing apoptosis and β-cell mass were conducted in collaboration with the Laboratoire of Biologie et Pathologie du Pancréas Endocrine at the University Paris-Diderot.

MicroRNAs (miRNAs) can regulate pancreas function and apoptosis, and miRNA expression is modulated by procyanidins in other tissues and cell types. Therefore, the miRNA profiles of the rat islets were analysed to determine whether miRNA modulation could be a mechanism of action for procyanidins in the pancreas.

The miRNA profile study was conducted with my colleague Anna Castell Auví. Only the results related to the effects on β-cell mass and apoptosis belong to this thesis.

Procyanidins Modulate MicroRNA Expression in Pancreatic Islets

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ABSTRACT

Procyanidins modulate glucose metabolism, partly due to its effects on pancreas. Given the role of microRNAs (miRNAs) in the regulation of diabetes and the fact that flavonoids modulate miRNAs in other tissues, we hypothesized that procyanidins might target miRNAs in the pancreas. We investigated the miRNA expression profile in pancreatic islets isolated from rats treated with a daily dose of grape seed procyanidin extract (GSPE) (25 mg/kg of body weight) for 45 days. The miRWalk database identified putative target genes of these miRNAs. We found that GSPE altered significantly the expression of miR-1249, miR-483, miR-30c-1*, and miR-3544. *In silico* prediction studies suggested that ion transport and response to glucose are among the regulated pathways. As a conclusion, this is the first study showing that procyanidins can also exert their bioactivity on pancreatic islets by modifying the miRNA expression pattern.

KEYWORDS

microRNA, procyanidins, pancreatic islets, apoptosis.

INTRODUCTION

MicroRNAs (miRNAs) are a family of small non-coding RNAs that post-transcriptionally regulate gene expression.[1] Each miRNA gene encodes a mature miRNA that is approximately 22 nucleotides in length. MiRNAs play predominantly inhibitory regulatory roles by binding to *cis*-elements in the 3'-untranslated region (UTR) of message-encoding RNAs.[2] This inhibition occurs by one of three mechanisms that are not mutually exclusive: target cleavage, repression of target translation, and message degradation in cytoplasmic P-bodies.[3] MiRNAs are important not only for normal organism development and physiology but also in cancer, heart disease and inflammation.[2]

MiRNAs are also involved in diabetes. Studies in the Goto-Kakizaki rat, a model of spontaneous lean type 2 diabetes, showed differential expression of fifteen miRNAs in the skeletal muscle of these animals compared to Wistar control rats.[4] Herrera et al. recently showed that the expression pattern of five miRNAs in insulin target tissues is modified by hyperglycemia, suggesting a role for these miRNAs in the pathophysiology of type 2 diabetes.[5] In addition, recent studies have demonstrated that miRNAs are required for pancreas development[6, 7] and the regulation of glucose-stimulated insulin secretion.[8, 9] The most studied miRNA molecule in the pancreas is miR-375. Studies have shown that miR-375 plays a role in pancreatic islet cell viability and function, and its knockdown or overexpression profoundly affects glucose metabolism.[2, 10] Other miRNAs have been shown to regulate pancreas function, including the regulation of insulin secretion by modulating the level of key components of exocytosis process and insulin biosynthesis (see Table 1). Some miRNAs have also been related to β-cell apoptosis. Given the role of miRNAs in regulating processes that are important in disease states, including diabetes, targeting miRNAs with bioactive compounds may be a potential therapeutic strategy.

Procyanidins are phenolic compounds that are found in fruits, vegetables, chocolate and beverages such as wine and tea.[11] Procyanidins modulate glucose metabolism by modifying both glycemia and insulinemia (reviewed in [12]). Procyanidins act as insulin-mimetic likely by targeting the liver and peripheral tissues. In glucose-altered metabolism, procyanidins prevent the induction of damage (fructose-feed models) and/or use alternative targets to exert their insulin-mimetic effects.[12] Our recent experiments also suggest that the pancreas is a target of procyanidins. We found that grape seed procyanidin extract (GSPE) affected insulinemia by modifying β-cell functionality and/or insulin degradation.[13] Some of these effects could be explained by its effect on membrane potential,[13] but we do not discard some other mechanistic explanation. Besides grape seed procyanidins modulate apoptosis in Zucker Fatty rats[14] and cafeteria-fed rats.[15]

A few recent studies have shown that flavonoids can alter miRNA expression profiles. It has been demonstrated that GSPE modulates miRNA expression profiles in the hepatocyte cell line HepG2,[16] and repress miR-122 and miR-33 in the liver.[17] Coffee polyphenols increase miR-122 expression in Hepa 1-6 cells,[18] and quercetin downregulates the proinflammatory miR-155 in murine RAW264.7 macrophages.[19] Recently Milenkovic described five hepatic miRNAs commonly modulated by nine different polyphenols.[20] Furthermore, flavonoids that have cancer preventative effects, such as genistein and epigallocatechin gallate, also modify miRNA expression profiles in human uveal melanoma cells[21] and in HepG2 cells,[22] respectively.

However, the majority of these studies were performed *in vitro* using cell lines, but to the best of our knowledge, there are no studies on the effects of flavonoids *in vivo* on the pancreas.

As miRNAs regulate pancreas function and apoptosis, we hypothesized that procyanidins effects in glucose homeostasis might also be mediated via modulating the pancreatic expression of miRNAs. Thus, the aim of this study was to investigate whether chronic procyanidin treatment modifies the expression of miRNAs in rat pancreatic islets and whether this is the mechanism that confers the effects of procyanidins on the pancreas.

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%). For a detailed composition at mg compound/ g of extract please check [23].

Animal procedures

Female Wistar rats weighing 225-250 g were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22 ºC with a 12 h light/12 h dark cycle. Treatment began after 1 week in quarantine. The animals were divided in two groups (10 animals per group): a control group and a group treated for 45 days with 25 mg GSPE per kg of body weight per day. The food (standard chow) was withdrawn at 8 a.m. every day, and at 8 p.m., the rats were treated with GSPE or vehicle (sweetened condensed milk diluted 1:6 with tap water, voluntary oral intake), after which the food was replaced. On the day of sacrifice, the animals, which had fasted overnight (fasting starts at 0.00 am), were anesthetized at 9 a.m. using sodium pentobarbital (75 mg/kg of body weight) and then sacrificed by exsanguination. The blood was collected, and pancreatic islets were isolated from ten animals per group. 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 the Government of Catalonia: 4250).

Islet isolation

The islets were prepared by collagenase digestion as previously described.[13] 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 its removal and incubation at 37 ºC for 15 min. The islets were purified on a Histopaque gradient (Sigma-Aldrich, St. Louis, MO) and selected by hand until a population of pure islets was obtained.

MiRNA profile analysis

The total RNA from freshly isolated islets was extracted with the Qiagen miRNeasy isolation kit (Qiagen, Barcelona, Spain) and stored at -80 ºC. The quality of the total RNA was determined with the Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit according to the manufacturer's instructions. All samples have a ratio of 260/280 absorbance of approximately 2.0 and a ratio of 260/230 absorbance greater than 1.4. We pooled the samples form 5 animals per treatment, i.e. two samples from a pool of two animals and one sample, only from one animal. Then we analyzed three samples from each treatment with a Geniom Realtime Analyzer (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*).[24] For each array, the RNA was suspended in febit's proprietary miRNA Hybridization Buffer (25 µL per array). Hybridization was performed automatically for 16 h at 42 ºC using the GRTA. Next, the biochip was stringently washed. Following the labeling procedure, febit was applied to the microfluidic-based primer extension assay.[25] This assay utilizes the bound miRNAs as a primer for enzymatic elongation using labeled nucleotides. The elongation was performed with Klenow fragment and biotinylated nucleotides at 37 ºC for 15 min. Finally, the Biochip was washed automatically. For maximum sensitivity, febit method used biotin and its detection with streptavidin-phycoerythrin (SAPE) in combination with febit's consecutive signal enhancement (CSE) procedure. The feature recognition (using Cy3 filter set) and signal calculation were performed automatically within milliseconds. The Geniom technology provides accurate detection of miRNA profiles. The microarray data were normalized by the variance stabilization normalization method,[26] and the statistics were analyzed with linear models as implemented in the limma Bioconductorpackage.[27] Fold changes with adjusted p-values less than 0.2 were considered significant.

Pathway analysis and prediction

The predicted target genes for rno-miRNAs that had statistically significant changes in expression after GSPE treatment were obtained from the online database miRWalk.[28] MiRWalk miRNA target prediction was performed 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 target genes, we used the DAVID (Database for Annotation, Visualization and Integrated Discovery).[29] 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 a p-value less than 0.05 after adjustment using the Benjamini method were considered significantly enriched.

To gain further knowledge of the pathways modified by procyanidins through miRNA the list of the common predicted target genes 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 the predicted target genes and genes 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 genes in a network could be generated randomly. A score of 3 indicates that there is a 1 in 1000 chance that the focus genes 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 (Ingenuity Systems, Inc., n.d.).

Measurement of apoptosis and proliferation marker expression

For gene expression experiments, the total RNA from freshly isolated islets was extracted as described above (Qiagen miRNeasy isolation kit). RNA (0.5-1 µg) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and cDNA was amplified for 40 cycles using a quantitative RT-PCR 7300 System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions with TaqmanMaster Mix and the following Taqman probes (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

Blocks of paraffin-embedded pancreases were serially sectioned (6 µm) and mounted on slides. One section of every 40 was deparaffinized, rehydrated and analyzed for apoptosis. Apoptotic cells were detected with the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA) as previously described.[30]

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

Analysis of the sections was performed with an Olympus BX40 microscope in conjunction with a video camera connected to a computer and 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. The rate of apoptosis is expressed as the number of apoptotic cells/total pancreas surface area (μm^2) multiplied by 10^7, and β-cell mass was determined as the percentage of the ratio between the β-cell surface and the total pancreatic surface area.

Calculations and Statistical Analysis

The results are expressed as the mean ± SEM. The effects were assessed using Student's *t*-test. All calculations were performed with SPSS software.

RESULTS

Global miRNA expression profiling in islets of GSPE-treated rats.

To analyze whether GSPE alters the miRNA expression profile in the pancreas, we treated rats with a daily dose of GSPE for 45 days. Total RNA was obtained from freshly isolated islets, and the expression of 680 miRNAs was analyzed. In Figure 1, the 50 miRNAs with the highest fold changes are shown. We found 4 miRNAs with significantly different expression after GSPE treatment (Table 2): miR-1249, miR-483, miR-30c-1*, and miR-3544. All of these were downregulated except for miR-3544, which was upregulated by GSPE treatment.

Integrative analysis of miRNA target genes and pathways

To investigate the function of these differentially expressed miRNAs, we analyzed their putative target genes using the miRWalk database.[28] MiR-1249 and miR-3544 do not have any predicted targets. MiR-483 has 1592 predicted targets, and miR-30c-1* has 2442 predicted targets.

To further define the effects of GSPE in the pancreas that are mediated by miRNAs, we explored whether the miRNAs modified by GSPE have common targets. We found that miR-483 and miR-30c-1* have 599 common targets. We next performed an *in silico* prediction to determine the functions of these predicted target genes using the ontology classification of genes based on gene annotation and summary information available through DAVID. This approach provides insights on which biological processes are modified by altered miRNA expression after GSPE treatment. We restricted the analysis to common predicted targets of miR-483 and miR-30c-1* (the other two miRNAs had no predicted targets) because it has been shown that the effects of binding multiple miRNA complexes to the 3′-UTR are cooperative; therefore, effects greater than those mediated by a single miRNA can be exerted.[31] As we are using predicted targets, we focused on the common targets that could suggest signaling pathways that are likely to be affected.

The significantly enriched Gene Ontology (GO) terms in the common predicted miRNA target genes are listed and classified in Table 3 according to the "biological processes" in which they are involved. The detailed list of predicted targets is provided as Table S1. The results indicate that most of the common miR-483 and miR-30c-1* target genes are involved in ion transport, response to stimulus such as hormones and organic substances, neuron differentiation and development, and transmission of nerve impulses. Moreover, the analysis of the GO "cellular component" category of the significantly enriched GO terms indicates that the majority of the common target genes are located on the plasma membrane, which could be predicted by their function.

To gain further knowledge we imported the common predicted miRNA targets into the Ingenuity Pathway Analysis software (Figure 2A, 2B, 2C and 2D). We selected the best scored pathways defined by the program, i.e. those with a score higher than 30, to identify the most suitable pathways regulated by miRNA in pancreatic islets. As indicated by GO analysis, most of proteins corresponding to predicted genes (the grey one's) were in plasma membrane. Figure 2A showed that most of them interact to AKT. On figure 2B the central focus of all the interactions was ERK 1/2; on figure 2C the central point was p38, and figure 2D showed a less clear unique central target, but it reinforces ERK target.

GSPE does not affect apoptosis in islets of healthy rats

MiRNA modify β-cell apoptosis[32] and we know that procyanidins can modulate apoptosis markers in Zucker Fatty rats[14] and cafeteria-fed rats.[15] Since we are working on healthy animals, we checked if there was some apoptotic effect due to GSPE treatment. Immunohistochemical analysis did not reveal apoptosis in islets, and apoptosis in the exocrine pancreas was not altered by treatment with GSPE (Figure 3A). The β-cell mass was not altered by treatment with GSPE (Figure 3B). We also analyzed the expression of the anti-apoptotic marker Bcl2 and the proliferation markers Cyclin D2 and MKI67. The islets of the rats treated with GSPE showed a trend of ($p < 0.1$) decreased expression of Bcl2 (0.75 ± 0.09 vs. 1.02 ± 0.10), while MKI67 expression did not change (0.71 ± 0.22 vs. 1.02 ± 0.11). However, Cyclin D2 was significantly downregulated by treatment with GSPE (0.77 ± 0.05 vs. 1.02 ± 0.10) ($p < 0.05$).

DISCUSSION

In this study we show for the first time that grape seed procyanidins modulate miRNA expression in pancreas: it down-regulated miR-1249, miR-483, miR-30c-1*, and up-regulated miR-3544. Most of previous studies, regarding regulation of miRNAs by flavonoids, in different cell lines, did not identify any of these miRNAs as regulated by flavonoids.[16, 19, 21, 22] However, recently, Milenkovic, working on mice, showed five miRNAs (miR-291b-5p, miR-296-5p, miR-30c-1*, miR-467b* and miR-374*) being commonly modulated by nine different polyphenols in the diet.[20] Despite that the work of Milenkovic was centered on liver tissue, they also found one miRNA common to our results, miR-30c-1*. This reinforces that it is a target for flavonoids. Besides, another of the four miRNAs that we found, miR-483, was also identified by Milenkovic as a target of mice that ingested narangin or curcumin.

Previous studies have reported that miRNAs play a role in the regulation of insulin secretion in rodent pancreatic β-cell lines (MIN6, INS-1E, and MIN6 B1 cells). Specifically, miR-375 has been well characterized in this function, but more recent studies have also suggested a role for miR-9, miR-124a, and miR-96, among others.[8, 9, 33, 34] All of these miRNAs were present in our biochip, but GSPE did not alter their expression. Actually of the nine flavonoids tested in the study of Milenkovic, only quercetin, which is not found in our extract, modulated miR-375. Instead, GSPE modulated the expression of other miRNAs for which, to our knowledge, a role in the pancreas has not been previously described. There is very little available information on the roles of these miRNAs. Only miR-483, which is a malignancy marker in adrenocortical tumors in humans,[35] and

miR-30c-1*, which is associated with the recurrence of non-small cell lung cancer following surgical resection in humans, have been studied.[36] To overcome this limitation we used bioinformatic tools to elucidate the main β-cell functions modulated by these miRNAs.

MiRWalk database predicted 599 common targets of two of the miRNAs obtained. These targets were classified according to GO classification. Our results showed that the majority of the common target genes of the GSPE-modulated miRNAs are located on the plasma membrane. In this regard, our previous studies in pancreatic INS-1E cells have suggested that GSPE functions by altering membrane potential.[13] Indeed, we had previously shown that the experimental design used in the present study led to altered glucose-stimulated insulin secretion in the islets isolated from GSPE-treated rats.[13] This effect could be partially mediated by the modification of cell and mitochondrial membrane potentials by GSPE, which has been observed in the INS-1E cells. The role of other miRNAs in the regulation of pancreas function by modulating membrane proteins has previously been demonstrated. For example, miR-15a inhibits the expression of uncoupling protein-2,[37] a mitochondrial inner membrane uncoupler that modifies mitochondrial membrane permeability. Additionally, the expression levels of the plasma membrane monocarboxylaten transporter-1 are decreased in pancreatic β-cells at least in part by miRNAs (miR-29a,miR-29b, and miR-124), thereby affecting insulin release.[38] Therefore, the effects of GSPE on the function of islets could in part be due to the effects of GSPE on the expression of miRNAs, which would contribute to changes in the cell and mitochondrial membrane permeability by varying the expression of ion transport proteins. To gain further knowledge on these possibilities, we charged the 599 predicted genes in Ingenuity System Pathway Analysis. This approach depicted networks modulated by these miRNA with some very clear nodes. These nodes correspond to proteins that, in previous studies from our research group, have been showed as modulated by procyanidins: AKT in adipocytes,[39] p38 and ERK in human monocytes.[40, 41]

The amount of insulin production, i.e. the central function of β-cell is highly dependent on the right synthesis and secretion of insulin, but also on the amount healthy β-cells. Our results support that procyanidins, through miRNA, could alter insulin secretion. But most of the published studies on flavonoids and miRNAs have reported on the activity of these phenolic compounds in cancer, in which they regulated apoptosis and proliferation.[42, 22, 43-45, 21, 46] Besides, GSPE modulates apoptosis markers in genetically obese rats[14] and rats fed a cafeteria diet.[15] As above mentioned, we had restricted our bioinformatics analysis to the common predicted targets of the differentially expressed miRNAs, although this could leave out of the analysis other pathways modified by one of the miRNAs. Thus, given the evidences that flavonoids affect miRNAs involved in apoptotic processes, we also evaluated the possibility that the β-cell mass and apoptosis was affected by the current treatment. Our results showed that GSPE did not modulate the islet content or apoptosis in the pancreas. This reinforces the bioinformatics analysis that did not show apoptosis pathways controlled by the miRNAs.

In conclusion, we show that chronic GSPE treatment in rats modulates the miRNA expression profile in pancreatic islets, downregulating the expression of miR-1249, miR-30c-1*, and miR-483 and upregulating miR-3544. The limited amount of information available on these miRNAs makes it difficult to describe the consequences of their modulation by GSPE. Our *in silico* prediction studies combined with observations in previous cell culture studies suggest that ion transport and

responses to glucose might be among the pathways affected. Thus, we have described a new mechanism of the effects of procyanidins on the pancreas.

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TABLES

Table 1. MiRNA action on β-cells.

MicroRNA	Effects on β-cells	References
miR-9	Modify insulin secretion by modulating the level of key components of the exocytosis process	9, 47
miR-124a		33, 34
miR-96		33, 47
miR-375		2, 10
miR-130a		48
miR-200		48
miR-410		48
miR-33a		49
miR-30d	Modify insulin biosynthesis	50, 51
miR-7		52
miR-15a		37
miR-19b		53
miR-24		54
miR-26		54
miR-148		54
miR-182		54
miR-34a	Modify β-cell apoptosis	32
miR-146		32

Table 2. Differentially expressed miRNAs in pancreatic islets isolated from rats treated chronically with a daily dose of grape seed procyanidin extract.

miRNA	Chromosome localization	Fold Change	P Value
upregulated			
rno-miR-3544	6q32	1.40	0.0010
downregulated			
rno-miR-1249	7q34	-1.72	0.0001
rno-miR-483	1q41	-1.39	0.0010
rno-miR-30c-1*	5q36	-1.36	0.0009

Table 3. 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
		Multicellular organismal signalling						
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

FIGURES

Figure 1. Differential expression of miRNAs in control rats (left column) vs. GSPE-treated rats (right column) and a hierarchical clustering/heatmap of the 50 miRNA genes with the highest logFC values. The 4 miRNA genes with the most significantly altered expression are shown (*).The colorgram depicts high (red), average (black), and low (green) expression levels. Each row represents a miRNA, and each column represents a treatment.

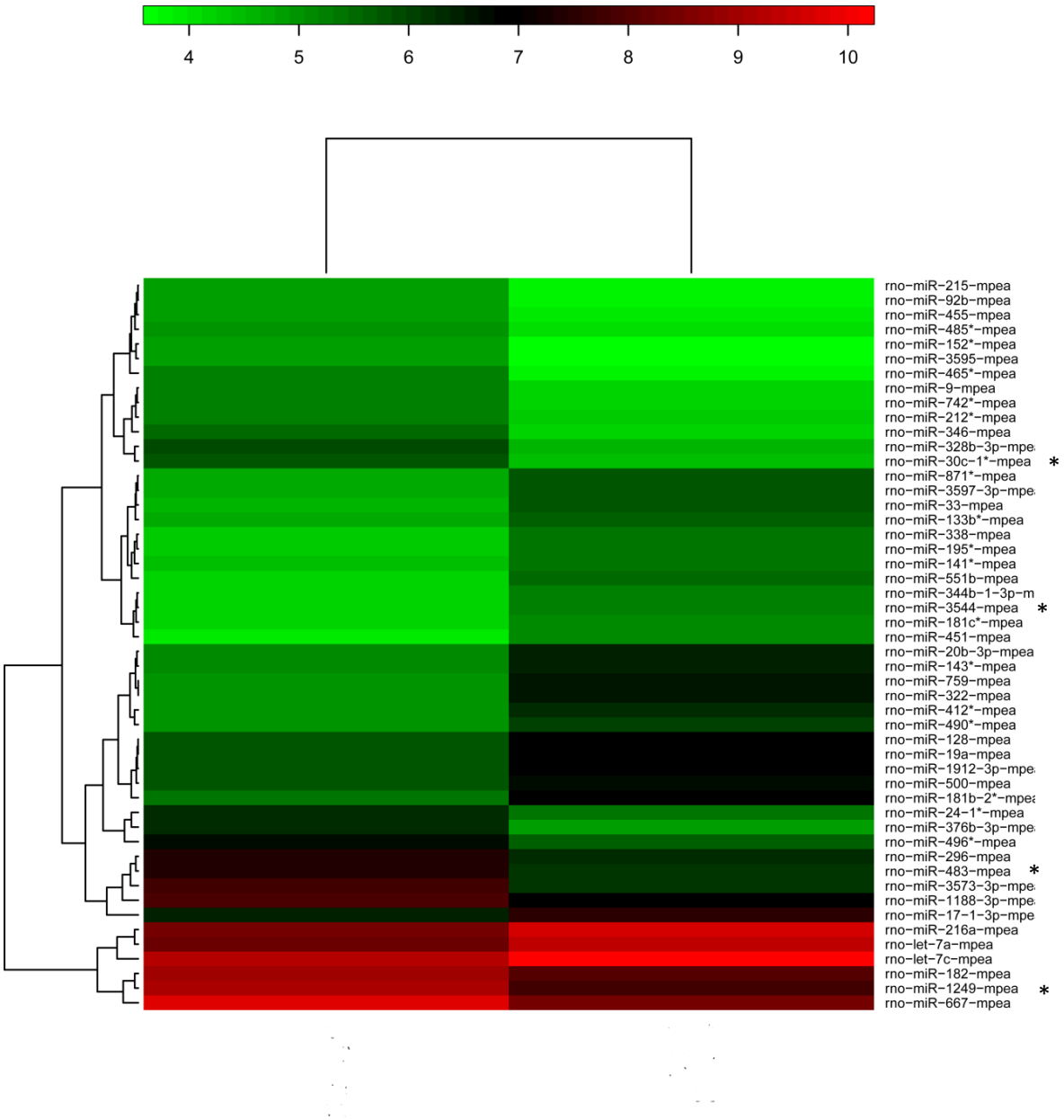
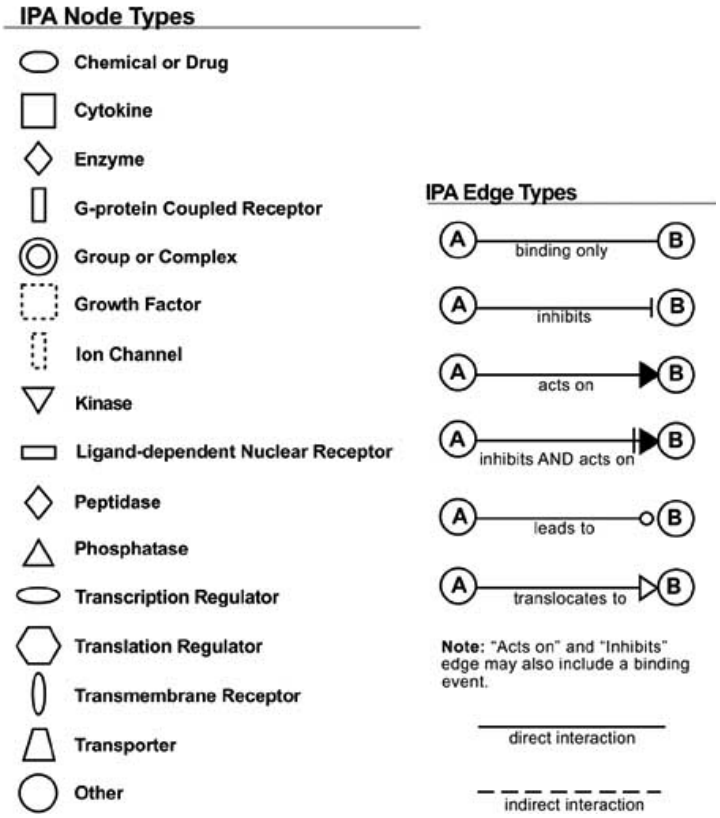
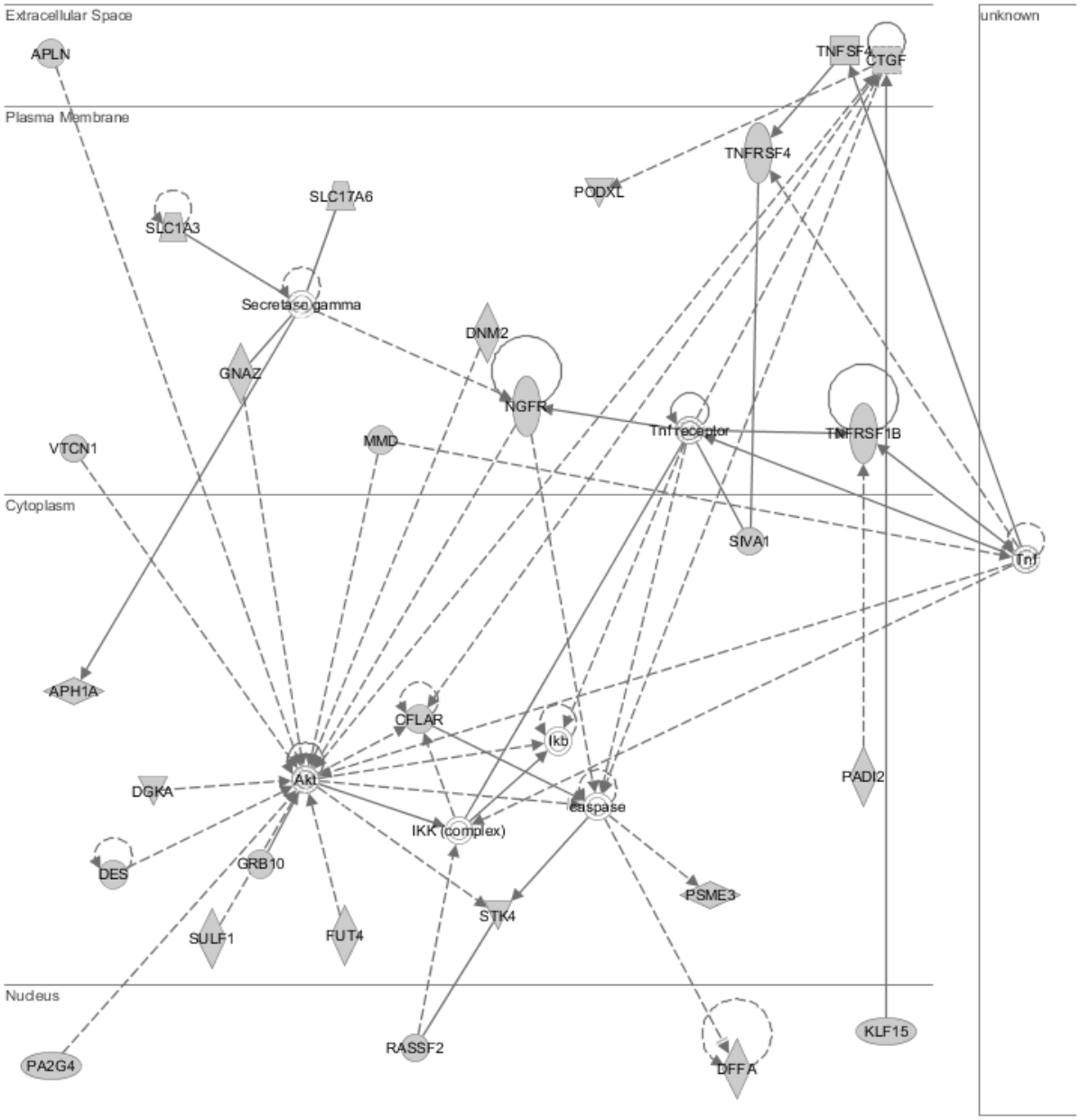


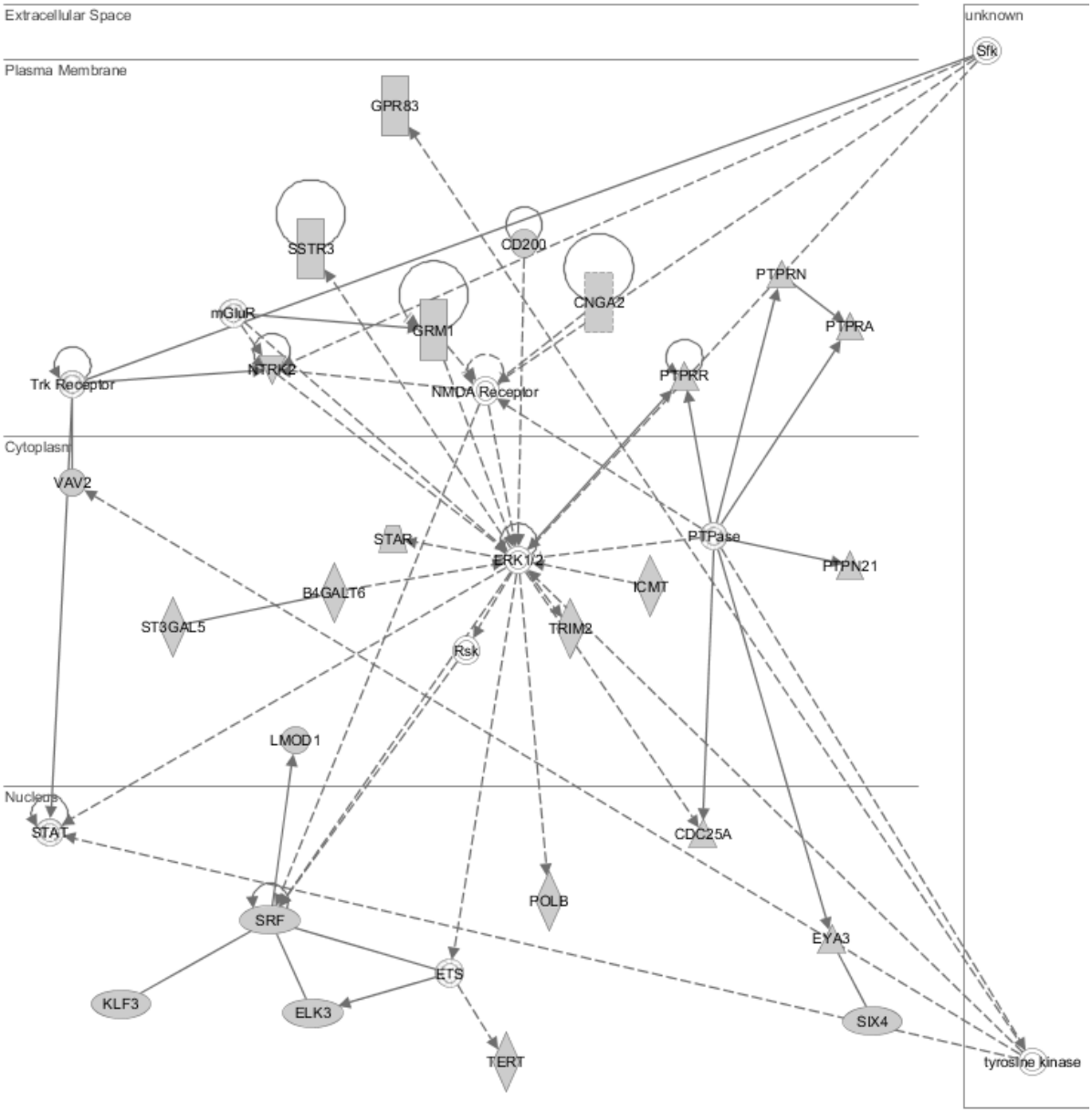
Figure 2. Hypothetical networks associated with the predicted target genes of the significantly altered miRNAs miR-483 and miR-30c-1* in the islets of the GSPE-treated rats generated by Ingenuity Pathway Analysis software. The best scored pathways were selected (a score higher than 30), A-D. Genes are represented as nodes with different shapes that represent different functional type of proteins. The predicted target genes are shown in grey and the genes depicted in white are genes from the Ingenuity database. The relationship between proteins is represented as a line, and the arrowheads indicate the direction of the interaction. A code description is included.



A


Extracellular Space
unknown
APLN
TNFSF4
CTGF
Plasma Membrane
TNFRSF4
PODXL
SLC17A6
SLC1A3
Secretase gamma
DNM2
GNAZ
NGFR
Tnf receptor
TNFRSF1B
VTCN1
MMD
Cytoplasm
SIVA1
Tnf
APH1A
CFLAR
Ikb
Akt
PADI2
DGKA
caspase
IKK (complex)
DES
GRB10
PSME3
STK4
SULF1
FUT4
Nucleus
KLF15
RASSF2
PA2G4
DFFA

B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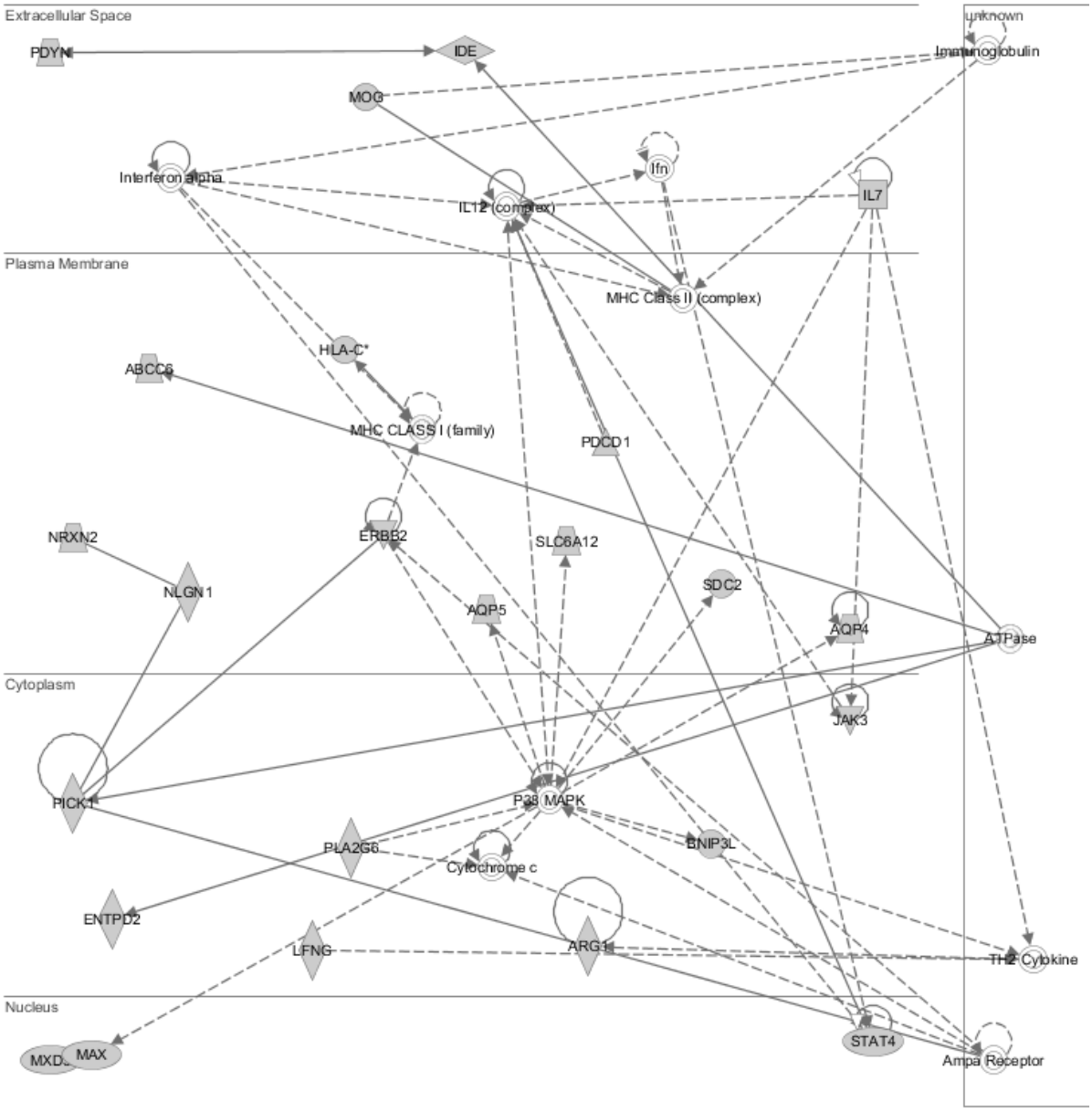
Plasma Membrane
Cytoplasm
Nucleus
unknown
Sfk
GPR83
SSTR3
CD200
CNGA2
PTPRN
PTPRA
mGluR
GRM1
NTRK2
Trk Receptor
NMDA Receptor
PTPRR
VAV2
STAR
ERK1/2
PTPase
PTPN21
B4GALT6
ICMT
ST3GAL5
TRIM2
Rsk
LMOD1
STAT
CDC25A
POLB
SRF
EYA3
ETS
KLF3
ELK3
SIX4
TERT
tyrosine kinase

C


Extracellular Space
unknown
PDYN
IDE
Immunoglobulin
MOG
Interferon alpha
Ifn
IL12 (complex)
IL7
Plasma Membrane
MHC Class II (complex)
HLA-C*
ABCC6
MHC CLASS I (family)
PDCD1
NRXN2
ERBB2
SLC6A12
NLGN1
SDC2
AQP5
AQP4
ATPase
Cytoplasm
JAK3
PICK1
P38 MAPK
PLA2G6
BNIP3L
Cytochrome c
ENTPD2
LFNG
ARG1
TH2 Cytokine
Nucleus
STAT4
MXD
MAX
Ampa Receptor

D

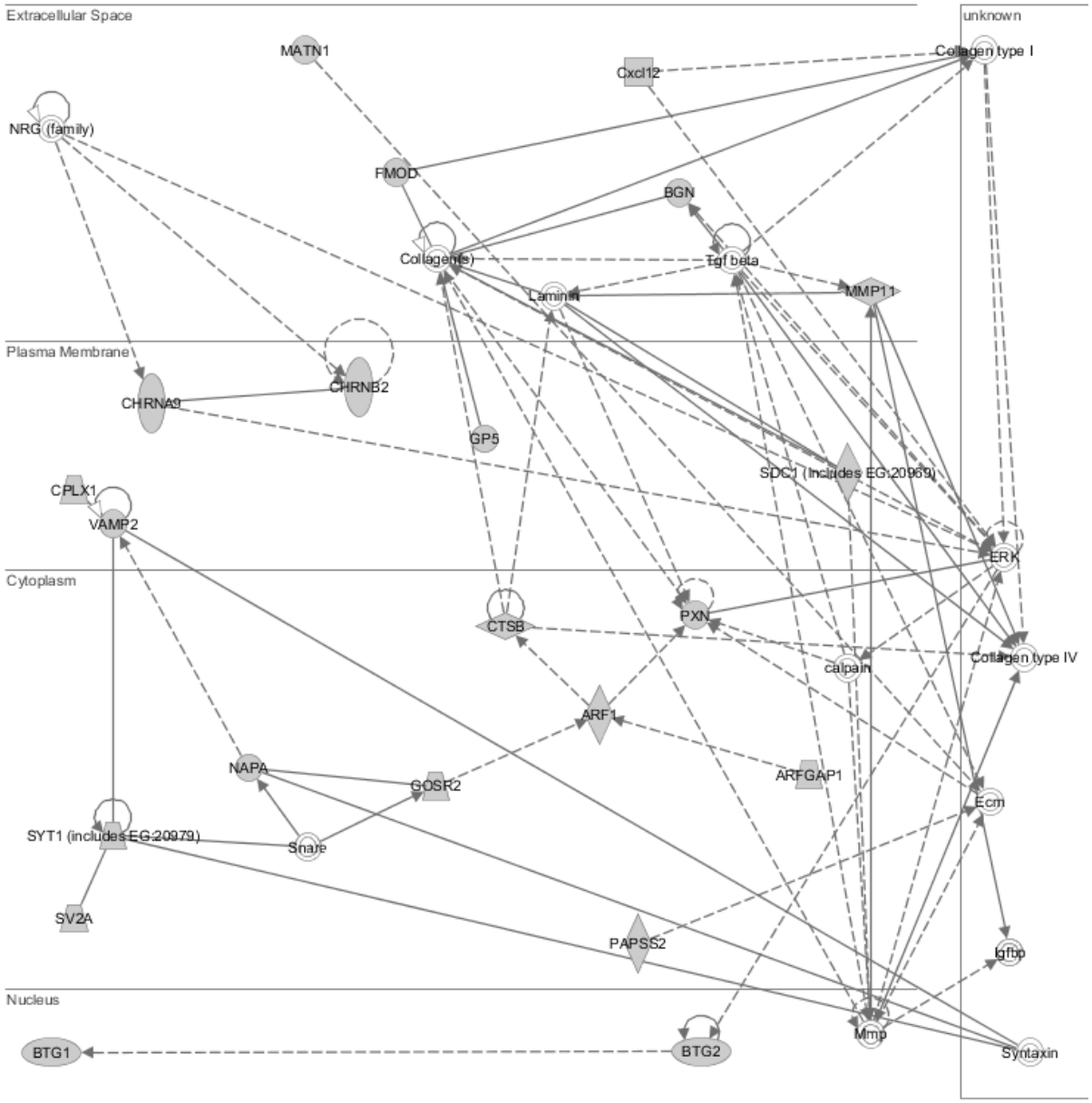
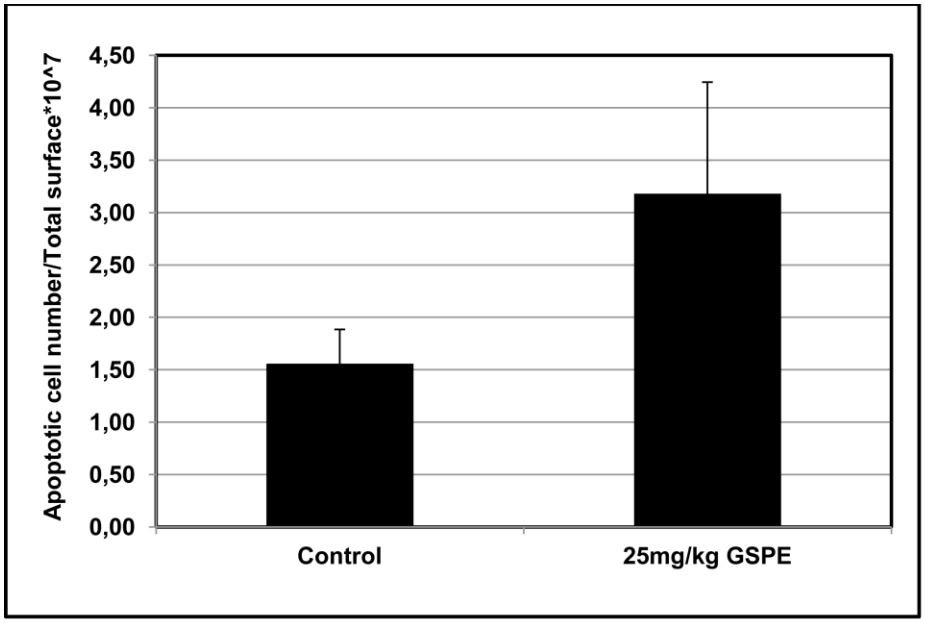

Extracellular Space
unknown
Plasma Membrane
Cytoplasm
Nucleus
MATN1
Cxcl12
Collagen type I
NRG (family)
FMOD
BGN
Collagens
Tgf beta
Laminin
MMP11
CHRNA9
CHRNB2
GP5
SDC1 (includes EG:20969)
CPLX1
VAMP2
ERK
PXN
CTSB
Collagen type IV
calpain
ARF1
NAPA
GOSR2
ARFGAP1
Ecm
SYT1 (includes EG:20979)
Snare
SV2A
PAPSS2
Igfbp
Mmp
Syntaxin
BTG1
BTG2

Figure 3. (A) Apoptosis in the exocrine pancreas measured by TUNEL assay. The rate of apoptosis is expressed as the number of apoptotic cells/total section surface area (μm^2) multiplied by 10^7. Data shown are the mean ± SEM from 3 samples per each group, and 5-6 sections were analyzed for each sample. (**B)** β-cell mass measured by insulin staining. The results were determined as the percentage of the ratio between the β-cell surface and the total pancreatic surface area. Data shown are the mean ± SEM from 4 samples per each group, and 4-10 sections were analyzed for each sample.

A



B

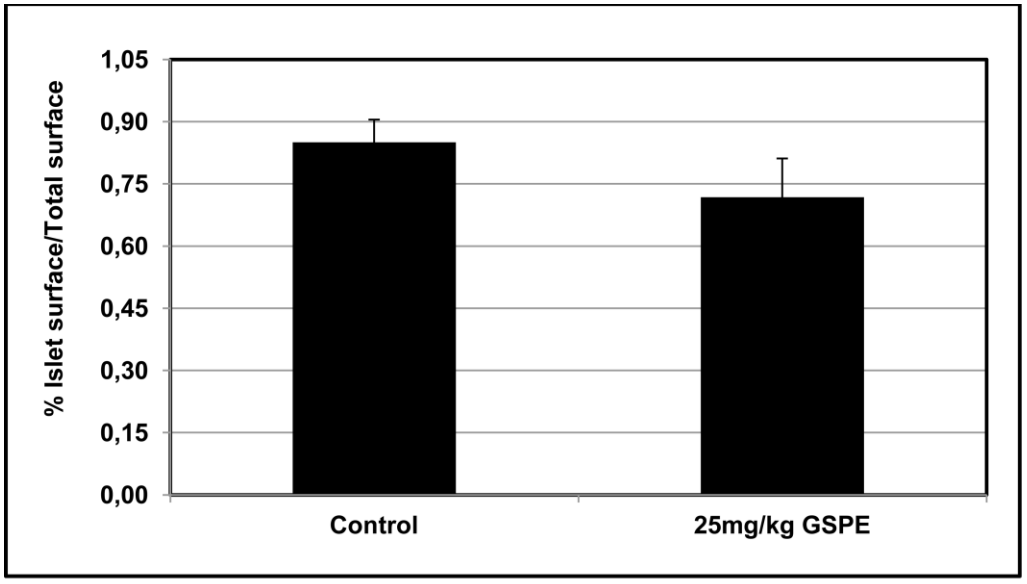


Table S1. Biological regulation

Regulation of cell morphogenesis
Cdc42se1
Cdh2
rnb2
Cspg4
Cxcl12
Dixdc1
Gas7
Lzts1
Mapt
Myh10
Ngfr
Sema4f
Smad3
Vegfa

Taula S1. Developmental process

Neuron differentiation	*Neuron development*
Acsl4	Acsl4
Btg2	
Cdh1	Cdh1
Chrnb2	Chrnb2
Clic5	Clic5
Cntn2	Cntn2
Cspg4	Cspg4
Cxcl12	Cxcl12
Dclk1	Dclk1
Dcx	Dcx
Dpysl5	
Dynll2	Dynll2
Efnb1	Efnb1
Erbb2	Erbb2
Gas7	Gas7
Lgi4	Lgi4
Mapt	
Myh10	Myh10
Napa	
Ngfr	Ngfr
Nlgn1	Nlgn1
Nnat	
Nrcam	Nrcam
Ntrk2	Ntrk2
Pip5k1c	Pip5k1c
Ptprr	
Sdc2	Sdc2
Sema4f	Sema4f
Slc1a3	Slc1a3
Vegfa	Vegfa

Taula S1. Cellular process

Cell migration	*Neuron differentiation*	*Neuron development*
	Acsl4	Acsl4
	Btg2	
Ccl22		
	Cdh1	Cdh1
Cdh2		
	Chrnb2	Chrnb2
Clasp2		
	Clic5	Clic5
Cntn2	Cntn2	Cntn2
Cspg4	Cspg4	Cspg4
Ctgf		
Cx3cl1		
Cxcl12	Cxcl12	Cxcl12
Dclk1	Dclk1	Dclk1
Dcx	Dcx	Dcx
	Dpysl5	
	Dynll2	Dynll2
Efnb1	Efnb1	Efnb1
	Erbb2	Erbb2
Foxe1		
	Gas7	Gas7
Icam1		
Lbp		
	Lgi4	Lgi4
	Mapt	
Myh10	Myh10	Myh10
	Napa	
	Ngfr	Ngfr
	Nlgn1	Nlgn1
	Nnat	
	Nrcam	Nrcam
	Ntrk2	Ntrk2
Pdgfrb		
	Pip5k1c	Pip5k1c
Plat		
Podxl		
	Ptprr	
	Sdc2	Sdc2
	Sema4f	Sema4f
	Slc1a3	Slc1a3
Vegfa	Vegfa	Vegfa

Taula S1. Localization

Ion transport	*Cation transport*	*Metal ion transport*	*Monovalent inorganic cation transport*	*Sodium ion transport*	*Neurotransmitter transport*
Accn4	Accn4	Accn4	Accn4	Accn4	
Atp1b3	Atp1b3	Atp1b3	Atp1b3	Atp1b3	
Atp1b4	Atp1b4	Atp1b4	Atp1b4	Atp1b4	
Atp6v1g2	Atp6v1g2		Atp6v1g2		
Cacnb1	Cacnb1	Cacnb1			
Camk2b	Camk2b	Camk2b			
Chrna9	Chrna9	Chrna9			
Chrnb2	Chrnb2	Chrnb2			
Clic5					
Cnga2	Cnga2	Cnga2	Cnga2		
					Cplx1
Fxyd6					
Itpr1	Itpr1	Itpr1			
Kcnab3	Kcnab3	Kcnab3	Kcnab3		
Kcnc3	Kcnc3	Kcnc3	Kcnc3		
Kcnd2	Kcnd2	Kcnd2	Kcnd2		
Kcnj6	Kcnj6	Kcnj6	Kcnj6		
Kcnk15	Kcnk15	Kcnk15	Kcnk15		
Kcnk6	Kcnk6	Kcnk6	Kcnk6		
Lasp1					
					Nlgn1
					Nrxn2
P2rx2					
Prkcd					
					Rab15
Scn3b	Scn3b	Scn3b	Scn3b	Scn3b	
Scn4b	Scn4b	Scn4b	Scn4b	Scn4b	
Slc17a6	Slc17a6	Slc17a6	Slc17a6	Slc17a6	Slc17a6
Slc17a8	Slc17a8	Slc17a8	Slc17a8	Slc17a8	Slc17a8
Slc1a3					Slc1a3
Slc24a2	Slc24a2	Slc24a2	Slc24a2	Slc24a2	
Slc25a1					
Slc25a10					
Slc34a2	Slc34a2	Slc34a2	Slc34a2	Slc34a2	
Slc4a10	Slc4a10	Slc4a10	Slc4a10	Slc4a10	
Slc6a8	Slc6a8	Slc6a8	Slc6a8	Slc6a8	Slc6a8
					Slc6a12
Slc8a3	Slc8a3	Slc8a3	Slc8a3	Slc8a3	
Slc9a1	Slc9a1	Slc9a1	Slc9a1	Slc9a1	
Slc9a5	Slc9a5	Slc9a5	Slc9a5	Slc9a5	
					Slc18a2
					Sv2a
					Sv2b
					Syt1
Trpm8	Trpm8	Trpm8			Vamp2

Taula S1. Locomotion

Cell migration
Ccl22
Cdh2
Clasp2
Cntn2
Cspg4
Ctgf
Cx3cl1
Cxcl12
Dclk1
Dcx
Efnb1
Foxe1
Icam1
Lbp
Myh10
Pdgfrb
Plat
Podxl
Vegfa

Taula S1. Multicellular organismal process and signalling

Transmission of nerve impulse
Chrnb2
Cplx1
Eif2b5
Erbb2
Grm1
Lgi4
Maob
Mylk2
Nlgn1
Nrcam
Nrxn2
P2rx2
Pdyn
Plat
Rab15
Slc17a6
Sv2b
Syt1
Ugt8

Taula S1. Response to stimulus

Res. to organic substance	*Res. to hormone stimulus*	*Res. to monosaccharide stimulus*	*Res. to hexose stimulus*	*Res. to glucose stimulus*	*Res. to oxygen levels*	*Res. to endogenous stimulus*
Abcc5	Abcc5					Abcc5
Abcg1						
Acsl1						
Acsl4						
Adcy6	Adcy6					Adcy6
Ak3l1						Ak3l1
Akap1	Akap1					Akap1
Akt2	Akt2					Akt2
Arnt2	Arnt2				Arnt2	Arnt2
Btg1	Btg1					Btg1
Btg2	Btg2					Btg2
Ccnd1	Ccnd1					Ccnd1
Cdh1						
Chrnb2					Chrnb2	
Cxcl12	Cxcl12				Cxcl12	Cxcl12
Eif2b5	Eif2b5	Eif2b5	Eif2b5	Eif2b5		Eif2b5
Erbb2	Erbb2					Erbb2
Gipr		Gipr	Gipr	Gipr		
Gnb2	Gnb2					Gnb2
Gnb3	Gnb3					Gnb3
Got2						
Gpr83	Gpr83					Gpr83
					Hyou1	
Igf2	Igf2					Igf2
Il1r1						
					Itpr1	
Lbp						
Mapt						
Mb	Mb				Mb	Mb
Nnat		Nnat	Nnat	Nnat		
Nr4a3	Nr4a3					Nr4a3
P2rx2					P2rx2	
Pdgfra	Pdgfra				Pdgfra	Pdgfra
Pdgfrb	Pdgfrb				Pdgfrb	Pdgfrb
Pfkfb2		Pfkfb2	Pfkfb2	Pfkfb2		
Pklr	Pklr	Pklr	Pklr	Pklr	Pklr	Pklr
Pla2g5						
Plat	Plat				Plat	Plat
Prkcd	Prkcd	Prkcd	Prkcd	Prkcd	Prkcd	Prkcd
Ptpra	Ptpra					Ptpra
Ptprn	Ptprn	Ptprn	Ptprn	Ptprn		Ptprn
Sdc1	Sdc1					Sdc1
Sdc2					Sdc2	
Slc18a2						Slc18a2
Slc34a2	Slc34a2	Slc34a2	Slc34a2			Slc34a2
Slc9a1						
					Smad3	
Sord	Sord					Sord
Star	Star					Star
Stat4	Stat4					Stat4
Sult1a1	Sult1a1					Sult1a1
Txn2	Txn2	Txn2	Txn2	Txn2	Txn2	Txn2
Txnip	Txnip	Txnip	Txnip	Txnip		Txnip
Vamp2		Vamp2	Vamp2	Vamp2		
					Vegfa	

5. Gallic acid is a major active constituent in the anti-carcinogenic activity of grape seed procyanidin extract in pancreatic adenocarcinoma cells

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To evaluate the effects of GSPE on proliferation and apoptosis in a pancreatic cancer cell model, the adenocarcinoma cell line MIA PaCa-2 was used. Pancreatic adenocarcinoma is one of the most aggressive cancers and is highly resistant to treatment. Moreover, pancreatic adenocarcinoma represents the most frequent neoplasm of the pancreas, accounting for 80-90% of all pancreatic neoplasms.[1] Insulinomas are very rare tumours, and surgery is generally successful for these tumours.[2]

There are numerous human adenocarcinoma cell lines, and MIA PaCa-2 is used in many studies investigating the effects of phenolic compounds on proliferation and apoptosis. These cells contain three frequent mutations in K-ras, p53 and p16.[3]

In this study, the effects of GSPE on proliferation and apoptosis of MIA PaCa-2 cells were evaluated. We studied the mechanism by which the extract modulates apoptosis and the components of the extract responsible for these effects. These studies utilised pure monomeric catechins and gallic acid, as well as fractions of the extract enriched for monomers, dimers or procyanidin oligomers.

Finally, MIA PaCa-2 cells were treated with basolateral media from Caco-2 cells treated with GSPE and with GSPE-treated-rat serum. Therefore, the cells were treated with the extract metabolites that are found in plasma after procyanidin ingestion.

(1) Hansel, D. E. et al. Molecular pathogenesis of pancreatic cancer. *Annu Rev Genomics Hum Genet.* **2003**, *4*, 237–256.

(2) Vaidakis, D. et al. Pancreatic insulinoma: current issues and trends. *HBPD INT.* **2010**, *9* (3), 234–241.

(3) Deer, E. L. et al. Phenotype and genotype of pancreatic cancer cell lines. *Pancreas.* **2010**, *39* (4), 425–435.

Gallic acid is a major active constituent in the anti-carcinogenic activity of grape seed procyanidin extract in pancreatic adenocarcinoma cells

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RUNNING TITLE

GA as a GSPE active constituent in anti-carcinogenesis

FUNDING

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KEYWORDS

procyanidins; gallic acid; MIA PaCa-2 cells; proliferation; apoptosis

ABSTRACT

Grape seed procyanidins have been described as anti-carcinogenic in different models, in part, by increasing apoptosis. Moreover, grape seed procyanidin extract (GSPE) has been described to modulate apoptosis in pancreatic islets and β-cells in altered conditions and to modulate β-cell functionality, demonstrating that the pancreas is a target of procyanidins. In this study, we analysed the effects of GSPE on the pancreatic carcinoma cell line MIA PaCa-2, and we found that the extract inhibits cell proliferation and increases apoptosis, which is primarily mediated by a down-regulation of the anti-apoptotic protein Bcl-2 and a depolarisation of the mitochondrial membrane. We also determined the component of the extract that possesses the highest antiproliferative and pro-apoptotic activity and found that gallic acid is a major active constituent in the anti-carcinogenic activity of GSPE.

INTRODUCTION

Procyanidins are the major group of polyphenols in the human diet because of their widespread occurrence in fruits, berries, nuts, beans, cocoa-based products, wine, and beer.[1,2] Procyanidins provide several benefits, including anti-inflammatory and antioxidant properties, and protect against cardiovascular diseases.[2] Moreover, procyanidins modulate glucose metabolism by modifying both glycaemia and insulinemia.[3] This modulation might be partly mediated by acting on the pancreas, because grape seed procyanidin extract (GSPE) modulates β-cell functionality and insulin degradation.[4] Furthermore, GSPE induces changes in apoptosis markers in the pancreas of cafeteria-fed rats[5] and in pancreatic islets in Zucker Fatty rats.[6] *In vitro*, using the INS-1E β-cell line, we found that GSPE enhanced the pro-apoptotic effect of high glucose and demonstrated clear antiproliferative effects under high glucose, insulin, and palmitate conditions, likely due to high molecular weight forms contained in the extract.[5]

The pro-apoptotic and antiproliferative effects of procyanidins are of great interest because they highlight the anti-carcenogenic effect of these compounds. Proanthocyanidins, and specifically, grape seed proanthocyanidins, have been reviewed to act as anti-carcinogenic agents *in vivo* and *in vitro*, and the chemopreventive activities include reduced proliferation, increased apoptosis, cell cycle arrest in tumour cells and the modulation of the expression and activity of nuclear factor-κB (NF-κB) and NF-κB-targeted genes.[7] Proanthocyanidins have been reported to have inverse associations with the risk of stomach,[8] colorectal,[9] and lung[10] cancers. In addition, other flavonoids have been described to increase the action of cytostatic drugs, which might allow a decrease in the dosage of these drugs to reduce the negative side effects of the treatment.[11]

That the pancreas is a target of procyanidins suggests that they could play a role against pancreatic carcinoma. Pancreatic ductal adenocarcinoma is one of the most aggressive cancers in developed countries, with a 5-year survival rate of only 5% and a median survival of less than 6 months;[12] furthermore, it is the 10th most common cause of cancer and the 4th cause of cancer-related deaths in the United States.[12,13] This cancer is characterised by invasiveness, rapid progression, and profound resistance to treatment.[12] Some evidence suggests that the consumption of fruits and vegetables protects against pancreatic cancer.[14,15] Proanthocyanidin intake has been found to be inversely related to the risk of pancreatic cancer in a case-control study in Italy,[16] and a flavonoid-rich diet is suggested to decrease pancreatic cancer risk in male smokers who do not consume supplemental α-tocopherol and/or β-carotene.[17] In addition, grape seed procyanidins have been recently reported to inhibit the *in vitro* growth and invasion of pancreatic carcinoma cells.[18]

Considering that GSPE modulates apoptosis and proliferation in pancreatic β-cells and given the anti-carcinogenic activity of procyanidins described in different models, this study was conducted to further analyse the effect of GSPE on the proliferation and apoptosis of the pancreatic carcinoma cell line MIA PaCa-2 and to identify the components of the extract with high antiproliferative and pro-apoptotic activity.

METHODS AND MATERIALS

Reagents

GSPE contained monomeric (16.6%), dimeric (18.8%), trimeric (16.0%), tetrameric (9.3%), and oligomeric procyanidins (5 to 13 units, 35.7%) in addition to phenolic acids (4.2%). Fractions II, VI, and X were obtained by a chromatographic separation of GSPE according to size from which 11 major fractions with increasing degrees of polymerisation were identified (described in [19]). Fraction II contained monomeric structures, fraction VI, dimeric, and fraction X, oligomeric. These fractions were vacuum dried and stored at -20 ºC for subsequent use in the biological studies. The monomeric procyanidins catechin (C), epicatechin (EC), and epigallocatechin gallate (EGCG) in addition to the phenolic acid gallic acid (GA) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium).

Basolateral media and GSPE-treated rat serum

Basolateral media were obtained by seeding Caco-2 cells onto culture inserts (Merck KGaA, Darmstadt, Germany) at a cell density of $5.3 \cdot 10^4$ cells/cm^2 and stored in Dulbecco's modified eagle medium (DMEM) supplemented with 20% foetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin. After 21 days, once the confluent monolayer had formed, 0.4 mL of DMEM with 750 mg/L GSPE was added to the apical side, and 1 mL of DMEM alone was added to the basolateral side and allowed to incubate for 4 or 24 h. Afterwards, the basolateral media were harvested and stored at -20 ºC for future experiments.

The content of the monomeric forms C, EC, and EGCG in addition to GA in the basolateral media was analysed by liquid chromatography tandem mass spectrometry (MS/MS). Prior to the chromatographic analysis, a solid phase extraction of the analytes was performed from basolateral media using OASIS HLB *µElution Plate* 30 µm cartridges (Waters Corp., Milford, MA). These cartridges were conditioned sequentially with 250 µL of methanol and 250 µL of Milli-Q water at pH 2.0. Three hundred microlitres of 4% phosphoric acid and 50 µL of catechol (IS) at a concentration of 20 mg/L were added to 350 µL of basolateral media, and this solution was loaded onto the conditioned cartridges. The loaded cartridges were washed with 200 µL of Milli-Q water and 200 µL of Milli-Q water at pH 2.0, for the monomeric forms, and with 150 µL of Milli-Q water at pH 2.0 for GA. Then, the retained compounds were eluted with 2 x 50 µL of an acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution for the monomeric forms, and with 2 x 50 µL of methanol for GA. The eluted solution was loaded onto the chromatographic system, and the compounds were identified by MS/MS and quantified as previously described.[20]

Female Wistar rats weighing 175–200 g were housed in animal quarters at 22 °C with a 12 h light/dark cycle, and after 1 week in quarantine and an overnight (o/n) fasting, 1 g/kg of body weight (bw) of GSPE dissolved in tap water was administered using an intragastric catheter. After 1 h, the animals were anesthetised using sodium pentobarbital (Sigma-Aldrich, St. Louis, MO) at 75 mg/kg of bw and sacrificed by abdominal aorta exsanguination. Serum was obtained by collecting blood in tubs without anticoagulant followed by centrifugation at 2000 g for 10 min.

Cell culture and treatment

Pancreatic carcinoma MIA PaCa-2 cells were kindly provided by Dr. Carlos J. Ciudad from the Universitat de Barcelona and were cultured in DMEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. When cells reached between 80-100% confluence, they were treated for 24 h with GSPE at a concentration from 1 to 150 mg/L, GSPE fractions and monomers at a concentration from 1 to 25 mg/L, basolateral media, or GSPE-treated rat serum diluted 1:2 with DMEM medium. In proliferation assays, cells were previously depleted of serum and maintained in 0.1% BSA o/n.

Proliferation and apoptosis assays

MIA PaCa-2 cells were cultured in 96-well plates and were treated as described. Bromodeoxyuridine (BrdU) was added to each well and incubated o/n. At the end of the treatment, proliferation (quantified as BrdU incorporation during DNA synthesis) was measured using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. In parallel, at the end of the treatment, the cells were lysed and 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.

Reactive oxygen species (ROS) determination

MIA PaCa-2 cells were cultured in 96-well plates with opaque bottom and were treated as described. At the end of the treatment, intracellular ROS were determined using DCFH-DA (2',7'-dichlorofluorescin diacetate). DCFH-DA (Sigma-Aldrich, St. Louis, MO) at a concentration of 20 µM in PBS was added to the cells and incubated for 30 min. Then, cells were washed with PBS, and the fluorescence was measured after 2 h at λ_{ex} = 485 nm and λ_{em} = 530 nm. The values were normalised with the protein content, as analysed by the Bradford method.[21]

Mitochondrial membrane potential (MMP) measurement

MIA PaCa-2 cells were cultured in 24-well plates and were treated as described. At the end of the treatment, the MMP was measured using the probe Rhodamine 123. Cells were incubated for 20 min with 10 mg/L of Rhodamine 123 (Invitrogen, Eugene, OR) in PBS. Then, cells were washed with PBS, and the fluorescence was measured at λ_{ex} = 485 nm and λ_{em} = 520 nm after 15 min. The values were normalised with the protein content, as determined by the Bradford method.[21]

Western blot

Protein was extracted from MIA PaCa-2 cells using RIPA lysis buffer (15 mM Tris-HCl, 165 mM NaCl, 0.5% Na-deoxycholate, 1% Triton X-100 and 0.1% SDS) containing a protease inhibitor cocktail (1:1000; Sigma-Aldrich, St. Louis, MO) and 1 mM phenylmethanesulfonyl fluoride

(PMSF) (Sigma-Aldrich, St. Louis, MO). Total protein levels of the lysates were determined using the Bradford method.[21] After boiling for 5 min, 24 µg of protein was loaded and electrophoresed through a 4-15% SDS-polyacrylamide gel. The samples were then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) and blocked at room temperature for 1 h using 5% (w:v) non-fat milk in TTBS buffer (Tris-buffered saline [TBS] plus 0.5% (v:v) Tween-20). The membranes were incubated o/n at 4 ºC with rabbit polyclonal Bax or Bcl-2 primary antibody (Cell Signaling Technology, Beverly, MA) at a 1:1500 dilution in blocking solution or rabbit anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:1000. After washing with TTBS, the blots were incubated with peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK) at a 1:10000 dilution at room temperature for 1 h. The blots were then washed thoroughly in TTBS followed by TBS. Immunoreactive proteins were visualised with the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) by chemiluminescence, and densitometric analysis of the immunoblots was performed using ImageJ 1.44p software. All of the proteins were quantified relative to the loading control.

Calculations and statistical analysis

The results are expressed as the mean ± SEM. Effects were assessed by Student's *t*-test or ANOVA. All calculations were performed with SPSS software v.19.

RESULTS

GSPE inhibits proliferation and enhances apoptosis in MIA PaCa-2 cells involving depolarisation of the mitochondrial membrane and decreased Bcl-2 protein expression

We evaluated the effects of GSPE on proliferation in the pancreatic adenocarcinoma cell line MIA PaCa-2. As shown in Figure 1, the proliferation rate decreased in a dose-dependent manner after 24 hours of treatment when compared with untreated cells. Proliferation was slightly but significantly reduced at 10 mg/L of GSPE, and the effect of the extract was clearly antiproliferative at doses higher than 50 mg/L.

The apoptotic effects of GSPE on MIA PaCa-2 cells were assessed. Because a dose of 50 mg/L GSPE demonstrated clear antiproliferative effects, cells were treated with 50 or 100 mg/L of GSPE for 24 hours. No apoptosis was detected at 50 mg/L of GSPE, whereas there was an increase in the apoptosis of MIA PaCa-2 cells treated with 100 mg/L of GSPE (Figure 2A).

Because mitochondrial production of ROS is involved in the induction of apoptosis,[22] we quantified the levels of ROS by a DCFH assay. However, the levels of ROS decreased by approximately 70% when cells were treated with 50 or 100 mg/L of GSPE for 24 hours compared with the control group (Figure 2B).

Conversely, using Rhodamine 123 dye as a marker, fluorimetric analysis revealed a depolarisation of the mitochondrial membrane in MIA PaCa-2 cells treated with GSPE at a

concentration of 100 mg/L of GSPE (Figure 2C), which were the identical dose at which apoptosis was detected. In contrast, no changes in the MMP were observed when cells were treated with 50 mg/L of GSPE (Figure 2C), a dose at which apoptosis was also not detected.

Finally, Bcl-2 and Bax protein expression was assessed by Western blot. As shown in Figure 2D, GSPE treatment down-regulated the expression of the anti-apoptotic marker Bcl-2 approximately 50% compared with the vehicle-treated cells. Despite Bax expression that remained unchanged, the ratio of Bcl-2/Bax tended to decrease with GSPE treatment.

GA is one of the components of GSPE with higher antiproliferative and pro-apoptotic effects

Because the studies were performed with a whole extract containing a mixture of procyanidins with differing degrees of polymerisation, in addition to other flavonoids in minor amounts, we analysed the component/s of the mixture that was/were responsible for the measured effects. GSPE was chromatographically separated into fractions representing different degrees of polymerisation. Fraction II (enriched in procyanidin monomers), fraction VI (enriched in dimers), and fraction X (enriched in oligomeric forms) were used in this study. As shown in Table 1, fraction II did not inhibit the proliferation of MIA PaCa-2 cells; on the contrary, at the lower concentration, it slightly but significantly increased the proliferation. Fraction VI also had no effect on proliferation at any dose tested. Fraction X slightly decreased the proliferation of MIA PaCa-2 cells at 10 and 25 mg/L (Table 1).

The effects of purified monomers and GA, the most abundant phenolic acid in GSPE,[23] on proliferation were examined. C and EC showed a slight antiproliferative effect but only at the higher concentration tested. EGCG and GA showed the strongest antiproliferative effects (Table 1).

Because the chemopreventive effects of EGCG have already been extensively studied and its concentration in GSPE is low (more than 40 times lower than GA),[23] we selected EC and GA at the highest and most effective dose to study their effects on apoptosis. Both compounds demonstrated antioxidant properties, with a decrease in the production of ROS (Figure 3A). However, whereas GA demonstrated pro-apoptotic effects with an increase in Bax protein expression (Figure 3B), EC did not. Moreover, GA induced the depolarisation of the mitochondrial membrane (1.44 ± 0.1 versus 1.00 ± 0.0, $p < 0.01$), similarly to what we observed for treatment with the whole extract.

Proliferation rate of MIA PaCa-2 cells treated with basolateral media and the serum of GSPE-treated rats

Following their ingestion, dietary procyanidins are hydrolysed and metabolised in the intestine and in the liver.[24] To approximate an *in vivo* situation, two different approaches were used.

First, intestinal Caco-2 cells were used to simulate the gut barrier. The culture of Caco-2 cells in permeable inserts possesses many of the morphological and functional characteristics of

intestinal enterocytes and is able to metabolise and absorb natural bioactive compounds.[25,26] Caco-2 cells were treated with 750 mg/L of GSPE for 4 or 24 hours and basolateral media were collected. Then, MIA PaCa-2 cells were treated with these media for 24 hours. We found that the molecules present in the basolateral media were not able to decrease the proliferation rate of MIA PaCa-2 cells (1.02 ± 0.0 versus 1.00 ± 0.0 for the basolateral media after GSPE treatment for 4 hours and 1.00 ± 0.0 versus 1.00 ± 0.0 for the basolateral media after GSPE treatment for 24 hours). The basolateral media collected after 4 hours of GSPE treatment contained the monomer C and metabolites of C and EC (previously shown in [24]) but no EGCG or GA. The polyphenol content in the basolateral media collected after 24 hours of GSPE treatment was quantified. Only C was significantly increased after GSPE treatment; EC, EGCG, and GA were not (Table 2).

The second approach exposed MIA PaCa-2 cells to serums obtained from rats treated with 1 g of GSPE/kg of bw for 1 hour. After a 24-hour treatment with the serum, the proliferation rate was slightly but significantly decreased by the serum of GSPE-treated rats compared with the cells exposed to the serum of rats treated with the vehicle (0.96 ± 0.0 versus 1.00 ± 0.0, $p \leq 0.05$).

DISCUSSION

Pancreatic adenocarcinoma is an aggressive and devastating disease characterised by a profound resistance to treatment.[12] A wide variety of botanicals, mostly dietary flavonoids, have been reported to possess anti-carcinogenic and antimutagenic activities and provide new options for the development of chemopreventive and chemotherapeutic strategies.[7] In previous studies, the pancreas was described to be a target of procyanidins,[4] which modulate apoptosis in the pancreas of cafeteria-fed rats[5] and in pancreatic islets of Zucker Fatty rats[6] in addition to modulating apoptosis and the proliferation of cultured β-cells in altered culture conditions.[5] Given these effects, this study was conducted to further analyse the effect of GSPE on the proliferation and apoptosis of the pancreatic carcinoma cell line MIA PaCa-2.

We first tested the effects of GSPE on proliferation, and our results indicate that the extract inhibits this process in a dose-dependent manner. These results are consistent with a previous recently published study in which GSPE inhibited the cell survival rate in MIA PaCa-2 cells.[18] In that experiment, GSPE inhibited cell growth by inducing apoptosis rather than inhibiting the cell cycle. In our experiments, the reduction in BrdU incorporation in the GSPE-treated cells at higher doses could be due to a reduction in the cell number, because apoptosis is also increased. However, at a dose of 50 mg/L of GSPE, the proliferation rate was inhibited without any modification to apoptosis; therefore, another mechanism for the anti-carcinogenic activity of GSPE could be the reduction of the proliferation rate. The differences in the results concerning the effective doses of GSPE compared with those obtained by Chung et al. could be due to the length of time the cells were in culture prior to treatment. In our case, cells were cultured at higher concentration, as was reported in other studies using these cells,[27–29] and were allowed to grow until near confluence, in contrast to the 18 hours used by Chung et al. If cells were treated with GSPE in the early stages of confluence, the effects of GSPE might be more aggressive. GSPE-enhanced apoptosis in MIA PaCa-2 cells is accompanied by a decrease in the anti-apoptotic

protein Bcl-2. GSPE-induced apoptosis in cancer cells is regulated through the Bcl-2 family of proteins, and more specifically, the pro-apoptotic marker Bax and the anti-apoptotic protein Bcl-2.[7,30,31] Bcl-2 protects cells against apoptosis by preventing the release of cytochrome c into the cytoplasm and, thereby, preventing cytochrome c from promoting apoptotic protease activating factor-1 (Apaf-1)-mediated caspase-9 activation maintaining intact mitochondrial membranes.[32] Therefore, GSPE-induced apoptosis in MIA PaCa-2 cells might be mediated by decreased levels of the anti-apoptotic Bcl-2, which promotes an activation of the caspase-9 pathway and loss of the integrity of the mitochondrial membrane. In fact, fluorimetric analysis revealed a depolarisation of the mitochondrial membrane in GSPE-treated MIA PaCa-2 cells at the doses that caused apoptosis in the cells.

Despite the ability of procyanidins to increase ROS which may cause cell death in other cancer cell types,[33,34] in our study, GSPE reduced the production of ROS in MIA PaCa-2 cells. Thus, these results exclude the possibility that GSPE induces apoptosis via an increase in ROS.

Because GSPE contains a mixture of different flavonoids, it is important to determine which of the extract compounds is responsible for the antiproliferative and pro-apoptotic effects. We tested fractions of GSPE enriched in catechins with different degrees of polymerisation and found that the only fraction that showed slight antiproliferative effects was the fraction containing the higher-order polymers. This finding is in agreement with that of a study using the colon carcinoma cell line HT29 in which grape oligomers were concluded as the most efficient antiproliferative agents[35] and with our results in β-cells in which the GSPE-antiproliferative effects are likely due to high molecular weight compounds contained in the extract.[5] However, the structures found in plasma after consumption of GSPE mostly include monomers in addition to dimers and trimers in lower proportion.[36,37] Therefore, oligomers are unlikely to reach the target organ because they are poorly absorbed in the gut due to their high molecular weight.[38] We also studied the effect of purified monomers and GA, the most abundant phenolic acid in GSPE,[23] and we found that EGCG and GA exerted higher antiproliferative effects. EGCG is the major polyphenol in green tea, and although it is also present in GSPE, its concentration is very low in contrast to the other monomers and GA.[23] The anti-carcinogenic effects of EGCG in MIA PaCa-2 cells have been widely studied;[28,39–41] therefore, we do not exclude it as an effector of GSPE effects, but its concentration in GSPE is very low, indicating that other molecules may be responsible for these effects. Therefore, we focused our study on GA. We found that as in the case of GSPE, GA inhibits proliferation, increases apoptosis, induces the depolarisation of the mitochondrial membrane, and demonstrates antioxidant activity. A recent publication described GA as a cancer-selective agent that induces apoptosis in pancreatic cancer cells.[42] Consistent with our results, that study also demonstrated GA as an inhibitor of cell growth and as a pro-apoptotic agent in addition to promoting the loss of the MMP and the increase in the pro-apoptotic marker Bax. The Bax protein is one of the primary targets of p53 and controls cell death through its participation in the disruption of mitochondria with the subsequent release of cytochrome c, which, in turn, activates caspase-9 and caspase-3.[30] GA has been described to increase ROS in pancreatic adenocarcinoma cells and other cancer cell lines.[42–44] Many tumour cells are characterised by increased ROS generation compared with their noncancerous counterparts, as has been reported in HEL cells, having approximately eight times more ROS content than HUVEC.[45] In that study, other phenolic acids, at low concentrations that are comparable to those

present in human plasma, were able to decrease basal ROS in the cancer cell line HEL.[45] In our study, GA exerts antioxidant effects, as observed in GSPE. Therefore, taking together all the results, GA is one of the major active constituents in the anti-carcinogenic activity of GSPE. This conclusion is in agreement with what was observed in another study using the human prostate carcinoma cell line DU145.[46]

To identify the structures that could be useful *in vivo*, we must consider that following their ingestion, not all components of procyanidins are absorbed in the intestine. Initially, they are hydrolysed in the small intestine and metabolised in the small intestine and in the liver. Moreover, procyanidins and their metabolites that are not absorbed in the small intestine can be absorbed in the large intestine after metabolism by colonic microflora.[24] Therefore, to approach an *in vivo* situation, MIA PaCa-2 cells were exposed to the components of GSPE absorbed and metabolised by the intestinal Caco-2 cells. However, the proliferation of MIA PaCa-2 cells remained unchanged after exposure to the basolateral media. The components of the extract that pass to the basolateral media are mainly C and the metabolites of C and EC,[25] whereas GA and EGCG, the molecules that produce a higher antiproliferative effect, were not increased in the media. GA has been reported to be less absorbable than catechins due to the reduced transport of GA by the monocarboxylic acid transporter relative to other phenolic compounds.[37] Furthermore, the transepithelial transport across the Caco-2 monolayer is very low, suggesting transport *via* the paracellular pathway.[47] Given these findings, the use of Caco-2 basolateral media is not effective for the study of the effects of GA but it is for the procyanidin monomers such as C and EC, indicating that these molecules and their metabolites might not be the main anti-carcinogenic molecules in the GSPE.

Finally, MIA PaCa-2 cells were exposed to the serum from rats treated with GSPE, an approximation that permits the exposure of the cells with the polyphenols absorbed and metabolised in the organism that reached the target organ *in vivo*. In this case, the proliferation of the cells slightly but significantly decreased. In several studies, the presence of C, EC, and GA has been reported in the plasma of rats treated with GSPE, peaking between 1 and 2 hours after treatment.[20,37,48] These studies indicated that EC demonstrated enhanced bioavailability relative to C, and that GA, despite its reduced transport by some transporters, is absorbed in the intestine. Therefore, the observed reduction in the proliferation of the cells treated with the serum of rats treated with GSPE could be, in part, due to the GA present in the serum.

CONCLUSION

This study demonstrates that GSPE treatment inhibits cell proliferation and increases apoptosis in the pancreatic adenocarcinoma cell line MIA PaCa-2, by depolarisation of the mitochondrial membrane and reduction of the anti-apoptotic marker Bcl-2, confirming its potential role as a chemopreventive or therapeutic agent. Furthermore, the direct treatment with monomers and the treatment with basolateral medium and serum from GSPE-treated rats allow us to postulate that GA is one of the major active constituents in the anti-carcinogenic activity of GSPE.

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TABLES

Table 1. Effects of 24-hour treatment of MIA PaCa-2 with purified monomers, the phenolic acid GA and GSPE fractions on proliferation assessed as the incorporation of BrdU. Data are normalised with respect to the control, treated with the vehicle, and are shown as the mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and # $p \leq 0.1$ versus the control (1.00 ± 0.0).

Dose	1 mg/L	5 mg/L	10 mg/L	25 mg/L
Catechin	0.99 ± 0.0	0.98 ± 0.0	0.97 ± 0.0	0.96 ± 0.0 *
Epicatechin	0.99 ± 0.0	0.99 ± 0.0	0.97 ± 0.0 *	0.95 ± 0.0 ***
EGCG	0.97 ± 0.0 **	0.98 ± 0.0	0.94 ± 0.0 **	0.88 ± 0.0 ***
Gallic acid	0.97 ± 0.0	0.98 ± 0.0	0.93 ± 0.0 ***	0.87 ± 0.0 ***
Fraction II	1.06 ± 0.0 ***	1.03 ± 0.0	1.02 ± 0.0	1.00 ± 0.0
Fraction IV	1.02 ± 0.0	1.00 ± 0.0	0.99 ± 0.0	1.03 ± 0.0
Fraction X	1.00 ± 0.0	0.97 ± 0.0	0.96 ± 0.0 *	0.97 ± 0.0 #

Table 2. Content of C, EC, EGCG, and GA in the basolateral media collected after 24 hours. Data are shown as the mean of 4 samples ± SEM (μM). *** $p \leq 0.001$ versus the control.

[GSPE] on apical media	750 mg/L
Catechin	0.27 ± 0.0 ***
Epicatechin	0.40 ± 0.0
EGCG	0.15 ± 0.0
Gallic acid	0.00 ± 0.0

FIGURES

Figure 1. Proliferation of MIA PaCa-2 cells after 24 h of treatment with GSPE assessed using a cell proliferation kit. Data are shown as the mean ± SEM. Different letters indicate significantly different groups ($p \leq 0.05$).

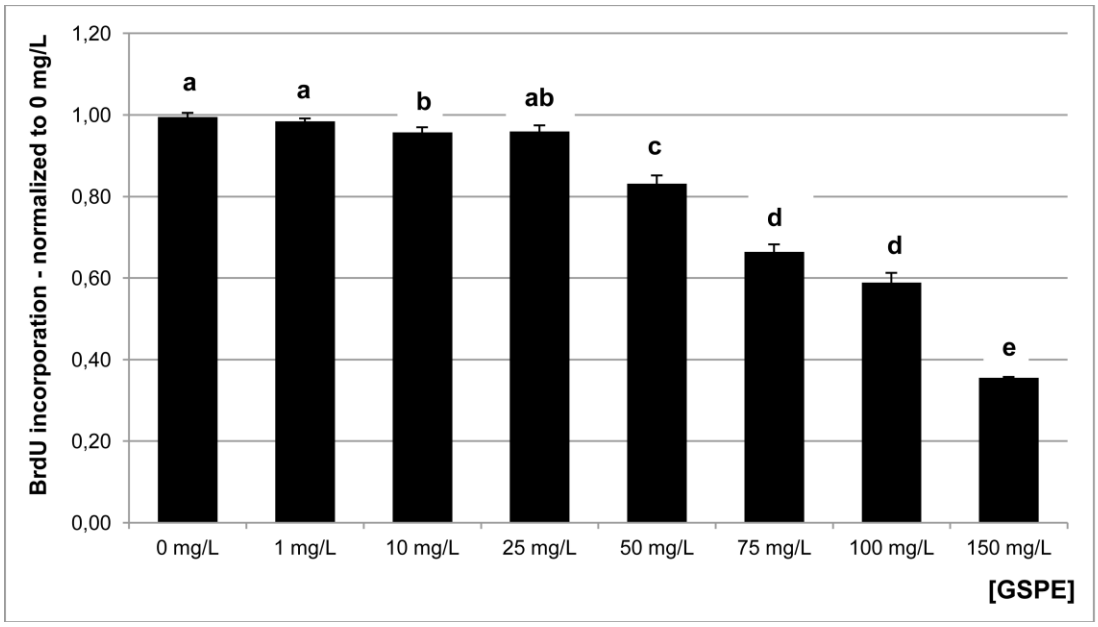
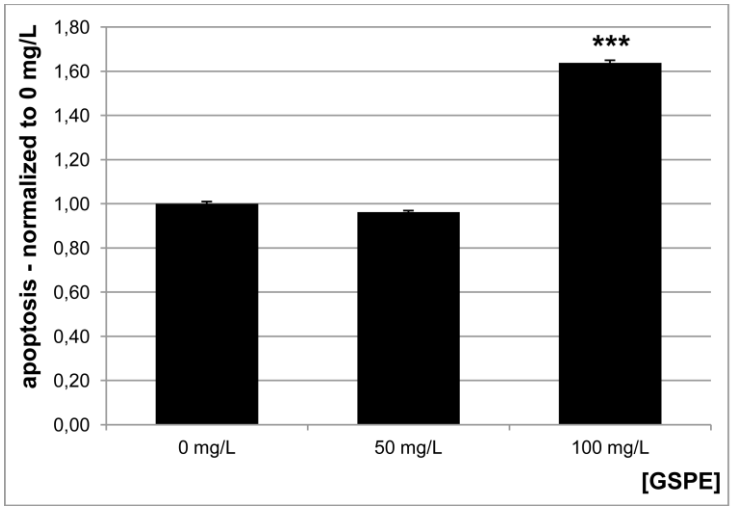
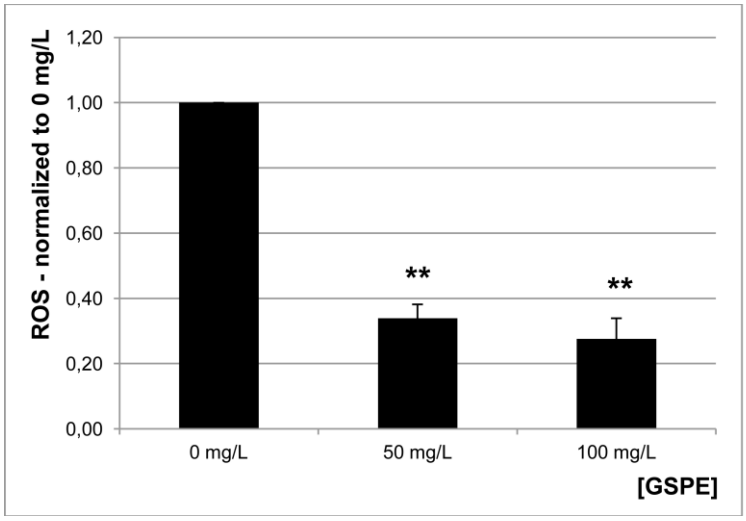


Figure 2. Apoptosis of MIA PaCa-2 cells after treatment with GSPE for 24 hours using a cell death apoptosis kit (A). The mechanisms of apoptosis were examined by determining ROS production (B), the mitochondrial membrane potential (MMP) (C), and the protein expression of apoptosis markers by Western blot (D). Data are shown as the mean ± SEM.
** $p \leq 0.005$, *** $p \leq 0.001$ and # $p \leq 0.1$.

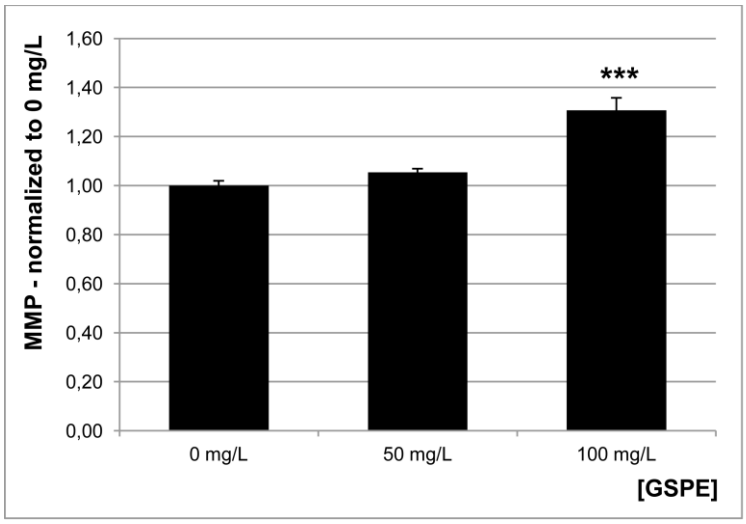
A



B


ROS - normalized to 0 mg/L
1,20
1,00
0,80
0,60
0,40
0,20
0,00
**
**
0 mg/L
50 mg/L
100 mg/L
[GSPE]

C


MMP - normalized to 0 mg/L
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
***
0 mg/L
50 mg/L
100 mg/L
[GSPE]

D

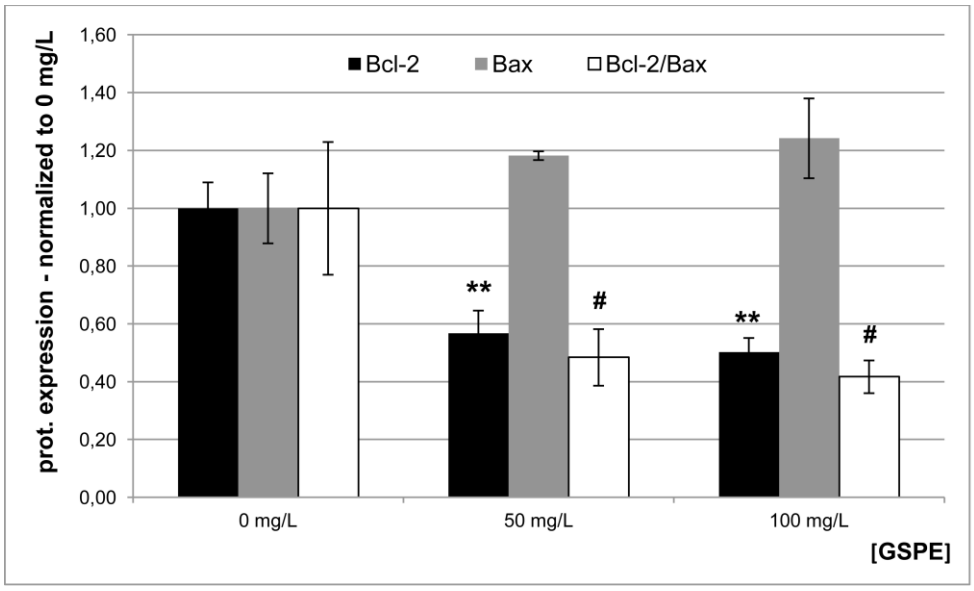
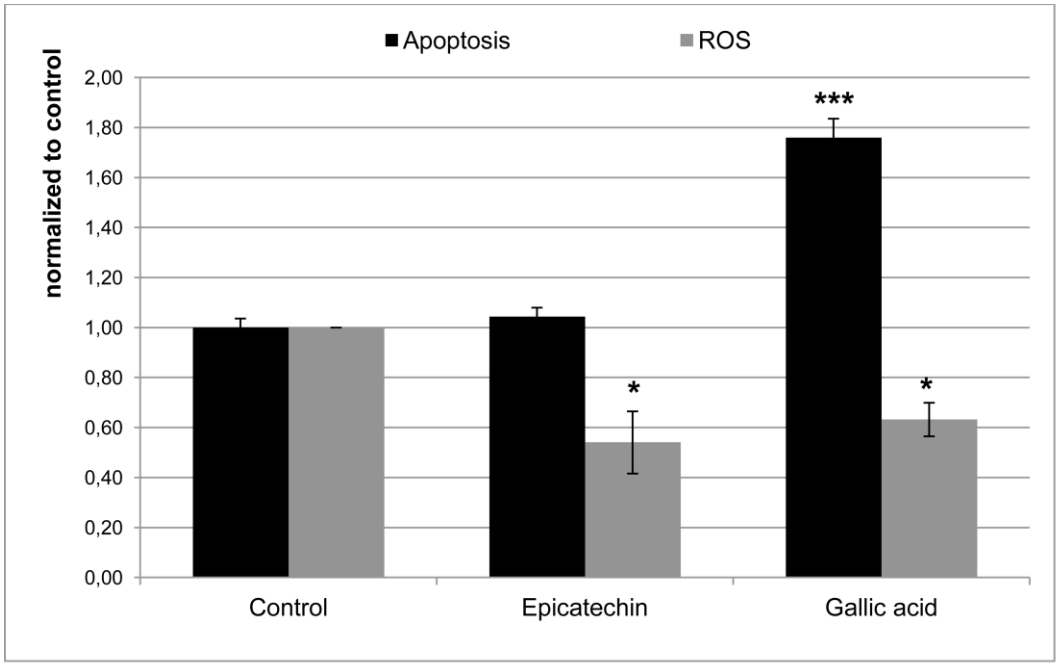
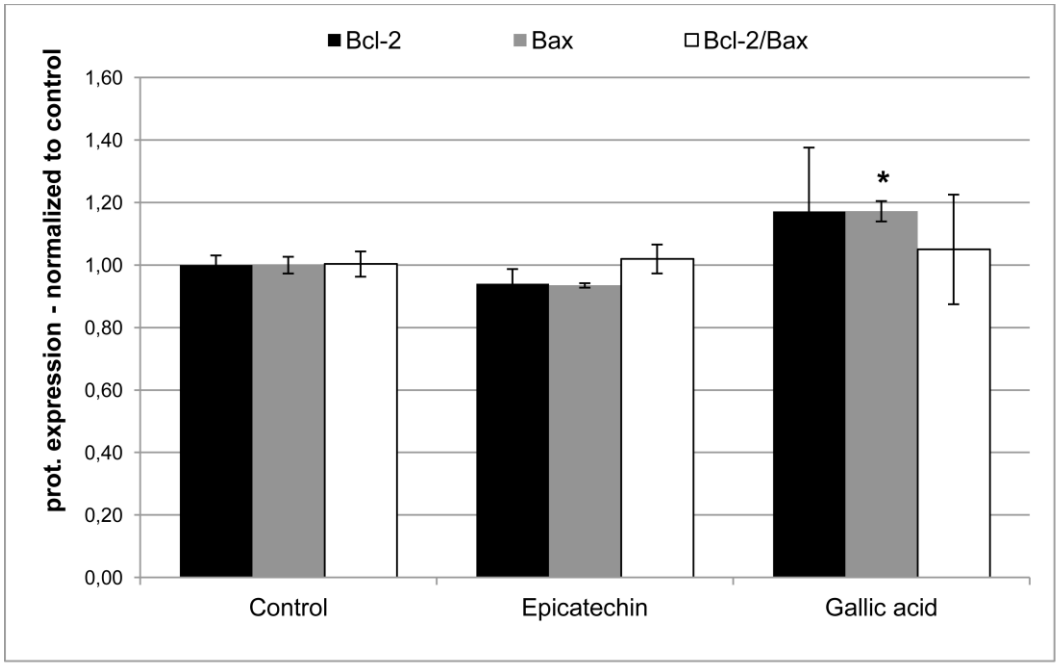

prot. expression - normalized to 0 mg/L
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Bcl-2
Bax
Bcl-2/Bax
**
#
**
#
0 mg/L
50 mg/L
100 mg/L
[GSPE]

Figure 3. Effects of 24-hour treatment of MIA PaCa-2 cells with 25 mg/L of epicatechin or gallic acid on apoptosis and ROS production (A). Bcl-2 and Bax protein expression was assessed by Western blot, and the ratio Bcl-2/Bax was calculated (B). Data are shown as the mean ± SEM. * $p \leq 0.05$ and *** $p \leq 0.001$.

A



B



IV. DISCUSSION

Procyanidins are able to modulate glucose homeostasis,[1] and other flavonoids act on insulin-secreting cells modulating insulin secretion, β-cell apoptosis and proliferation.[2] A previous study from our research group demonstrated that a grape seed procyanidin extract (GSPE) improved glycaemia and insulinemia in cafeteria-fed rats, suggesting that procyanidins could act on the endocrine pancreas.[3] To our knowledge, the effects of procyanidins on pancreatic cells were scarce; therefore, a new project was begun to investigate the effects of a GSPE on pancreas functionality and mass. In parallel to this doctoral thesis, a study of the effects of procyanidins on insulin synthesis and secretion was conducted and indicated that procyanidins modulate these processes.[4] Conversely, the present work focused on the study of the effects of GSPE on proliferation and apoptosis in pancreatic cells, studying the modulation of these processes in physiological and pathological states. To achieve our objectives, we used *in vivo* models of non-pathological rats, rats with obesity induced by a cafeteria diet and genetically obese Zucker fatty (ZF) rats; and *in vitro* models, using the insulinoma cell line INS-1E and the adenocarcinoma cell line MIA PaCa-2.

Because the effects of GSPE largely depend on the metabolic environment,[1] the effects of the extract were tested in altered conditions in which a protective effect could be beneficial as a therapeutic tool. One situation in which proliferation and apoptosis processes are disturbed in the pancreas is in prediabetes, in which hyperinsulinemia appears to compensate for insulin resistance that precedes the onset of type 2 diabetes mellitus (T2DM).[5] One of the adaptive responses that occur to maintain normal glycaemic levels is an increase in β-cell mass in the face of obesity and insulin resistance.[6] However, when β-cells are unable to compensate for the increased insulin demand, there is a decrease in β-cell mass characteristic of the onset of T2DM due to an increase in β-cell apoptosis.[7] Postprandial hyperglycaemia and dyslipidemia are common features that occur before the development of T2DM.[8] Chronic exposure to a hyperglycaemic environment and an elevated FFA concentration causes β-cell dysfunction and death, a phenomenon termed glucotoxicity and lipotoxicity, respectively.[6,9] Therefore, we exposed INS-1E cells to high concentrations of glucose and palmitate, and both increased apoptosis in β-cells. However, GSPE only enhanced the pro-apoptotic effect of high glucose, not demonstrating any effect in a condition of lipotoxicity. Several mechanisms by which glucose is toxic to β-cells have been described. One study reported that chronic exposure to high glucose induces β-cell apoptosis by decreasing glucokinase (GCK) and its association with mitochondria, increasing BAX oligomerisation, and causing the loss of the mitochondrial membrane potential (MMP) and cytochrome c release, all of which occur in the intrinsic apoptosis pathway.[9] In fact, GSPE has been previously described to stimulate glucose uptake and down-regulate GCK gene expression,[4] which could enhance the deleterious effects of glucose, inducing Bax protein translocation to the mitochondrial membrane causing apoptosis.

Once the effects of GSPE on apoptosis *in vitro* were assessed, its effects *in vivo* in altered conditions were also investigated. There are several animal models to study obesity and insulin resistance, including both genetically and diet-induced obesity models. First, we analysed the effects of GSPE on apoptosis in a model of diet-induced obesity, using cafeteria-fed female rats, as a continuation of a previous study. We investigated the effects of the cafeteria diet and confirmed that feeding rats with a cafeteria diet mimicked a prediabetic state and modulated the

expression of markers of apoptosis, indicating an increase in the apoptosis of the pancreas of the cafeteria-fed rats.[10] When a palliative treatment of GSPE was administered to these rats (two doses administered for a short or a long period), glycaemia and insulinemia were improved.[3] However, despite GSPE modulation of apoptosis markers in the pancreas of these rats, the exact effects depended on the dose and period of treatment. Nevertheless, the results appear to exclude the possibility that the decrease in plasma insulin could be due to an increase in β-cell apoptosis because, in general, GSPE treatment appeared to counteract the increase in the apoptosis of the pancreas of the cafeteria-fed rats.

In contrast, when the effects of the extract were analysed in male Wistar rats, GSPE treatment did not reproduce the previously observed results but enhanced the increase in the pro-apoptotic marker BAX caused by the cafeteria diet. In this case, rats were fed with a cafeteria diet for a shorter time period, and although the dose was equal to one of the doses tested in the previous study in females, the experimental design was not identical between the two studies.

Therefore, the modulation of apoptosis biomarkers by GSPE in cafeteria-fed rats appears to be dependent on the dose, treatment period and/or gender. In fact, the effects of the extract on plasma glucose and insulin levels were also dependent on the dose and treatment period.[3] This dependence on the experimental design used in the study of the effects of procyanidins on glucose homeostasis has already been reviewed.[1] For insulin, although all the doses and treatment periods tend to decrease its plasma levels when compared with the cafeteria-fed rats, only certain doses were significantly modified.[3] However, these changes in insulin do not appear directly related with the changes in apoptosis.

Considering the effects of GSPE on proliferation *in vitro*, we exposed INS-1E cells to high glucose and high palmitate levels, as in the study of apoptosis, and also to high insulin levels, which is a potential β-cell mitogen.[11] All these conditions are factors that modify β-cell mass. In fact, as expected, the results indicated that both glucose and insulin induced proliferation in INS-1E cells, whereas palmitate inhibited it. The procyanidin extract demonstrated clear antiproliferative effects under the three conditions tested.

These antiproliferative effects observed for the extract were likely due to high molecular weight compounds. After administration of GSPE in rats, the structures found in plasma are primarily monomers, with dimers and trimers at lower concentrations;[12,13] however, oligomeric procyanidins are not absorbed in the intestine. Therefore, because the most antiproliferative compounds are the higher molecular weight forms, caution is required when interpreting *in vitro* data and comparing such data with results found *in vivo*.

In fact, in the previously mentioned experiments with cafeteria-fed rats, the proliferation marker cyclin D2 was not modified by GSPE treatment. However, cyclin D2 was also not modified by the cafeteria diet, suggesting that although β-cell mass is increased in a model of prediabetes to counter insulin resistance and to maintain normal glucose levels[6], at the time point of the analysis, β-cell mass had likely already increased. These results are consistent with that in high fat-fed rats, in which no changes in the proliferation marker Ki67 were observed, despite showing

an increase in β-cell mass.[14] Therefore, these results indicate a possible more effective preventive role of procyanidins instead of a palliative role in proliferation.

As mentioned above, a genetically induced obesity model was also used to evaluate the effects of GSPE on proliferation and apoptosis in the pancreas in a pathological state. In this way, ZF rats were chronically treated with GSPE. In this case, the dose of GSPE used was higher than in other experiments because the ZF model is a model of a more severe diseased state and we expected mild GSPE action. In fact, although GSPE counteracted the expression of some markers of apoptosis and proliferation compared with untreated ZF rats, the molecular changes induced by procyanidins were not strong enough to counteract the genetic background of the ZF model at a physiological level because both apoptosis and the plasma levels of glucose and insulin in treated rats were elevated as those of the ZF control group.

Another objective raised in this experiment was to analyse the proteomic profile of the islets treated with GSPE. Proteomic techniques provide insight into global protein expression from identification to quantification, taking into account the highly dynamic status of the proteome.[15] In this way, we detected differentially expressed proteins in the GSPE-treated ZF rats and identified the biological processes in which they were implicated. One of these biological processes was apoptosis and cell death, and the levels of most of the proteins included in this group counteracted the effects of the ZF genotype, as was observed in the markers analysed at the gene expression level. Therefore, using ZF rats as a reference for apoptosis, GSPE tended to improve the process, although it did not induce changes in the final apoptosis levels. Considering the *in vitro* results in which GSPE enhanced the pro-apoptotic effects of glucose, although plasma glucose levels were increased in ZF rats in contrast to the lean rats, GSPE did not enhance the increased apoptosis levels observed in ZF rats; on the contrary, GSPE appeared to counteract some markers of apoptosis at the gene and protein level. This finding might suggest that other pathways were involved in addition to the pathway involved in the deleterious effects of glucose; however, the proteomic analysis did not reveal a clear pathway by which GSPE could exert its protective effects, although we have identified individual targets (e.g., Eef1a1, Vdac1 or chaperones such as Hspd1 and Hspa5).

Concerning the physiological state, the effects of GSPE on proliferation and apoptosis were tested *in vitro*, by treating INS-1E cells with GSPE, and *in vivo*, in an experiment in which rats were chronically treated with a moderate dose of the extract. The tested dose, translated to humans according to Reagan-Shaw et. al,[16] would correspond to an intake of 240 mg of procyanidins/day, which could be achieved in regular wine drinkers and fruit eaters. *In vitro*, the treatment of the cells with the extract modified neither the apoptosis and proliferation processes nor their respective analysed markers. The observed results *in vitro* were consistent with those observed *in vivo* in which GSPE treatment modified neither apoptosis nor β-cell mass.

The lack of effects of GSPE on proliferation and apoptosis could be explained by the slow renewal of β-cells in a healthy state, with steady low levels of proliferation and apoptosis.[17] β-cell mass slightly increases over the lifespan of an organism, due to age-related increases in body

weight and insulin demand to maintain glucose homeostasis.[17] However, in rats, this increase in β-cell mass is due to an increase in cell size, rather than to an increase in proliferation.[18]

Moreover, the effects of flavonoids in apoptosis and proliferation in pancreatic islets and β-cells that have been previously studied (reviewed in the introduction and in [2]) primarily demonstrate that flavonoids are protective prior to damage and toxic assaults. Only a few studies demonstrated the effects of flavonoids under healthy conditions, as in the case of INS-1E cells treated with resveratrol, which exerted pro-apoptotic and antiproliferative effects,[19] or genistein, which inhibited the proliferation of cultured mouse islet cells at high concentrations and long treatment periods.[20] In contrast, at lower concentrations, genistein enhanced β-cell proliferation,[21] and quercetin increased the number of pancreatic islet cells in rats.[22] However, other studies demonstrated no effect of flavonoids on cell viability or mass,[21,23,24] as we observed in the case of GSPE. Therefore, the effects of flavonoids in apoptosis and proliferation depend on the molecule and dose tested.

Although GSPE did not modify proliferation and apoptosis processes under healthy conditions, some of the markers showed signs of modulation by the extract. These changes could suggest possible protective and preventive effects against a subsequent damage. In fact, polyphenols, which are synthesised by plants in response to stress, have been hypothesised to activate the animal's own stress defence pathways when ingested and confer stress resistance and survival benefits. This hypothesis is named xenohormesis.[25] In this way, procyanidins could activate rat stress defence pathways, inducing the modulation of genes involved in cell apoptosis and proliferation, without affecting these processes at a physiological level but preparing the cells for a future situation of stress. Taking all of this into consideration, it would be interesting to study the protective effects of GSPE in the context of damaged β-cells.

Finally, the microRNA (miRNA) profile was analysed from the islets isolated from these rats chronically treated with GSPE because miRNAs have been shown to regulate pancreatic function, including the regulation of insulin synthesis[26,27] and secretion[28,29] and β-cell apoptosis[30]. Moreover, procyanidins have been reported to modulate miRNA expression in other cells.[31,32] We found that pancreatic islet miRNAs are targets of procyanidins. However, the miRNAs modified by GSPE did not correspond with those described to regulate insulin synthesis and secretion and apoptosis. Moreover, *in silico* analysis of these miRNAs did not indicate any involvement in proliferation and apoptosis, supporting the idea that GSPE did not alter these processes under normal conditions.

Another pathological situation in which proliferation and apoptosis are disturbed in pancreatic cells is in cancer in which proliferation is enhanced and apoptosis inhibited. Pancreatic ductal adenocarcinoma is one of the most aggressive cancers, with poor prognosis and a low rate of survival. Therefore, procyanidins, which exert anti-carcinogenic effects in other cancer cells,[33] could be a potential candidate for pancreatic cancer prevention and treatment. Moreover, the pro-apoptotic and antiproliferative effects of GSPE found *in vitro* using the INS-1E cell line also indicate their possible anti-carcinogenic effect in pancreatic ductal adenocarcinoma. Several transgenic and xenograft mouse models of pancreatic exocrine neoplasia have been developed. However, the *in vivo* models remain limited and present different molecular features that must be

considered in the studies, in addition to cost, time and reproducibility.[34] Therefore, as an alternative, we selected an adenocarcinoma cell line, MIA PaCa-2, to test the effects of GSPE on proliferation and apoptosis *in vitro*.

As expected, the extract inhibited cell proliferation and increased apoptosis. In addition, this effect was found to be mediated by a down-regulation of the anti-apoptotic protein Bcl-2 and a depolarisation of the MMP.

In this case, the components of the extract with higher antiproliferative and pro-apoptotic activity were also identified. Epigallocatechin gallate (EGCG) and gallic acid were the components with the strongest antiproliferative effects, but considering that the concentration of gallic acid is more than 40 times higher than EGCG,[35] gallic acid was considered the component of the extract that was more responsible for the observed effects. Similar to the whole extract, the phenolic acid also induced apoptosis in MIA PaCa-2 cells, promoting the depolarisation of the mitochondrial membrane.

In addition, in this study, two different approaches were used to approximate *in vivo* conditions. On the one hand, cells were treated with basolateral media containing the components of the extract absorbed and metabolised by Caco-2 cells. This assay system of treating cells with basolateral media was first used by Eguchi et al. in 2005 to study the antioxidant activity of fresh food preparations.[36] Moreover, Caco-2 cells have been used as a model to study the intestinal absorption or secretion of various drugs and to determine the transport kinetics, absorption characteristics and metabolism of dietary polyphenols.[37–40] On the other hand, cells were treated with GSPE-treated rat serum, an approximation that permitted the exposure of the cells to the polyphenols that were absorbed and metabolised in the organism, reaching the target organ *in vivo*, which, to our knowledge, has not been described before in the study of the effects of polyphenols *in vitro*. The results indicated that the Caco-2 basolateral media is effective for the study of procyanidin catechins but not for the study of gallic acid, which was not absorbed by Caco-2 cells. However, despite a reduced transport in the intestine, gallic acid is found in the plasma of rats treated with GSPE, reinforcing the conclusion that the effects of GSPE in MIA PaCa-2 cells could be due in part to gallic acid.

Comparing the results in INS-1E cells with those obtained in MIA PaCa2 cells, the high molecular weight components of the whole extract demonstrated higher antiproliferative activity in the β-cell line. The fraction enriched with molecules with the highest degree of polymerisation also demonstrated more effective antiproliferative effects compared with other fractions tested in MIA PaCa-2 cells, yet gallic acid was the molecule that best correlated with the effects obtained using the whole extract. However, this molecule showed high toxicity in the INS-1E cell line (results not shown).

In contrast, the pro-apoptotic effects of GSPE observed in INS-1E cells involved the glucose pathway, as mentioned above, implying the involvement of the mitochondrial apoptotic pathway. In MIA PaCa-2 cells, the pro-apoptotic effects of GSPE also suggested the involvement of the intrinsic apoptotic pathway but possibly by other mechanisms than in INS-1E cells. p53 was mutated and inactivated in pancreatic cancer, which allows cell survival in a situation of DNA damage.[41] p53 can regulate the expression of the Bcl-2 family members, inducing BAX and

inhibiting Bcl-2 expression.[42] Our results indicated that GSPE and gallic acid modulated the expression of these proteins, suggesting that procyanidins could be inducing apoptosis through p53. In fact, green tea catechins were shown to inhibit the expression of mutant p53 and induce apoptosis in the pancreatic adenocarcinoma cell line HPAF-II.[43]

In summary, in this doctoral thesis, we analysed the effects of GSPE both *in vitro*, using cell lines, and *in vivo*, using rats. We have used proteomic techniques and different approaches to approximate an *in vivo* situation, in addition to identifying the molecules from the extract that exert the observed effects. However, the repercussion of these results in humans is unknown. Flavonoid intake was associated with lower risk of T2DM[44,45] and pancreatic cancer.[46] In addition, some studies of the effects of procyanidins on glucose homeostasis in humans have been conducted. As reviewed by Pinent et al., only some studies found effects on glycaemia, with improvement in insulin sensitivity, either in physiological or pathological situations.[1] In such human trials, only parameters in the blood can be measured, predominantly glucose and insulin but also TAG, free fatty acids or other lipid metabolism related biomarkers, inflammatory markers and factors related to the redox status.[47] Although it is obviously more difficult to study in detail the physiological β-cell turnover in humans than in rodents, there are nevertheless enough indications that similar phenomena occur (reviewed in [48]). However, the structural organisation of the islets of Langerhans has a species difference that is considered to have considerable functional significance.[49,50] Therefore, these differences should be taken into consideration when extrapolating the results obtained in rats concerning the effects of flavonoids on apoptosis and proliferation of pancreatic cells to humans.

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V. CONCLUSIONS

1. **GSPE modulates β-cell proliferation**

   - GSPE does not affect proliferation under healthy conditions at the doses and duration of the treatment tested, either *in vivo* or *in vitro*.

   - GSPE is antiproliferative under altered conditions in INS-1E cells, counteracting the increase in proliferation due to high glucose and high insulin levels. GSPE also enhances the inhibition of this process due to high palmitate levels.

   - There is no evidence that the antiproliferative effects of GSPE observed *in vitro* can be reproduced *in vivo*.

2. **GSPE modulates β-cell apoptosis**

   - GSPE does not affect apoptosis under healthy conditions at the doses and duration of the treatment tested, either *in vivo* or *in vitro*.

   - GSPE enhances the pro-apoptotic effects of high glucose in the insulinoma cell line INS-1E.

   - GSPE modulates apoptosis markers *in vivo*, generally counteracting the effects of cafeteria diet-induced apoptosis and the Zucker Fatty genotype, but the efficacy of procyanidins depends on the dose, the time of treatment, and/or the gender.

3. **The mechanism by which GSPE modulates β-cell apoptosis involves the modulation of Bcl-2 and Bax expression, implying the involvement of the intrinsic apoptosis pathway. GSPE does not affect the regulation of miRNAs that regulate β-cell apoptosis.**

4. **GSPE inhibits proliferation and enhances apoptosis in the pancreatic adenocarcinoma cell line MIA PaCa-2. The mechanisms by which the extract exerts the anti-carcinogenic effects involve the alteration of mitochondrial membrane potential but not ROS production.**

5. **The main components of the extract responsible for the effects have been identified.**

   - The antiproliferative effects of GSPE in the beta-cell line INS-1E are likely due to high molecular weight forms.

   - Gallic acid is one of the components of the extract with higher antiproliferative and pro-apoptotic activity in MIA PaCa-2 cells.

1. **El GSPE modula la proliferació de les cèl·lules β pancreàtiques**

   - El GSPE no modifica la proliferació en condicions fisiològiques a les dosis i durades de tractament analitzats, ni en els experiments *in vivo* ni *in vitro*.

   - El GSPE és antiproliferatiu sota condicions alterades *in vitro* en les cèl·lules INS-1E, contrarestant l'augment de proliferació causat per nivells de glucosa i insulina elevats, i incrementant la inhibició d'aquest procés degut a un alt nivell de palmitat.

   - No hi ha evidències que els efectes antiproliferatius del GSPE observats *in vitro* es reprodueixin *in vivo*.

2. **El GSPE modula l'apoptosi de les cèl·lules β pancreàtiques**

   - El GSPE no afecta l'apoptosi en condicions fisiològiques a les dosis i durades de tractament analitzats, ni en els experiments *in vivo* ni *in vitro*.

   - El GSPE incrementa els efectes pro-apoptòtics de l'elevada glucosa *in vitro* en la línia cel·lular d'insulinoma de rata INS-1E.

   - El GSPE modula marcadors d'apoptosi *in vivo*, en general, contrarestant els efectes de l'apoptosi induïda per la dieta de cafeteria o pel genotip de les rates *Zucker Fatty*, però l'eficàcia de les procianidies depèn de la dosi, la durada del tractament, i/o el gènere.

3. **El mecanisme pel qual GSPE modula l'apoptosi de les cèl·lules β involucra la modulació de l'expressió de Bcl-2 i Bax, implicant la via intrínseca de l'apoptosi. GSPE no afecta la modulació de miRNAs que regulen l'apoptosi de les cèl·lules β.**

4. **El GSPE inhibeix la proliferació i incrementa l'apoptosi *in vitro* de la línia cel·lular d'adenocarcinoma de pàncrees MIA PaCa-2. Els mecanismes pels quals l'extracte realitza els efectes anticarcinogènics inclouen l'alteració del potencial de membrana mitocondrial, però no la producció d'espècies reactives d'oxigen.**

5. **S'han identificat els principals components de l'extracte responsables dels efectes de l'extracte.**

   - Els efectes antiproliferatius del GSPE en la línia cel·lular INS-1E són probablement a causa de les molècules d'alt pes molecular.

   - L'àcid gàl·lic és un dels components de l'extracte amb més activitat antiproliferativa i pro-apoptòtica en les cèl·lules MIA PaCa-2.

VII. ANNEXES

1. Currículum Vitae

LÍDIA CEDÓ GINÉ

Cognoms:	**CEDÓ GINÉ**
Nom:	**LÍDIA**
DNI:	**77788696M**
Data de naixement:	**16/08/1986**
Sexe:	**Dona**
Telèfon fix:	**977558465**
Correu electrònic:	**lidia.cedo@urv.cat**

Situació professional actual, només si treballa (no situació d'atur)

Nom de l'entitat: Universitat Rovira i Virgili
Departament, Servei, etc.: Facultat d'Enologia, Departament de Bioquímica i Biotecnologia
Categoría / lloc de treball o càrrec: Becari predoctoral - DGR
Data d'inici: 01/01/2010
Tipus de dedicació: Temps complet

CURRÍCULUM VÍTAE NORMALIZADO

Formació rebuda

Formació acadèmica universitària

Diplomatures, llicenciatures i enginyeries

1 **Titulació oficial:** Diplomatura / Llicenciatura / Grau
Nom del títol: Diploma d'Especialització en Fonaments de Química
Entitat que expedeix el títol: Universitat Rovira i Virgili
Data de la titulació: 18/11/2008

2 **Titulació oficial:** Diplomatura / Llicenciatura / Grau
Nom del títol: Bioquímica
Entitat que expedeix el títol: Universitat Rovira i Virgili
Data de la titulació: 07/07/2008
Nota mitjana de l'expedient: Excel·lent

3 **Titulació oficial:** Màster
Nom del títol: Nutrició i metabolisme
Entitat que expedeix el títol: Universitat Rovira i Virgili
Data de la titulació: 30/06/2009
Nota mitjana de l'expedient: Notable

Formació especialitzada, continuada, tècnica, professionalitzada (diferent a la formació acadèmica reglada i a la sanitària)

1 **Tipologia de la formació:** Curs
Títol específic: Estadística Aplicada a l'Experimentació Bioquímica
Entitat que expedeix el títol: Centre Tecnològic de Nutrició i Salut (CTNS) **Tipus d'entitat:** Universitat
Data de finalització de la formació: 03/10/2012, 2 dies

2 **Tipologia de la formació:** Curs
Títol específic: Formació bàsica sobre radiacions ionitzants i protecció radioógica (6 h.)
Entitat que expedeix el títol: Servei de recursos científics i tècnics - URV (Tarragona) **Tipus d'entitat:** Universitat
Data de finalització de la formació: 04/08/2010, 4 mesos - 7 dies

3 **Tipologia de la formació:** Curs
Títol específic: Curs d'Aptitud Preventiva URV 2010 (18h.)
Entitat que expedeix el títol: Institut de Ciències de l'Educació de la URV **Tipus d'entitat:** Universitat
Data de finalització de la formació: 26/02/2010, 3 dies

4 **Tipologia de la formació:** Curs
Títol específic: Formació preventiva i primers auxilis en el treball amb productes corrosius
Entitat que expedeix el títol: Intitut de Ciències de l'Educació de la URV **Tipus d'entitat:** Universitat
Data de finalització de la formació: 11/06/2009, 1 dia

5 **Tipologia de la formació:** Curs
Títol específic: Formació de personal investigador usuari d'animals d'experimentació
Entitat que expedeix el títol: Universitat de Barcelona **Tipus d'entitat:** Universitat
Data de finalització de la formació: 12/05/2009

6 **Tipologia de la formació:** Curs
Títol específic: Real time PCR y secuenciación masiva de DNA Aplicaciones
Entitat que expedeix el títol: Roche Diagnostics, S.L. - Barcelona **Tipus d'entitat:** Universitat
Data de finalització de la formació: 12/11/2008, 1 dia

Coneixement d'idiomes

Idioma	Parla	Lectura	Escriptura
Català	Bé	Bé	Bé
Espanyol	Bé	Bé	Bé
Anglès	Suficient	Bé	Suficient

Experiència docent

Docència impartida

1 **Tipologia de la docència:** Docència no oficial
Nom de l'assignatura: BioDocentia: Curs d'actualització en Biologia Molecular i Biotecnologia (3 hores) (Cursos i Seminaris Impartits)
Tipus de programa: Cursos i Seminaris Impartits **Tipus de docència:** Teòrica i pràctiques presencials
Data de l'últim cop: 25/05/2012
Entitat de realització: Institut de Ciències de l'Educació de la URV i l'Associació de Biotecnòlegs de Catalunya

2 **Tipologia de la docència:** Docència oficial
Nom de l'assignatura: Pràctiques de Biologia Molecular de Sistemes (1,5 crèdits) - Grau de Bioquímica i Biologia Molecular - Curs 2011/12 (Docència a segon cicle)
Tipus de programa: Llicenciatura **Tipus de docència:** Pràctiques presencials
Data de l'últim cop: 27/04/2012
Entitat de realització: Universitat Rovira i Virgili

3 **Tipologia de la docència:** Docència oficial
Nom de l'assignatura: Pràctiques de Metodologia i Experimentació en Biociències Moleculars I (3 crèdits) - Grau de Bioquímica i Biologia Molecular - Curs 2011/12 (Docència a segon cicle)
Tipus de programa: Llicenciatura **Tipus de docència:** Pràctiques presencials
Data de l'últim cop: 11/10/2011
Entitat de realització: Universitat Rovira i Virgili

4 **Tipologia de la docència:** Docència oficial
Nom de l'assignatura: Pràctiques d'Enginyeria Genètica Molecular (4,5 crèdits) - Llicenciatura en Biotecnologia - Curs 2010/11 (Docència a segon cicle)
Tipus de programa: Llicenciatura **Tipus de docència:** Pràctiques presencials
Data de l'últim cop: 25/10/2010
Entitat de realització: Universitat Rovira i Virgili

5 **Tipologia de la docència:** Docència oficial
Nom de l'assignatura: Pràctiques d'Enginyeria Genètica Molecular (4,5 crèdits) - Llicenciatura en Biotecnologia - Curs 2009/10 (Docència a segon cicle)
Tipus de programa: Llicenciatura **Tipus de docència:** Pràctiques presencias
Data de l'últim cop: 30/10/2009
Entitat de realització: Universitat Rovira i Virgili

6 **Tipologia de la docència:** Docència oficial
Nom de l'assignatura: Pràctiques de Regulació del Metabolisme (1,5 crèdits) - Llicenciatura en Bioquímica i Llicenciatura en Biotecnologia - Curs 2008/09 (Docència a segon cicle)
Tipus de programa: Llicenciatura **Tipus de docència:** Pràctiques presencials
Data de l'últim cop: 31/03/2009
Entitat de realització: Universitat Rovira i Virgili

7 **Tipologia de la docència:** Docència oficial
Nom de l'assignatura: Pràctiques d'Enginyeria Genètica Molecular (4,5 crèdits) - Llicenciatura en Biotecnologia - Curs 2008/09 (Docència a segon cicle)
Tipus de programa: Llicenciatura **Tipus de docència:** Pràctiques presencials
Data de l'últim cop: 24/10/2008
Entitat de realització: Universitat Rovira i Virgili

Experiència científica i tecnològica

Activitat científica o tecnològica

Participació en projectes d'R+D+I finançats en convocatòries competitives d'entitats públiques o privades

1 **Denominació del projecte:** Interacción de los flavonoides con el sistema endocrino. Repercusiones sobre la funcionalidad pancreática y el control de la ingesta.
Entitat de realització: Universitat Rovira i Virgili
Investigador/s responsable/s: Ana Maria Ardèvol Grau
Nombre d'investigadors: 3
Entitats finançadores: Ministerio de Ciencia e Innovación
Codi de projecte segons l'entitat finançadora: AGL2011-23879
Data d'inici: 2012
Finançament del projecte, quantia total: 100.000

2 **Denominació del projecte:** Nutrigenòmica
Entitat de realització: Universitat Rovira i Virgili
Investigador/s responsable/s: Luis Maria Arola Ferrer
Nombre d'investigadors: 18
Entitats finançadores: Universitat Rovira i Virgili
Codi de projecte segons l'entitat finançadora: 2010PFR-URV-B2-03
Data d'inici: 2010
Finançament del projecte, quantia total: 99.229

3 **Denominació del projecte:** Acción de las procianidinas de los alimentos sobre la producción pancreática de insulina
Entitat de realització: Universitat Rovira i Virgili
Investigador/s responsable/s: Ana Maria Ardèvol Grau
Nombre d'investigadors: 3
Entitats finançadores: Ministerio de Educación y Ciencia
Codi de projecte segons l'entitat finançadora: AGL2008-01310
Data d'inici: 2009
Finançament del projecte, quantia total: 115.000

4 **Denominació del projecte:** Nutrigenòmica
Entitat de realització: Universitat Rovira i Virgili
Investigador/s responsable/s: Luis Maria Arola Ferrer
Nombre d'investigadors: 18
Entitats finançadores: Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR)
Codi de projecte segons l'entitat finançadora: 2009 SGR 526
Data d'inici: 2009
Finançament del projecte, quantia total: 53.040

Activitats científiques i tècniques generals

Producció científica

Publicacions, documents científics i tècnics de caràcter tecnològic i qualsevol altra expressió

1 Cedó, L.; Castell-Auví, A.; Pallarès, V.; Blay, M.; Ardévol, A.; Arola, L.; Pinent, M. Grape seed procyanidin extract modulates proliferation and apoptosis of pancreatic beta-cells. Food Chemistry.138, pp. 524 - 530. 2013.
Tipus de producció: Article **Tipus de suport:** Revista

2 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 insulinemia by affecting insulin production and degradation. Journal of Nutritional Biochemistry. 2012 (in press).
Tipus de producció: Article **Tipus de suport:** Revista

3 Pallarès, V.; Calay, D.; Cedó, L.; Castell-Avuí, A.; Raes, M.; Pinent, M.; Ardévol, A.; Arola, L.; Blay, M. Additive antagonistic and synergistic effects of procyanidins and polyunsatuurated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide. Nutrition. 28, pp. 447 - 457. 2012.
Tipus de producció: Article **Tipus de suport:** Revista

4 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. Critical Reviews in Food Science and Nutrition. 52, pp. 569 - 584. 2012.
Tipus de producció: Review **Tipus de suport:** Revista

5 Cedó, L.; Castell-Auví, A.; Pallarès, V.; Ubaida Mohien, C.; Baiges, I.; Blay, M.; Ardévol, A.; Pinent, M. Pancreatic islet proteome profile in Zucker fatty rats chronically treated with a grape seed procyanidin extract. Food Chemistry. 135, pp. 1948 - 1956. 2012.
Tipus de producció: Article **Tipus de suport:** Revista

6 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. 108, pp. 1155 - 1162. 2012.
Tipus de producció: Article **Tipus de suport:** Revista

7 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. 108, pp. 1562 - 1573. 2012.
Tipus de producció: Article **Tipus de suport:** Revista

8 Castell-Auví, A.; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M.; Ardévol, A. Grape seed procyanidins improve ß-cell functionality under lipotoxic conditions due to their lipid-lowering effect. Journal of Nutritional Biochemistry. 2012 (in press)
Tipus de producció: Article **Tipus de suport:** Revista

9 Guasch, L.; Sala, E.; Castell-Auví, A.; Cedó, L.; Liedl, K.R.; Wolber, G.; Muehlbacher, M.; Mulero, M.; Pinent, M.; Ardévol, A.; Valls, C.; 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. 2012 (in press)
Tipus de producció: Article **Tipus de suport:** Revista

10 Castell-Auví, A.; Cedó, L.; Movassat, J.; Portha, B.; Sánchez-Cabo, F.; Pallarès, V.; Blay, M.; Pinent, M.; Ardévol, A. Procyanidins modulate microRNA expression in pancreatic islets. Journal of Agricultural and Food Chemistry. 2012 (in press).
Tipus de producció: Article **Tipus de suport:** Revista

11 Castell, A.; Cedó, L.; Pallarès, V.; Blay, MT.; Pinent, M.; Motilva, MJ.; Arola, Ll.; Ardèvol, A. Development of a Coculture System to Evaluate the Bioactivity of Plant Extracts on Pancreatic ß-Cells. Planta Medica. 76, pp. 1576 - 1581. 2010.
Tipus de producció: Article **Tipus de suport:** Revista

Treballs presentats en congressos nacionals o internacionals

1 **Títol:** Procyanidins modify ß-functionality. Its effectiveness on insulinaemia depends on the physiological situation.
Nom del congrés: Advanced Technologies & Treatments for Diabetes
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Localitzaci· i data: Barcelona, Espanya. 2012
Castell-Auví, A.; Cedó, L.; Pallarès, V.; Blay, M.; Pinent, M.; Ardévol, A.

2 **Títol:** Pancreatic islet proteome profile in Zucker fatty rats chronically treated with a grape seed procyanidin extract
Nom del congrés: Doctoral Workshop on Molecular Nutrition
Tipus d'esdeveniment: Congrés
Tipus de participació: Ponència
Ciutat de realització: Tarragona, Espanya
Data: 2012
Cedó, L.

3 **Títol:** Pancreatic islet proteome profile in Zucker fatty rats chronically treated with a grape seed procyanidin extract
Nom del congrés: The Southern Catalonia Nobel Campus
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster i ponència
Ciutat de realització: Tarragona, Espanya
Data: 2012
Cedó, L.; Castell-Auví, A.; Pallarès, V.; Baiges, I.; Blay, M.; Ardévol, A.; Pinent, M.

4 **Títol:** Procyanidins affect insulinaemia due to their effects on insulin synthesis and insulin degradation sensitivity depends on the physiological situation
Nom del congrés: 4th International Congress on Prediabetes and the metabolic syndrome
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Madrid, Espanya
Data: 2011
Ardèvol, A.; Castell-Auvi, A.; Cedó, L.; Pallarés, V.; Blay, MT.; Pinent, M.

5 **Títol:** GSPE Inhibits proliferation of pancreatic ß-cells
Nom del congrés: 4th International Congress on Prediabetes and the metabolic syndrome
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Madrid, Espanya
Data: 2011
Cedó, L.; Castell-Auvi, A.; Pallarés, V.; Blay, MT.; Ardèvol, A.; Pinent, M.

6 **Títol:** Synergistic effects of polyphenols and polyunsaturated fatty acids (PUFAs) on RAW 264.7 macrophages activated by lipopolysaccharide (LPS)
Nom del congrés: 36th FEBS CONGRESS Biochemistry for Tomorrow's Medicine
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Torino, Itàlia
Data: 2011
Pallarès, V.; Calay, D.; Cedó, L.; Castell-Auví, A.; Raes, M.; Pinent, M.; Ardévol, A.; Arola, L.; Blay, M.

7 **Títol:** GSPE effects on apoptosis of pancreatic ß-cells in conditions of glucotoxicity
Nom del congrés: 36th FEBS CONGRESS Biochemistry for Tomorrow's Medicine
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Torino, Itàlia
Data: 2011
Cedó, L.; Castell-Auví, A.; Pallarès, V.; Blay, MT.; Ardèvol, A.; Pinent, M.

8 **Títol:** Efecto de las procianidinas de pepita de uva (GSPE) sobre la inflamación en ratas Zucker fa/fa
Nom del congrés: XXXIV Congreso de la Sociedad Española de Bioquímica y Biología Molecular
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Barcelona, Espanya
Data: 2011
Pallarès, V.; Cedó, L.; Castell-Auvi, A.; Pinent, M.; Ardévol, A.; Blay, M.

9 **Títol:** El Páncreas como diana de los efectos de las procianidinas de la uva.
Nom del congrés: XXXIV Congreso de la Sociedad Española de Bioquímica y Biología Molecular. SEBBM BCN2011
Tipus d'esdeveniment: Congrés
Tipus de participació: Ponència
Ciutat de realització: Barcelona, Espanya
Data: 2011
Pinent,M.; Ardèvol, A.; Arola, L.; Bladé, C.; Blay, M.T.; Arola, A.; Castell-Auví, A.; Cedó, L.; Fernández-Larrea, J.; García-Vallvé, S.; Mulero, M.; Pujadas, G.; Salvadó, M.J.

10 **Títol:** GSPE effects on proliferation and apoptosis markers in pancreas and mesenteric adipose tissue.
Nom del congrés: XXV International Conference on Poliphenols
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Montpellier, França
Data: 2010
Cedó, L.; Castell, A.; Pallarés, V.; Blay, MT.; Ardèvol, A.; Pinent, M.

11 **Títol:** Screening of the effects of grape seed procyanidins (GSPE) on different adipose tissue depots in Zucker fa/fa rats.
Nom del congrés: 7th Nutrigenomics Conference - NuGOweek2010
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Glasgow, Regne Unit
Data: 2010
Ardèvol, A.; Matas, A.; Castell, A.; Cedó, L.; Pallarès, V.; Blay,M.; Pinent, M.

12 **Títol:** A Paracrine loop between adipocytes and machrophages in adipose tissue: An in Vitro model to study obesity-induced inflamation.
Nom del congrés: 4th Congress of the International Society of Nutrigenetics/Nutrigenomics (ISSN)
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Pamplona, Espanya
Data: 2010
Pallarès, V.; Castell-Auví, A.; Cedó, L.; Pinent, M.; Ardèvol, A.; Blay, M.

13 **Títol:** GSPE Effects on proliferation an apoptosis on pancreatic B-cells in genetically obese rats.
Nom del congrés: 4th Congress of the International Society of Nutrigenetics/Nutrigenomics (ISSN)
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Pamplona , Espanya
Data: 2010
Cedó, L.; Castell, A.; Pallarés, V.; Blay, M., Ardèvol. A.; Pinent, M.

14 **Títol:** Development of co-culture system to evaluate the bioactivity of plant extracts on pancreatic beta cells.
Nom del congrés: Nugoweek
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Montecatini, Itàlia
Data: 2009
Castell, A.; Cedó, L.; Pallarès, M.; Blay, M.; Pinent, M.; Motilva, MJ.; Arola, L.; Ardèvol, A.

15 **Títol:** GSPE modify insulin-degrading enzyme expression
Nom del congrés: Bioactive food components, mitochondrial function and health
Tipus d'esdeveniment: Congrés
Tipus de participació: Pòster
Ciutat de realització: Palma Mallorca, Espanya
Data: 2009
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Altres mèrits

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Compendi d'altres mèrits

1 **Descripció del mèrit:** Assistència a Reunions Científiques (sense presentació ni publicació): Sessió científica sobre Aliments Funcionals
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3 **Descripció del mèrit:** Assistència a Reunions Científiques (sense presentació ni publicació): 2ème Jounée Diabéto-Diderot: 'De la Manipulation des Cellules Souches à la Thérapie Cellulaire des Diabètes'.
Entitat que concedeix: Université Paris-Diderot
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4 **Descripció del mèrit:** Línies de recerca: Effects of grape seed procyanidin extract on proliferation and apoptosis in pancreatic cells.
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Data de la concessió: 08/10/2008

5 **Descripció del mèrit:** Assistència a Reunions Científiques (sense presentació ni publicació): XXXI Congreso de la Sociedad Española de Bioquímica i Biología Molecular - Bilbao
Data de la concessió: 10/09/2008

6 **Descripció del mèrit:** IDIOMES
- First Certificate in English. Universitat de Cambridge. Desembre 2006.
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- Estudis de mecanografia

7 **Descripció del mèrit:** BEQUES, AJUTS
- Beca predoctoral URV de la Universitat Rovira i Virgili, 08/10/2008 - 31/12/2008
- Beca de formació de personal investigador (modalitat A). Generalitat de Catalunya. 01/01/2009 - 31/12/2009.
- Beca de formació de personal investigador (modalitat B). Generalitat de Catalunya. 01/01/2010 - 31/12/2012.
- Beca M3C 2010 - Borses de mobilitat de 3r cicle per assistir al XXVth International Conference on Polyphenols.
- Premi per incorporació a beca FI. 2010.
- Beca de vitge per realitzar una estada a un centre d'inverstigació a l'estranger. Generalitat de Catalunya. Curs 2010/2011.
- Beca M2D 2011 - Borses de mobilitat de 3r cicle per assistir al 36th FEBS Congress.

8 **Descripció del mèrit:** BEQUES I AJUTS
- Becària de règim general. Ministeri d'Educació i Ciència. Cursos 2004/2005, 2005/2006, 2006/2007 i 2007/2008.
- Beca per realitzar un curs d'anglès a l'estranger: estada de tres setmanes a Dublín a l'escola Aspect College Dublin, assolint el nivell Advanced. Juliol 2007.
- Beca per assistir al Curs d'Iniciació a la Investigació i al Congrés de la Sociedad Española de Bioquímica y Biotecnologia Molecular. Bilbao 2008.

2. Other articles derived from this doctoral thesis

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The effects of a cafeteria diet on insulin production and clearance in rats

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Abstract

The aim of the present study was to determine the effects of a cafeteria diet on the function and apoptosis of the pancreas, and the activity and expression of the insulin-degrading enzyme (IDE). Female Wistar rats were fed either with a cafeteria diet or a control diet for 17 weeks, and blood and tissues were then collected for analysis. The cafeteria diet-treated rats had higher plasma insulin and C-peptide levels ($P<0·05$), showing increased insulin secretion by the pancreas. Insulin protein and gene expression levels were higher in the pancreas of obese rats, as was its transcriptional controller, pancreatic duodenal homeobox 1 ($P<0·05$). Feeding a cafeteria diet down-regulated the gene expression of the anti-apoptotic marker B-cell/lymphoma 2 (BCL2), and up-regulated the protein levels of BCL2-associated X protein, a pro-apoptotic marker ($P<0·05$). The cafeteria diet caused lipid accumulation in the pancreas and modified the expression of key genes that control lipid metabolism. To assay whether insulin clearance was also modified, we checked the activity of the IDE, one of the enzymes responsible for insulin clearance. We found increased liver IDE activity ($P<0·05$) in the cafeteria diet-fed animals, which could, in part, be due to an up-regulation of its gene expression. Conversely, IDE gene expression was unmodified in the kidney and adipose tissue; although when the adipose tissue weight was considered, the insulin clearance potential was higher in the cafeteria diet-treated rats. In conclusion, treatment with a cafeteria diet for 17 weeks in rats mimicked a pre-diabetic state, with ectopic lipid accumulation in the pancreas, and increased the IDE-mediated insulin clearance capability.

Key words: Cafeteria diet: Pancreas: Insulin-degrading enzyme: Apoptosis: Insulin

The prevalence of overweight and obesity is quickly increasing to epidemic proportions around the world[1]. Obesity is associated with a higher incidence of a number of diseases, including CVD, cancer[2] and diabetes[3]. There are several causes of obesity, some of which are genetically defined, but some others are related to environmental factors. To study obesity, several animal models have been used, including both genetic and diet-induced obesity models. Among them, the cafeteria diet has been used as a robust model because it is a good reproduction of the diet in Western society[4].

The cafeteria diet model has been described in terms of the effects of the diet on increasing body weight and modulating adipogenesis and inflammation[4–6]. The diet consists of feeding animals with a substantial amount of salt, sugar and fat; thus, mimicking the diet consumed by Western cultures[4]. The diet promotes voluntary hyperphagia that results in rapid weight gain, increases fat pad mass and leads to a pre-diabetic state[4,7]. Different studies have shown that the cafeteria diet increases plasma insulin levels and alters glucose metabolism[4,8–11]. Despite the key role of the pancreas in glucose metabolism, scarce work has been performed to study the chronic effects of this type of high-fat diet on this organ.

Insulin resistance induced by the diet might ultimately lead to the impairment of β-cell function and reduced β-cell mass, in part due to an increase in the apoptosis of these cells, and thus might lead to diabetes[12]. The impairment of β-cell function partly results from the accumulation of TAG in the pancreas, as in other non-adipose tissues, when a positive net energy balance occurs[13]. Studies in human islets have confirmed that insulin secretion at high glucose concentrations is impaired in a time-dependent fashion by exposure to NEFA, and NEFA also produce a decrease in insulin biosynthesis[14]. Yet, few *in vivo* studies concerning the effects of the cafeteria diet on the pancreas are available in the literature. In female rats, feeding the cafeteria diet for 14 weeks has been reported to diminish the glucose (and other depolarising agent)-stimulated insulin secretion of isolated islets, probably

Abbreviations: BAX, B-cell/lymphoma 2-associated X protein; BCL2, B-cell/lymphoma 2; CPT1a, carnitine palmitoyltransferase-1a; DM2, type 2 diabetes mellitus; FASn, fatty acid synthase; HOMA-IR, homeostatic model assessment for insulin resistance; IDE, insulin-degrading enzyme; PDX1, pancreatic duodenal homeobox 1; TTBS, Tris-buffered saline (TBS) plus 0·5% (v/v) Tween-20; UCP2, uncoupling protein 2.

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through a defect in the Ca^{2+} mobilisation by these islets[11]. Additionally, in male rats, treatment with the cafeteria diet for 15 weeks has been shown to induce changes in pancreatic islet morphology[4]. More studies concerning the effects of this diet in the pancreas would be beneficial for the description of this widely used obesity model, which allows the analysis of the environmental effects of the diet, free from possible genetic effects.

Plasma insulin levels are highly dependent on pancreas functionality and the number of islet β-cells, but insulin clearance is also of importance in determining the levels in plasma. *In vivo*, a major role in the clearance and degradation of insulin is played by the metalloproteinase insulin-degrading enzyme (IDE)[15], and the IDE has been identified as a candidate gene for diabetes susceptibility in the Goto–Kakizaki rat, a genetic model of non-insulin-dependent diabetes[16]. These animals exhibit elevated blood glucose and insulin levels[16] due to a mutated form of the IDE, which provokes reduced insulin degradation and causes symptoms typical of the human type 2 diabetes mellitus (DM2) syndrome[17]. The evidence for a putative influence of the IDE on the pathogenesis of DM2 has been confirmed with human genetic studies that have linked polymorphisms in the IDE gene to an increased risk for insulin resistance and DM2[18–20]. Furthermore, genome-wide association studies in human subjects have revealed that the IDE region of chromosome 10q contains a variant that confers DM2 risk[21]. Lastly, IDE knockout mice are both glucose-intolerant and hyperinsulinaemic, supporting the concept that the IDE is important in the maintenance of normal blood glucose and insulin levels[22].

Despite the data provided above, the specific effects of the cafeteria diet on insulin production and clearance remain unclear. Given the high prevalence of diet-induced obesity and the importance of this model of study, we aimed to investigate the effects of the cafeteria diet by analysing the pancreas functionality and apoptosis. Moreover, we also evaluated the effects of this diet on the activity and expression of the IDE, which, to our knowledge, have not been studied previously.

Materials and methods

Animal experimental procedures

Wistar female rats (Charles River Laboratories), weighing between 160 and 175 g, were housed in animal quarters at 22°C with a 12 h light–12 h dark cycle, and after 1 week in quarantine, the animals were treated as described previously[23]. Briefly, twelve rats were divided in two groups (*n* 6): a control group fed with a standard diet and a group fed with a cafeteria diet (Table 1) and water in addition to the standard diet. The animals were fed *ad libitum*, and the food was renewed daily. At the end of the treatment (17 weeks), the food was removed and 3 h later, the animals were killed by beheading. Blood was collected using heparin, and animal tissues were excised, frozen immediately in liquid N_2 and stored at −80°C until analysis. All procedures were approved by the Experimental Animals Ethics Committee of the Rovira i Virgili University.

Table 1. Cafeteria diet composition

Components	Quantity per rat
Bacon	15–20 g
Sweets	1/2
Biscuit with pâté	1
Cheese	1–2 g
Muffins	1/2
Carrots	3 g
Milk with sugar (220 g/l)	50 ml
Water	*Ad libitum*
Standard diet	*Ad libitum*

Intraperitoneal glucose tolerance test and plasma parameters

Intraperitoneal glucose tolerance tests were carried out (2 g glucose/kg body weight) after overnight fasting at week 15 and also 3 d before killing at week 17. Glucose was measured with a glucometer after blood samples had been collected by tail bleeding (Menarini).

Insulin and C-peptide plasma levels at killing were assayed using ELISA methodology (Mercodia) following the manufacturer's instructions. Glucose plasma levels were determined using an enzymatic colorimetric kit (QCA).

Homeostatic model assessment for insulin resistance (HOMA-IR) and the HOMA-β index were calculated using the fasting values of glucose and insulin with the following formulas:

$$\text{HOMA-IR} = \frac{\text{insulin}\,(\mu\text{U/ml}) \times \text{glucose}\,(\text{mM})}{22{\cdot}5},$$

$$\text{HOMA-}\beta = \frac{20 \times \text{insulin}\,(\mu\text{U/ml})}{\text{glucose}\,(\text{mM}) - 3{\cdot}5}.$$

Insulin content in the pancreas

For insulin extraction, the pancreas was homogenised with an acid–ethanol solution (75 % ethanol, 1·0 M-glacial acetic acid and 0·1 M-HCl), and the extracts were kept overnight at 4°C and then centrifuged. The insulin levels from the extracts were measured using ELISA methodology (Mercodia).

TAG content in the pancreas

TAG from the pancreas were extracted using PBS containing 0·1 % Triton X-100 (Sigma-Aldrich), and the levels of TAG were determined using an enzymatic colorimetric kit (QCA). The protein content of each sample was measured using the Bradford method[24] and was used to normalise the TAG values.

Insulin-degrading enzyme activity assay

Liver extracts were prepared by homogenising the tissue in Cytobuster Protein Extraction Reagent (Novagen) according to the manufacturer's recommended protocol. IDE activity was assessed with the InnoZyme Insulysin/IDE Immunocapture Activity Assay Kit (Calbiochem/Merck) and is expressed as relative fluorescent units.

Western blot

Protein was extracted from the whole frozen pancreas using RIPA (radio-immunoprecipitation assay) 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 (1:1000; Sigma-Aldrich) and 1 mM-PMSF (phenylmethanesulfonyl fluoride solution). Total protein levels of the lysate were determined using the Bradford method[24]. After boiling for 5 min, 100 μg of protein were loaded and electrophoresed through a 4–15 % SDS-polyacrylamide gel. The samples were then transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories) and blocked at room temperature for 1 h using 5 % (w/v) non-fat milk in TTBS buffer (Tris-buffered saline (TBS) plus 0·5 % (v/v) Tween-20). The membranes were incubated overnight at 4°C with rabbit polyclonal B-cell/lymphoma 2 (BCL2)-associated X protein (BAX) primary antibody (Cell Signaling Technology) at a 1:1000 dilution in blocking solution or rabbit anti-β-actin antibody (diluted 1:750; Sigma-Aldrich). After washing with TTBS, the blots were incubated with peroxidase-conjugated monoclonal anti-rabbit secondary antibody (Sigma-Aldrich) at a 1:10 000 dilution at room temperature for 2 h. The blots were then washed thoroughly in TTBS, followed by TBS. Immunoreactive proteins were visualised with the ECL Plus Western Blotting Detection System (GE Healthcare) using a FluorChem system (Alpha Innotech) and software version 6.0.2. Densitometric analysis of the immunoblots was performed using ImageJ 1.44p software (National Institutes of Health); all of the proteins were quantified relative to the loading control.

Quantitative RT-PCR

Total RNA from the pancreas, liver and mesenteric adipose tissue was extracted using the TRIzol reagent, following the manufacturer's instructions. Complementary DNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit (Applied Biosystems), and it was subjected to quantitative RT-PCR amplification using the Taqman Master Mix (Applied Biosystems). Specific Taqman probes (Applied Biosystems) were used for different genes, as follows: Rn01774648-g1 for insulin; Rn00565839-m1 for IDE; Rn00755591_m1 for pancreatic duodenal homeobox 1 (PDX1); Rn01754856-m1 for mitochondrial uncoupling protein 2 (UCP2); Rn00569117_m1 for fatty acid synthase (FASn); Rn00580702_m1 for carnitine palmitoyltransferase-1a (CPT1a); Rn99999125_m1 for BCL2; Rn01480160_g1 for BAX. β-Actin was used as the reference gene (Rn00667869-m1). The reactions were performed using a quantitative RT-PCR 7300 System (Applied Biosystems) according to the manufacturer's instructions. The relative mRNA expression levels were calculated using the $\Delta\Delta C_t$ method.

Calculations and statistical analysis

The results are expressed as means with their standard errors, and the effects were assessed by Student's *t* test. All of the calculations were performed using SPSS software (SPSS, Inc.).

Results

Cafeteria diet increases insulin production in the pancreas

We first examined the effects of the cafeteria diet on pancreatic insulin production after 17 weeks of the treatment. The cafeteria diet-fed rats showed significantly higher plasma insulin (cafeteria: 0·94 (SEM 0·2) nM *v.* control: 0·18 (SEM 0·0) nM) and C-peptide levels (cafeteria: 2·54 (SEM 0·4) nM *v.* control: 0·77 (SEM 0·0) nM), demonstrating an increase in insulin secretion by the pancreas. After the intraperitoneal glucose tolerance tests at 15 and 17 weeks of the treatment, HOMA-IR and the HOMA-β index were calculated (Table 2). After 15 weeks of cafeteria-diet feeding, the HOMA-IR index indicated that the animals had peripheral insulin resistance, as we mentioned above, and the HOMA-IR index was even higher after two additional weeks of the treatment. In contrast, the values of the HOMA-β index at 15 weeks indicated that there were no significant differences in pancreas functionality, despite peripheral insulin resistance. However, after two more weeks of the treatment (at week 17), the HOMA-β values were significantly increased in the cafeteria-diet treatment group, suggesting that pancreas functionality in terms of the response to glucose was higher than that in the controls to counteract peripheral insulin resistance. We also determined insulin content in the pancreas, which showed that there was significantly more insulin accumulation in the pancreata of the cafeteria-fed rats (cafeteria: 69·90 (SEM 17·2) ng/mg tissue *v.* control: 8·76 (SEM 0·9) ng/mg tissue). The increased insulin secretion and pancreatic insulin content agree with the observed effect on insulin gene expression, which was significantly higher in the obese animals (Table 3). In addition,

Table 2. Effects of the cafeteria diet on homeostatic model assessment for insulin resistance (HOMA-IR) and the homeostatic model assessment-β (HOMA-β) index
(Mean values with their standard errors)

	HOMA-IR				HOMA-β			
	Control		Cafeteria		Control		Cafeteria	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
15 weeks	0·14	0·0	6·24*	2·2	−33·04	34·6	781·11	812·5
17 weeks	0·27	0·6	9·06*	3·6	858·42	1107·4	7009·43*	1058·5

* Mean values were significantly different between the treatments ($P \leq 0·05$).

Table 3. Gene expression in the pancreas
(Mean values with their standard errors)

	Control		Cafeteria	
Gene	Mean	SEM	Mean	SEM
Insulin	1·50	0·6	5·11*	1·4
PDX1	1·43	0·5	4·07*	1·0
UCP2	0·89	0·1	1·64**	0·3
BCL2	1·00	0·0	0·72*	0·1
BAX	1·07	0·2	0·86	0·1
CPT1a	1·03	0·1	1·63**	0·2
FASn	1·08	0·2	0·75	0·1

PDX1, pancreatic duodenal homeobox 1; *UCP2*, uncoupling protein 2; *BCL2*, B-cell/lymphoma 2; *BAX*, BCL2-associated X protein; *CPT1a*, carnitine palmitoyltransferase-1a; *FASn*, fatty acid synthase.
Mean values were significantly different between the treatments: *$P \leq 0{\cdot}05$, **$P \leq 0{\cdot}1$.

the cafeteria diet increased the expression of PDX1, an important regulator of insulin transcription (Table 3).

Cafeteria diet activates apoptosis biomarkers

To examine the effects of the cafeteria diet on apoptosis in the pancreas, we analysed the anti-apoptotic marker BCL2 and the pro-apoptotic marker BAX. The cafeteria-fed rats showed a decrease in the expression of BCL2 (Table 3). Concerning BAX expression, although the mRNA levels of this gene were not altered (Table 3), we did observe an increase in the protein levels of BAX in the cafeteria diet-fed group (Fig. 1).

Cafeteria diet increases pancreatic TAG content

Feeding a cafeteria diet leads to a higher amount of TAG and NEFA in the plasma. Under physiological conditions, most

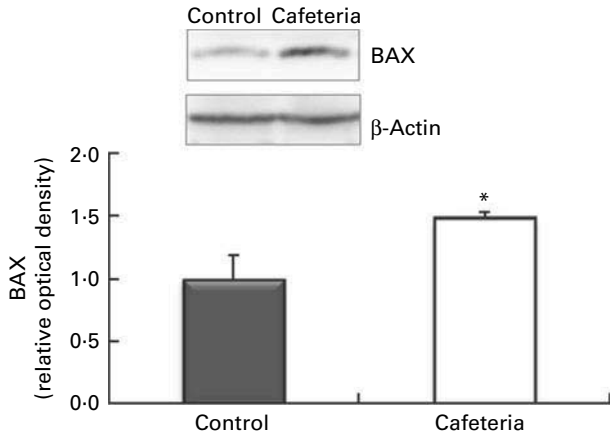

Fig. 1. Effects of the cafeteria diet on B-cell/lymphoma 2-associated X protein (BAX) protein levels in the pancreas. BAX protein levels were quantified by Western blot analysis. A representative Western blot is provided. Protein expression was quantified relative to the β-actin loading control using ImageJ 1.44p software (National Institutes of Health). Values are means of six animals, with their standard errors represented by vertical bars. *Mean values were significantly different from the control group ($P < 0{\cdot}05$).

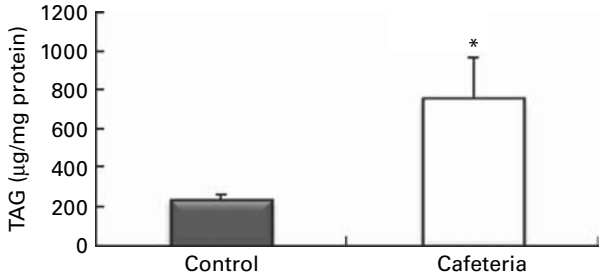

Fig. 2. Effects of the cafeteria diet on pancreatic TAG content. After 17 weeks of the cafeteria diet, the animals were killed, and the pancreas was obtained. The TAG content in the pancreas was measured using an enzymatic colorimetric kit. Values are means of six animals, with their standard errors represented by vertical bars. *Mean values were significantly different from the control group ($P < 0{\cdot}05$).

TAG are stored in adipocytes, but in animals with obesity, increased stores of TAG are detectable in other tissues. To determine whether the cafeteria diet treatment led to an accumulation of lipids in other tissues, we examined the TAG content in the pancreas, and found that it was four times higher in the obese animals than in the control group (Fig. 2). To better understand lipid metabolism in the pancreas, we also analysed the expression of key regulatory genes. We selected the *FASn* gene, the key enzyme of *de novo* fatty acid synthesis[25], and the *CPT1a* gene, the key controller of NEFA oxidation[26]. The cafeteria-fed rats showed a slight increase in CPT1a mRNA levels (Table 3). In contrast, the cafeteria diet tended to reduce the mRNA levels of FASn (Table 3).

It has previously been reported that UCP2 expression is regulated in tandem with the level of NEFA[27,28]; similarly, we observed that the cafeteria-fed rats showed a slight increase in the levels of UCP2 mRNA in the pancreas (Table 3).

Cafeteria diet modifies the activity and expression of insulin-degrading enzyme

The cafeteria diet-fed animals showed higher levels of plasma insulin due to a higher insulin production and secretion, but we speculated that insulin clearance could also have contributed to this effect. Thus, we analysed the gene expression and activity of one of the main factors responsible for insulin clearance: the IDE. Because the liver is an organ with high IDE mRNA and protein levels[29–31], we determined IDE gene expression and activity in this tissue. Fig. 3 shows that IDE enzyme activity in the liver was increased by the cafeteria diet, and IDE mRNA levels were also slightly increased. This effect was stronger when the whole tissue weight was considered, which better correlates with the real capacity of liver IDE to remove insulin (Table 4). To analyse further the capacity of the cafeteria-fed rats to degrade insulin, we also determined IDE gene expression in the kidney, an important site of insulin clearance from the systemic circulation. The results did not show any effect of the cafeteria diet on renal IDE or when considering the total tissue weight. Furthermore, we did not observe any effect due to the cafeteria diet in white

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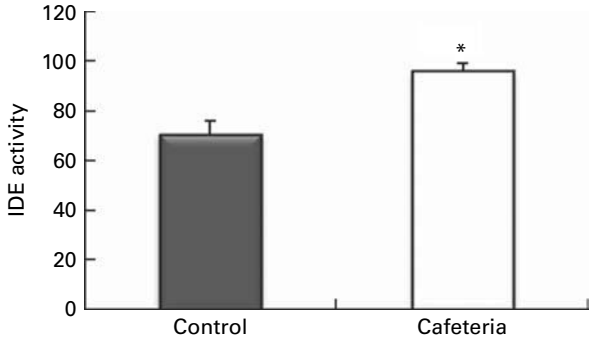

Fig. 3. Effects of the cafeteria diet on insulin-degrading enzyme (IDE) activity in the liver. IDE activity from liver samples was determined with an immunocapture-based fluorometric assay. Values are means of six animals, with their standard errors represented by vertical bars. * Mean values were significantly different from the control group ($P<0·05$).

adipose tissue, where the IDE is also expressed (Table 4). However, when considering the total tissue weight, the cafeteria-fed rats had a higher capacity to remove insulin due to the IDE in the adipose tissue.

Discussion

Obesity, driven by an excess of fat intake, leads to a toxic lipid accumulation in non-adipose tissues, which is accompanied by insulin resistance. Concurrently, the increased insulin demand promotes a β-cell compensation that involves increased pancreas functionality and/or increased β-cell mass. Insulin resistance may develop into DM2, as driven by β-cell failure[12,32,33].

The present study was designed to examine the effects of the cafeteria diet on insulin production by evaluating pancreas functionality and apoptosis and the activity and expression of the IDE, as an estimation of insulin clearance. We had previously shown that 17 weeks of feeding a cafeteria diet led to insulin resistance[23]. The fact that plasma TAG and fatty acids are increased by the cafeteria diet[4,34,35] indicates a failure in the adipose tissue, which is unable to remove the lipids from the circulation and might not respond to the insulin inhibition of lipolysis. In the present study, we found that TAG content in the pancreas was already increased by 17 weeks. The source of lipids for this increased TAG storage might be plasma NEFA, as under conditions of high NEFA, *de novo* fatty acid synthesis is inhibited, a condition that is in agreement with the present gene expression results for *FASn*. The lack of malonyl-CoA, secondary to the inhibited *de novo* fatty acid synthesis, avoids the main CPT1a-inhibitory regulation, thus allowing the entrance of fatty acids into the mitochondria for oxidation. In fact, we found a higher gene expression of *CPT1a*, suggesting that, in the pancreas, increased fatty acid β-oxidation occurred to counteract lipid accumulation. Increased fatty acid oxidation has also been observed in the rat insulinoma cell line INS-1 treated with oleate and palmitate for 72 h[36], and has been associated with impaired glucose-induced insulin secretion in INS-1 β-cells[36,37] and islets[38]. Defects in glucose-stimulated insulin secretion in INS-1 cells after chronic fatty acid treatment could have been related, in part, to an increase in UCP2 mRNA that is associated with uncoupled mitochondria[39,40]. We observed a tendency for the up-regulation in pancreatic *UCP2* gene expression after feeding the cafeteria diet. Based on the present results, we cannot discern whether the individual β-cell insulin-secretory functionality was modified due to the cafeteria diet; however, we did find that, despite this increased lipid accumulation, the pancreata of the cafeteria diet-treated rats were still able to respond to an acute glucose load, with a similar increment in plasma insulin as the control rats, and, in fact, the HOMA-β index in the cafeteria-fed rats was better. To counteract peripheral insulin resistance, the cafeteria diet-treated animals exhibited increased insulin synthesis and secretion. We found higher insulin protein and gene expression in the pancreata of the cafeteria-treated rats and an up-regulation of *PDX1*, a transcriptional controller of insulin gene expression[41]. Altogether, it is likely that the increased amount of insulin and up-regulated insulin and *PDX1* gene expression in the pancreas is due to an increase in β-cells. In mice, a high-fat diet leads to increased β-cell mass[42,43], and treatment with a cafeteria diet in rats has been shown to lead to larger pancreatic islets, although their functionality was not evaluated in that study[4]. Despite the results showing that pancreas still responded to a glucose load after the cafeteria diet treatment, we also observed that there were signs of apoptosis in the pancreas, specifically the down-regulation of BCL2 expression and increased BAX protein levels. Although we found that the *BAX* gene expression was not altered, changes in BAX protein levels without changes in gene expression have previously been shown to correlate with apoptosis[44]. *In vitro* studies have

Table 4. Insulin-degrading enzyme gene expression in the liver, white adipose tissue (WAT) and kidney (Mean values with their standard errors)

		Control		Cafeteria	
Tissue		Mean	SEM	Mean	SEM
Liver	Gene expression relative to the control	0·94	0·1	1·23**	0·1
	Relative gene expression × tissue weight	9·74	1·1	16·46*	1·9
WAT	Gene expression relative to the control	1·00	0·0	0·91	0·0
	Relative gene expression × tissue weight	3·99	0·5	18·52*	3·0
Kidney	Gene expression relative to the control	1·01	0·1	0·94	0·1
	Relative gene expression × tissue weight	1·62	0·2	1·95	0·1

Mean values were significantly different between the treatments: *$P\leq0·05$, **$P\leq0·1$.

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reported that chronic hyperglycaemia and high NEFA can induce β-cell apoptosis[45,46]. In Zucker diabetic fatty rats, obesity, chronic hyperglycaemia and worsening insulin resistance have been shown to lead ultimately to β-cell apoptosis[47,48]. In addition, male C57BL/6J mice fed a high-fat diet (60 % fat) for 8[42] or 12 weeks[43] have shown increased islet β-cell apoptosis. However, different results have also been reported, as a 60 % high-fat diet did not induce pancreatic apoptosis in mice[49]. In the present study, we showed that in rats, hyperglycaemia, increased fatty acids and/or pancreatic lipid accumulation derived from a 17-week cafeteria diet triggered the apoptosis process in the pancreata of rats, although this pancreatic damage was not reflected in insulin plasma concentrations.

We also focused on another important step for the regulation of glucose metabolism, the insulin clearance, specifically, one of its main regulators, the IDE. Alterations in gene coding for this enzyme have been linked with diabetes susceptibility[16–21]. In adipocytes isolated from human patients, insulin degradation (probably mediated by the IDE) has been demonstrated to be reduced in pre-diabetic and diabetic states[50]. In mice, the lack of the IDE leads to glucose intolerance and hyperinsulinaemia[22]. The effects of the diet on the expression of the IDE in the brain have been studied in the context of insulin resistance as an underlying mechanism that is responsible for an increased risk of Alzheimer's disease and, thus, of the possible involvement of cerebral IDE in the development of β-amyloidosis. In APP/PS1 double transgenic mice (which develop memory deficits and amyloid plaques), providing 10 % sucrose-sweetened water *ad libitum* has been reported to not statistically modify brain IDE protein levels[51]. In another model of Alzheimer-disease-like neuropathology, the feeding of Tg2576 mice with a high-fat diet for 9 months led to insulin resistance and decreased IDE activity and protein expression in the brain[52]. However, the effects of a high-fat diet on the IDE in tissues responsible for insulin clearance and degradation have, to our knowledge, not been studied thus far. The present results showed that the liver activity of the IDE was increased in the cafeteria-fed animals, which could have been partially due to an up-regulation of its gene expression. We did not find the same effects in other tissues. Despite the role of the kidney in degrading insulin[15], we did not observe any effects of the cafeteria diet on renal IDE gene expression, nor did we find any modification of IDE gene expression in adipose tissue. Because we are studying obesity, we must also consider the tissue size, which is mainly relevant for the adipose tissue. When the adipose tissue weight is taken into consideration, the insulin clearance potential is higher in cafeteria diet-treated rats[53]. This has been described in fa/fa genetically obese animals and in obese human subjects, where the potential of insulin cleavage by adipose tissue in obese patients was higher than that in the controls, implying that both insulin secretion and turnover are increased in obese individuals[54]. When considering the whole amount of tissue, the cafeteria diet-fed rats in the present study exhibited a higher capacity to degrade insulin. This fact suggests that feeding a high-fat diet is accompanied by a mechanism to eliminate high plasma insulin levels, and at least with the experimental procedure used in the present study (cafeteria-diet for 17 weeks), it appears that hyperinsulinaemia was not due to impaired insulin clearance but that it was counteracted by enhanced insulin degradation. The present results are not in agreement with previous studies suggesting that hepatic insulin degradation may be reduced as an adaptive mechanism to relieve stress on pancreatic β-cells imposed by insulin resistance that is induced by a high-fat diet[55,56]. Remarkably, the experimental models differ vastly, suggesting that there could be different stages in diet-induced obesity and insulin resistance with different degrees of involvement of hepatic glucose clearance. Thus, the cafeteria diet acts on IDE expression and activity in the liver; it does not directly modulate IDE activity in adipose tissue. However, the increased amount of such tissue induced by the feeding of a cafeteria diet would contribute to the amount of insulin clearance.

In conclusion, we showed that the cafeteria diet treatment for 17 weeks in rats mimicked a pre-diabetic state with ectopic lipid accumulation in the pancreas. At this time point, the insulin content and gene expression in the pancreas were higher in the cafeteria diet-treated group than in the control rats, a condition that leads to hyperinsulinaemia. In addition, initial signs of apoptosis appeared in the pancreas. We also showed that IDE-mediated insulin clearance capability was higher in the cafeteria diet-treated rats than in the controls.

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Procyanidins Improve some Disrupted Glucose Homoeostatic Situations: An Analysis of Doses and Treatments According to Different Animal Models

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This review analyses the potential beneficial effects of procyanidins, the main class of flavonoids, in situations in which glucose homeostasis is disrupted. Because the disruption of glucose homeostasis can occur as a result of various causes, we critically review the effects of procyanidins based on the specific origin of each type of disruption. Where little or no insulin is present (Type I diabetic animals), summarized studies of procyanidin treatment suggest that procyanidins have a short-lived insulin-mimetic effect on the internal targets of the organism, an effect not reproduced in normoglycemic, normoinsulinemic healthy animals. Insulin resistance (usually linked to hyperinsulinemia) poses a very different situation. Preventive studies using fructose-fed models indicate that procyanidins may be useful in preventing the induction of damage and thus in limiting hyperglycemia. But the results of other studies using models such as high-fat diet treated rats or genetically obese animals are controversial. Although the effects on glucose parameters are hazy, it is known that procyanidins target key tissues involved in its homeostasis. Interestingly, all available data suggest that procyanidins are more effective when administered in one acute load than when mixed with food.

Keywords procyanidin, glucose, diabetes, insulin resistance, human studies, rat

LIST OF ABBREVIATIONS

bw = body weight
GSPE = Grape Seed Procyanidin Extract
Gck = Glucokinase
Gckr = Glucokinase-regulatory protein
G6pc = glucose-6-phosphatase
Glut4 = Glucose transporter type 4
Hk2 = hexokinase 2
STZ = streptozotocin
MPD = mean polymerization degree

INTRODUCTION

Abnormalities affecting glucose homeostasis, that is, in insulin secretion and/or insulin action, lead to the progressive deterioration of glucose tolerance and cause hyperglycemia, which in turn leads to a complex metabolic disorder of the endocrine system called diabetes mellitus (Type 2 diabetes). The disease is a major public health problem found in all parts of the world and its incidence is rapidly increasing (Bajaj and DeFronzo, 2003). This has led to a growing interest in the search for hypoglycemic agents from natural products, especially those derived from plants.

Flavonoids are naturally occurring phenolic compounds that are commonly found in plants. They are in fruits and vegetables, in chocolate, as well as in drinks such as wine and tea (Aron and Kennedy, 2008; Bhagwat et al., 2010). Their basic structure consists of three phenolic rings (Aherne and O'Brien, 2002). Depending on the structure and oxidation level of the C ring, flavonoids are further divided into several subclasses (Fig. 1). So, flavonoids include thousands of structures with different chemical, physical, and biological properties which perform a wide array of biochemical and pharmacological actions such as cardioprotective (Zern and Fernandez, 2005), antioxidant (Williams et al., 2004), anti-inflammatory (Rivera et al.,

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2008), antiallergic, antiviral, and anticarcenogenic (Amin and Buratovich, 2007; Kim et al., 2008) activities. Among their effects, 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 (Potenza et al., 2007; Park, 2008; Bose et al., 2008; Cao et al., 2007; Diepvens et al., 2006).

This review focuses on the effects of the largest and most ubiquitous class of flavonoids, the flavan-3-ols, which comprise the main constitutive units of condensed procyanidins. Homo-oligomeric proanthocyanidins with two (3′,4′) B-ring hydroxyl groups are called procyanidins and are one of the most common types of proanthocyanidins found in nature (Aron and Kennedy, 2008). Procyanidins are oligomeric structures formed by the polymerization of the monomeric flavan-3-ols (+)-catechin and (–)-epicatechin up to ten subunits. Procyanidins are found in a wide variety of foods in the human diet. They are present in the fruit, bark, leaves, and seeds of many plants and plant-derived

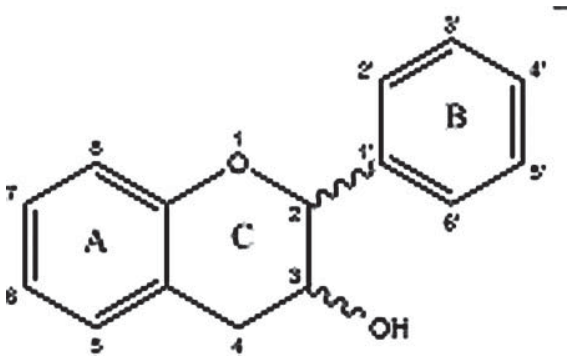

Figure 1a Representative structure of procyanidins. Basic structure of a flavonoid.

foods such as green tea, apples, cocoa, chocolate, grapes, apricots, and cherries. They are especially abundant in fruit juices and red wine (Aherne and O'Brien, 2002; Aron and Kennedy, 2008). Several authors have studied flavan-3-ol consumption

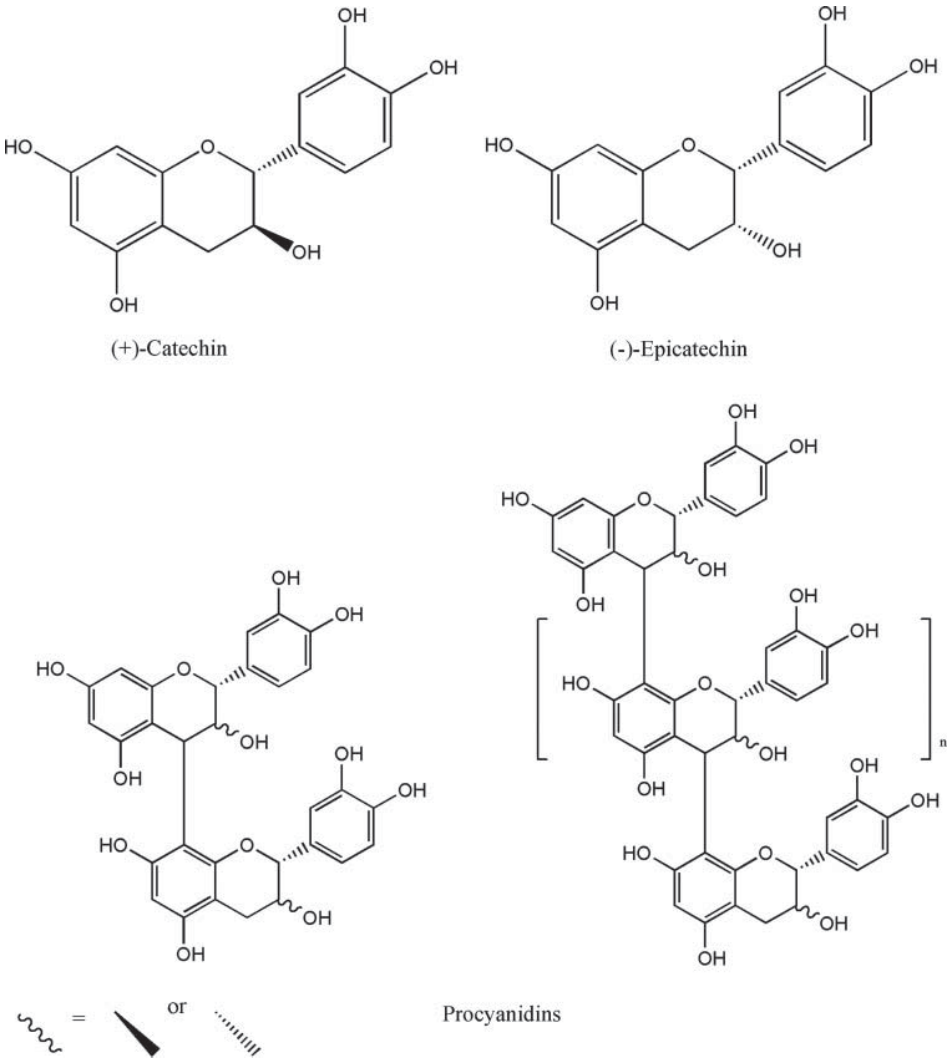

Figure 1b Structure of the flavanol monomers (+)-catechin and (-)-epicatechin, and their polymers called procyanidins, which include from dimers to oligomers.

and bioavailability, although the lack of reliable concentration data for procyanidin in foods has made it difficult to accurately evaluate their dietary intake. Gu et al. (2004) reported that daily proanthocyanidin intake could vary from 10 mg to 500 mg/day and they estimated that the mean daily proanthocyanidin intake in the United States was 53.6 mg/person/day. Arts and Hollman (2005) estimated that the mean intake of flavan-3-ol monomers in the Netherlands was 50 ± 56 mg/day, although the intake of oligomeric forms was not analyzed in that study. Due to the considerable intake of procyanidins through the diet, their potential beneficial effects have been widely studied. Procyanidins act against coronary heart diseases and atherosclerosis as well as several metabolic processes associated with the development of those disorders (Bladé et al., 2010). They are involved in the modulation of cholesterol and lipid metabolism (Bladé et al., 2010), induce changes in vascular events (Corder et al., 2006), have antigenotoxic (Llópiz et al., 2004) and cardiovascular effects (Karthikeyan et al., 2007), and improve oxidative or inflammatory states (Puiggros et al., 2009; Roig et al., 1999; 2002, Terra et al., 2009). They also have antiproliferative effects and have been studied as cancer preventive agents (Faria et al., 2006, Mantena et al., 2006). Several studies have investigated their effects on glucose homeostasis-disrupted situations; however, a clear consensus has not been reached on what exactly those effects are. Some evidence suggests that they might act as hypoglycemic agents, but hyperglycemia can result from various different causes. On the one hand, it might be the consequence of Type 1 diabetes in which there is a loss of beta cells due to an immune assault (Ichinose et al., 2007). On the other hand, high glucose levels can occur due to the ineffectiveness of insulin, that is, insulin resistance, a condition in which insulin levels initially increase to compensate for the lack of effect before reaching a state in which the pancreas is no longer functional, which is Type 2 diabetes (Kashyap and DeFronzo, 2007). Considering, therefore, that the causes that lead to glucose homeostasis disruption have different molecular origins, one could speculate that the effects of procyanidins might depend on the specific condition of glucose homeostasis disruption in which they are analyzed. Consequently, it is important to take into account all these situations before making general statements about these compounds. In this review we summarize the effects of procyanidins and their possible mechanisms of action, critically analyzing their role in different glucose homeostasis situations.

PROCYANIDIN BIOACTIVITY IN INSULIN DEFICIENCY

Hyperglycemia in Type 1 diabetes is the result of the body's inability to synthesize and/or secrete functional insulin. Several authors have assayed the ability of procyanidin-enriched extracts to ameliorate the physiological state caused by this situation (summarized in Table 1), which is easily reproduced with animal models through the destruction of the pancreas, mainly by means of streptozotocin (STZ).

In this model, the lowest dose of procyanidins that has been assayed, as far as we know, is 10 mg/kg body weight (bw). Maritim et al. (2003) showed that at this dose a procyanidin-rich extract from pine bark (Pycnogenol®) improved serum glucose after 14 days of treatment. This is the only model in which procyanidins were administered intraperitoneally; in other studies they are given orally, thus more closely reproducing the physiological ingestion of a procyanidin extract, since it can be digested, metabolized, not fully absorbed, etc. Lee et al. (2007) also administered 10 mg/kg bw of procyanidins orally once a day for three weeks and they describe an improvement in serum glucose and glycosylated protein due to oligomeric forms. In the same study the authors showed that polymeric procyanidins do not have the same effect. A similar dose of extract (in this case, polyphenol-enriched white wine and its derivative free ethanol) administered twice daily for a period of six weeks was used by Landrault et al. (2003), who found a tendency towards a decrease in plasma glucose, although not statistically significant. In that case, the dose of procyanidins was not given all at once in a daily bolus, but was added to feed pellets used in routine animal feeding. Similarly, in a study by Osakabe et al. (2004) a dose of roughly 50 mg/kg bw (in fact, it is difficult to clearly identify the administered dose in that study) of polyphenols in the form of cocoa liquor for a period of ten weeks did not show any significant effect either on plasma glucose or on body weight. However, slight improvements were found for the same parameters in the procyanidins-treated group. Al-Awwadi et al. (2004) tested a procyanidins dose of roughly 200 mg/kg bw which showed a clearer antihyperglycemic effect. This effect of reducing blood glucose was only found during daily measurements, whereas when measured during fasting without a previous daily administration of procyanidins, no effects on glycemia were recorded. Considering this, along with the fact that insulin levels were not modified in any of the procyanidin treatments, the authors explain that the effects of procyanidins are related to the reduction of food intake and/or absorption of nutriments and suggest a short-lived effect not involving the correction of the diabetic state. Our group worked with a similar amount (250 mg/kg) of a grape seed-derived extract enriched with oligomeric procyanidins (GSPE) administered acutely (five hours of treatment), which limited hyperglycemia in male STZ-diabetic Wistar rats in a fed state (Pinent et al., 2004). A parallel study with fasted animals showed that the reduction of glucose absorption was not the main mechanism used by procyanidins to decrease glycemia (Pinent et al., 2004). In a further analysis, we looked at the effects of the same dosage of GSPE in an oral sacarose tolerance test (supplementary data: Fig. S1) where we found no delay in the appearance of glucose in the blood in GSPE treated animals compared to controls, suggesting that grape procyanidins do not act primarily (or only) through disturbing glucose absorption. Instead, our results suggest that the antihyperglycemic effect of procyanidins is in part due to the fact that procyanidins have central insulin-mimetic effects.

Table 1 Summary of published effects of oligomeric procyanidins on glucose homeostasis in type I diabetic animal models

Estimated Dose*	Published dose	Extract	Duration	Animal model	Glucose measurement	Effects on glucose	References
	10 mg/kg bw; daily; intraperitoneally	Pycnogenol, a standarized extract from French maritime pine bark	14 days	Female Sprague-Dawley STZ-diabetic rat	On day 15, cardiac puncture halothane anaesthesia	Decreased serum glucose	(Maritim et al., 2003)
	10 mg/kg bw; daily; forced orally	Oligomeric procyanidins (MPD: 3.3) / polymeric From persimmon peel	3 weeks	Male Wistar STZ-diabetic rat	24 hours after last dose (not clear) pentobarbital anaesthesia	Decreased serum glucose and glicosilated protein	(Lee et al., 2007)
14.25 mg "gallic eq"/kg twice daily.	10 mg/kg bw; twice daily	Polyphenol enriched white wine (no anthocyanins); with and without etanol	6 weeks	Male Wistar STZ-diabetic rat	Not clear	Tendency to decrease glycaemia. Improved oxidative stress status	(Landrault et al., 2003)
Around 50 mg polyphenol/kg bw in the pellet	Diet containing 0.5% CLP (2.2% catechin+ epicatechin; 1.69% B2;2.37% C1; 2.01% cinnamtannin A2)	Polyphenol derived from cacao liquor	10 weeks	Male Sprague-Dawley STZ-diabetic rat	Some hours later than the polyphenols intake;from tail vein	No effects on glucose.	(Landrault et al., 2004)
	200 mg "gallic eq" /kg bw in a single dose at 9am	Red wine extract	6 weeks	Male Wistar STZ-diabetic rat	Monthly simultaneous to the procyanidins dose. End experiment and OGTT, 24 hours after last dose.	Decreased glucose linked to the extract administration	(Al-Awwadi et al., 2004)
	250 mg/kg bw forced orally	GSPE: monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 units) (31.7%).	1 dose	Male Wistar control and STZ-diabetic rat	5 hours after the dose	Decrease glucose	(Pinent,et al., 2004)
	250 mg/kg bw forced orally	GSPE	24 weeks	Male Wistar STZ-diabetic rat	After overnight fasting	No changes in glucose or glucosilated hemoglobin	(Li et al., 2009; 2008)
	50 and 100mg/kg bw; once daily; oral gavage	GSPE	At 24, 48 and 72 hours	Male Wistar alloxan-diabetic rat	After 18 h fasting	Decreased glucose (dose 100 after 48 h and both doses after 72 h) Decreased insulin at 72 hours	(El-Alfy et al., 2005)

*In order to compare the different studies we show the administered doses as mg procyanidins/kg body weight, therefore for the studies in which other units were used, we have estimated the amount of mg procyanidins/kg body weight from the published data.

They favor glucose uptake in the liver through increasing the mRNA expression of glucokinase (GcK) and decreasing the mRNA of glucokinase-regulatory protein (Gckr) and glucose-6-phosphatase (G6pc). Procyanidins might also act peripherally, since GSPE increases the mRNA expression of glucose transporter type 4 (Glut4) in white adipose tissue, although no significant changes in muscle of Glut4 or hexokinase 2 (Hk2) have been found (Fernandez-Larrea et al., 2007).

Finally, Li et al. (2008) tested the same dosage of GSPE (250 mg/kg), but for 24 weeks of treatment. They found neither a change in glycemia or in HbA1c, but they found a clear improvement in body weight gain and several other disturbed aspects linked to diabetes. In fact, almost all the previously mentioned studies also found improvements in inflammatory status (Lee et al., 2007), antioxidant effects (in almost all the studies), and improvements in diabetic nephropathy (Li et al., 2008).

Taken together, these studies suggest that procyanidins have a short-lived insulin-mimetic effect on internal targets of the organism (Al-Awwadi et al., 2004; Maritim et al., 2003; Pinent et al., 2004) and are useful in improving the general situation of the entire organism (Li et al., 2008) most likely due to oligomeric forms (Lee et al., 2007) and to a forced acute dose (almost all summarized works in Table 1). What remains unclear is the ability of procyanidins to ameliorate the actual cause of Type 1 diabetes, that is, to restore pancreas functionality (reviewed in Pinent et al., 2008). Of the studies mentioned above, only Al-Awwadi et al. (2004) analyzed plasma insulin, finding that procyanidins had no significant effect on it. This, in conjunction with the weak procyanidin effects found in other studies, does not support such amelioration of pancreas function. On the other hand, El-Alfy et al. (2005) used a different model for Type 1 diabetes, alloxan-induced diabetes, and found a reduction in glycemia due to procyanidins associated with an increase in insulinemia, but only after 72 hours of GSPE (50 and 100 mg/kg bw, daily) treatment. The reasons for the difference between the results of this study and those of other studies may include the time that the procyanidins were administered (El-Alfy et al., 2005) are the only ones who gave the first dose prior to drug treatment) but also to the different mechanisms used by streptozotozin and alloxan to impede pancreas functionality. Both diabetogens lead to beta-cell loss, but while in alloxan-induced diabetes reactive oxygen mediates the selective necrosis of beta-cells, in STZ-diabetes DNA alkylation might primarily mediate its toxic action, although production of reactive oxygen species may also be involved (Lenzen, 2008). Therefore, the antioxidant effects of procyanidins on the pancreas cannot be ruled out as a parallel mechanism for ameliorating Type 1 diabetes in some situations.

PROCYANIDIN BIOACTIVITY IN HEALTHY ANIMALS (NORMOINSULINEMIC)

The ability of procyanidins to mimic insulin effects in insulin sensitive targets when insulin is scarce or absent has been proved in vivo and shown, as well in vitro (see section titled Mechanism of Action). However, such effects are not so clear when there is a normal amount of insulin in the body.

Table 2 summarizes the results obtained from procyanidin treatments in healthy animals. Of all of them, only Al-Awwadi et al. (2004) describe a slight but significant positive effect of procyanidins on plasma glucose. They used male Wistar rats as a healthy model simultaneously with the above-mentioned Type 1 diabetic animals and they describe similar but milder procyanidin effects in the normal rats, which they explain as due to a reduction in food intake and/or absorption. Meanwhile, a study conducted by our group in which we administered an acute dose of GSPE similar to that used by Al-Awwadi to male Wistar rats for five hours, does not support this effect (Fernandez-Larrea et al., 2007). We found the same changes in the gene expression of liver Gck and Gkrp as those observed in diabetic animals, but on the other hand, we observed an up-regulation of G6pc gene expression, and remarkably plasma glucose was not modified. A higher acute dose (1 g/kg bw) did not have any effect on plasma glucose either (unpublished results). Agouni et al. (2009) report no changes in plasma glucose or in body weight after treating lean male Zucker rats with a much lower dose (20 mg/kg) of provinols (Agouni et al., 2009). Esteve et al. (personal communication) conducted a similar study with GSPE in lean Zucker rats using a similar dosage of procyanidins, and did not find any effects on glycemia, although a slight limitation in weight gain was noted. Higher doses were assayed in mice by Tomaru et al. (2007), but procyanidins were not found to affect glycemia.

If inhibition of glucose absorption were 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 shown here do not confirm that expectation. Therefore, all these data lead to the conclusion that when there is a lack of insulin, procyanidins may act as insulin-mimetic agents affecting some insulin targets. But under normal insulinemia, the results presented here suggest no clear effect of procyanidins on whole glucose homeostasis, probably due to the fact that insulin is more effective in terms of physiological effects.

PROCYANIDIN BIOACTIVITY IN INSULIN RESISTANT STATES

Procyanidins, therefore, do not seem to have a clear effect in normal conditions, but their interest as bioactive compounds lies in their beneficial effects against glucose homeostasis deregulation. Animal models for disturbed glucose homeostasis can be achieved by means of several different approaches, such as special diets. Table 3 summarizes studies in which procyanidins were given to animals simultaneously with the induction of insulin resistance by means of different diets. The first three summarized assays (Al-Awwadi et al., 2005; Yokozawa et al., 2008; Tsai et al., 2008) analyze the effects of procyanidins administered by oral gavage on fructose-induced insulin-resistant

Table 2 Summary of published effects of oligomeric procyanidins on glucose homeostasis in healthy animal models

Estimated dose	Published dose	Extract	Duration	Animal model	Glucose measurement	Glucose effects	References
	10 mg /kg bw; daily;intraperitoneally	Pycnogenol, a standarized extract from French maritime pine bark	14 days	Female Sprague-Dawley rat	Day 15; cardiac puncture; halothane anaesthesia	No change on serum glucose	(Maritim et al., 2003)
	20 mg/kg daily	provinols	8 weeks	Zucker lean rat	Not specified	No change glucose nor fructosamine	(Agouni et al., 2009)
19.5 mg/kg bw in sunflower oil		GSPE	4 weeks	Zucker lean rat	Not specified	No change glucose and slight decrease on body weight gain	unpublished results
Around 40 and 70 mg/kg bw in the pellet	Diet containing 0.5% or 1% CLP (2.2% catechin+ epicatechin; 1.69% B2;2.37% C1; 2.01% cinnamtannin A2)	Proanthocyanidins from cacao liquor	3 weeks	mice	Postprandial	No change	(Tomaru et al., 2007)
	200 mg "gallic eq"/kg; single dose at 9 a.m.	Red wine extract	6 weeks	Male Wistar rat	Monthly simultaneous to the procyanidins dose. End experiment and OGTT, 24 hours after last dose.	Reduced body growth and blood glucose slight but significant	(Al-Awwadi et al., 2004)
250 mg/kg bw forced orally		GSPE	1 dose	Male Wistar rat	5 after dose	No change	(Fernandez-Larrea et al., 2007)
	250 mg/kg bw forced orally	GSPE	24 weeks	Male Wistar rat	After overnight fasting	No effects in blood glucose	(Li et al., 2009)
1000 mg/kg bw forced orally		GSPE	1 dose	Male Wistar rat	4 after dose	No change	unpublished results

Table 3 Summary of preventive effects on different models of insulin resistance

Estimated dose	Published dose	Extract	Duration	Animal model	Glucose effects	References
	21.42 mg/kg oral gavage	Vitaflavan vs antocianins vs procyanidins	6 weeks	Sprague-Dawley simultaneous to 66% fructose	Decrease glucose. no changes insulin	(Al-Awwadi et al., 2005)
	10 or 20 mg/kg bw; oral gavage	Grape seed proanthocyanidin (gravinol)	2 weeks	Male Wistar simultaneous to 65% fructose	Lowers glucose and bw	(Yokozawa et al., 2008)
	125 mg/bw and 250 mg /bw; oral gavage at 6 pm	Proanthocyanidin-rich extract from longan flowers	14 weeks	Male Sprague-Dawley simultaneous to 60% fructose	Lowers glucose, insulin. Improves irs-1 and glut 4 protein in WAT mes	(Tsai et al., 2008)
21.7 mg gspe/kg bw included on the pellet	Nearly 7mg/animal/day	GSPE	19 weeks	Male Zucker lean simultaneous to 32% fat diet	Slight decrease on glucose, nor insulin, nor bw, adiponectin increased	(Terra et al., 2009)
20.15 mg gspe/kg bw included on the pellet		GSPE	19 weeks	Female Wistar Simultaneous to 45% fat diet	No changes	submitted to ICP
35.8 mg gspe/kg bw included on the pellet		GSPE	19 weeks	Female Wistar Simultaneous to 60% fat diet	No changes	submitted to ICP
35.7 mg/kg oral gavage	7.14 mg/kg bw	Chardonnay GSE	12 weeks	Male Hamster Simultaneous to 44.36% fat diet	Decrease glucose. No changes insulin and increase in adiponectin. Lowers bw	(Decorde et al., 2009)

This table summarizes the studies in animals in which insulin resistance was induced using different diets, and procyanidins were administered simultaneously to the beginning of this diet treatment.

animals. Even though these studies differ in terms of dosages, treatment times, and the procyanidin extracts, all of them found an improvement in hyperglycemia as a result of procyanidin treatment. On the other hand, other studies summarized here found that procyanidins had little effect on hyperglycemia. We assayed the preventive role of a final dose of approximately 20 mg GSPE/kg bw given concurrently with two similar medium-fat diets (31.8% fat and 45% fat) in lean male Zucker rats and female Wistar rats, respectively, for a period of 19 weeks. In these studies, the GSPE was included in the animal feed (pellet). Male Zucker rats showed a slight improvement in glycemia along with improved adiponectinemia (Terra et al., 2009). In contrast, in female Wistar rats glycemia tended to increase and insulinemia decrease as a result of GSPE treatment, with no changes in adiponectinemia (Terra et al., 2009). Since the GSPE extract used was the same in both studies, this different response could be partially explained by sex differences. In fact, female rats are more sensitive to high-fat treatments than males (Ribot et al., 2008) and this could mask the effects of GSPE, to which females would be more resistant. Glycemia was also not affected in a parallel group of female Wistar rats treated with a higher dosage of GSPE (35.8 mg/kg bw) included in a 60% fat diet, although increased insulinemia due to GSPE treatment was documented. Adiponectinemia also remained unchanged, despite an increase in its gene expression in mesenteric adipose tissue (Terra et al., 2009). The differences in the efficacy of procyanidin extracts in different insulin-resistance induction models may be explained by the origin of the disturbances in each model. Fructose-feed disruption begins with the lack of control of hepatic lipogenesis (Tran et al., 2009; Le, 2006), which procyanidins have been shown to repress (Baiges et al., 2010). This effect, together with the fact that GSPE limits VLDL assembly (Bladé et al., 2010) would inhibit the induction of pain in the animal, limiting the development of insulin resistance in this model. Meanwhile, in high-fat models, hepatic lipogenesis and VLDL assembly are not the key point leading to metabolic disruption (Varga et al., 2009; Storlien et al., 2000), so the effects of procyanidins on lipid metabolism would not directly lead to the amelioration of glucose homeostasis.

On the other hand, Decorde et al. (2009) (Table 3) show that a GSPE dose similar to those previously discussed and administered to hamsters for 12 weeks can reduce hyperglycemia in high-fat (44.4%) treated animals. Again, the differential effects of procyanidins on high-fat treated animals could be due to their effects on lipid metabolism, considering that hamsters have a very different lipid metabolism. But it is also important to bear in mind that in this study GSPE was not included in the feed pellets of the animals, but was administered daily by means of oral gavage. This suggests that procyanidins could also partially prevent high-fat diet-induced hyperglycemia, but only when they are acutely administered. Serra et al. (2010) claim that plasma procyanidin levels depend on the vehicle. GSPE administered orally to rats along with carbohydrate enriched food led to lower plasma levels of oligomeric procyanidins than when GSPE was dissolved in water. And it is important to note that higher amounts of procyanidins reach the circulation when they are administered with a daily bolus than if they are included in feed pellets, which animals consume small amounts of throughout the day. Because this daily oral gavage was also used in the fructose-feed models in which GSPE showed positive effects, the mode of administration seems to be important, suggesting that a single dose of GSPE may be effective in lowering glycemia; however, this single dose might not always be effective. Zhang et al. (2009) also administered procyanidins once daily by oral gavage (80 mg/kg bw for 6 weeks) and found no amelioration of plasma glucose levels. This study was performed on mice, so the use of a different species could also explain the different results compared to the above-mentioned studies.

Another situation in which procyanidins might have beneficial effects is their possible role in improving or correcting an already disturbed glucose homeostasis. Table 4 compiles the studies that analyze these effects in different animal models. The cafeteria diet is a successful model for inducing a clear dysfunction in the metabolism of rats (Petry et al., 2001). In this model, an acute daily dose of 25 mg GSPE/kg bw dissolved in semi-skimmed milk for 30 days improved the glucose homeostasis of diet-induced (high fat, high sucrose) insulin resistant animals. However, the effects of GSPE are dependent on the dosage and time of treatment, since neither shorter treatments nor a higher dosage were found to have a clear positive effect on glucose homeostasis (Montagut et al., 2009).

Glucose homeostasis disruption can also be studied using genetically altered models. Table 4 also contains experiments performed with such models, in which the effects of procyanidin extracts are more difficult to understand. A similar dose (19.5 mg/kg bw) of the same GSPE as that previously shown to be effective on cafeteria-diet fed animals, daily force-fed (sunflower oil as vehicle) to male Zucker fa/fa rats did not improve glycemia at all (M. Esteve, personal communication). In contrast, orally force-fed provinols at 20 mg/kg bw have been found to improve glycemia in the same animal model (no information concerning animal sex is provided in this case) (Agouni et al., 2009). There are numerous differences between these studies, such as the vehicle, the duration of the treatment, and the flavonoid extract. The main difference between the two extracts is their monomeric components—anthocyanidins in provinols and flavanols in GSPE. One might speculate that the different compositions are the source of the different responses. In fact, in the above-mentioned study by Al-Awwadi (2005), slight differences were observed between extracts rich in either anthocyanidins or procyanidins, although in that case the procyanidin-enriched extract was the most effective at ameliorating insulin sensitivity. Therefore, the conclusion cannot be drawn that a particular type of flavanol is responsible for the effects of the extracts.

In db/db mice studies, GSPE improved glucose homeostasis at very low doses: approximately 10 mg/kg bw daily forced (Lee et al., 2008) and 40 or 70 mg/kg bw in feed pellets (Tomaru et al., 2007). The same genetic alteration is found in db/db mice

Table 4 Summary of corrective effects on glucose homeostasis in animal models

Estimated dose	Published dose	Extract	Duration	Animal model	Glucose effects	References
	25 or 50 mg/kg bw voluntarily in the morning	GSPE	10 days	Female Wistar rat kafeteria treated	No change glucose, slightly higher insulin	(Montagut et al., 2009)
	25 or 50 mg/kg bw voluntarily in the morning	GSPE	4 weeks	Female Wistar rat kafeteria treated	Slight decrease on glucose simultaneous to insulin	(Montagut et al., 2009)
Around 40 and 70 mg/kg bw in the pellet	Diet containing 0.5% or 1% CLP (2.2% catechin+ epicatechin; 1.69% B2;2.37% C1; 2.01% cinnamtannin A2)	Proanthocyanidins from cacao liquor	3 weeks	Db/db mouse	Decreased glucose, dose-dependent and fructosamine	(Tomaru et al., 2007)
	10 mg /kg bw orally forced	Oligomeric procyanidins (MPD: 3.3)/ polymeric All from persimmon peel	6 weeks	Db/db mouse	Decreased serum glucose, and glicosilated protein	(Lee et al., 2008)
	20 mg/kg	provinols	8 weeks	Zucker fa/fa rat	Decreased serum glucose, fructosamine	(Agouni et al., 2009)
19.5 mg/Kg bw. vehicle:sunflower oil, forced orally,		GSPE	4 weeks	Male Zucker fa/fa rat	No changes	Esteve: personal communication

This table summarizes the studies in animals with genetically-induced insulin resistance and studies in which insulin resistance was induced using different diets, and procyanidins were administered afterwards, once the animals were already damaged.

as in obese Zucker fa/fa rats (Tartaglia, 1997), but the genetic background of each species gives rise to metabolic differences in their disturbed metabolic profiles, such as different b-cell characteristics. According to Shafrir et al. (1999), fa/fa rats have "sturdy" b-cells that maintain a robust, lifelong insulin secreting capacity. Meanwhile, db/db mice are characterized by "brittle" b-cells that only allow transient insulin hypersecretion with short-term obesity. Little research has been done into the effects of procyanidins on beta-cells (Pinent et al., 2008), and preliminary results from our research group indicate that beta-cells are a target for procyanidins (Castell et al., 2009). So, differences in b-cell characteristics may be one explanation for the different responses to GSPE in fa/fa rats and db/db mice, although more work needs to be done to get a more complete picture of its effect on genetically altered animal models.

In conclusion, GSPE might 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.

MECHANISMS USED BY PROCYANIDINS TO IMPROVE GLUCOSE HOMEOSTASIS

The collected data show that procyanidins might be able to ameliorate glucose homeostasis disturbances in specific situations. The mechanisms used by procyanidins to exert such effects are diverse, as summarized here.

First, after the ingestion of food, limiting post-prandial glycemia increases would be beneficial against insulin resistance. In this respect, inhibitors of the enzymes that participate in carbohydrate digestion would have a positive effect. In fact, procyanidins derived from different plant origins can inhibit alpha-glucosidases and alpha-amylases (Lee et al., 2007; Loo and Huang, 2007; Schäfer and Högger, 2007; Yuste et al., 1992). Such effects are observed mainly in polymeric forms and are not reproduced with monomers (Loo and Huang, 2007; Schäfer and Högger, 2007) and involve a mechanism that is not fully elucidated. Most of these studies analyze the inhibitory capacity in vitro, but little evidence has been found that reinforces their actual role in vivo. Another possible mechanism for reducing the level of glucose entering the organism is the inhibition of its absorption via inhibiting the transporters involved in glucose uptake to the intestine. Some monomeric flavonoids have been shown to inhibit the sodium-coupled glucose transporter (Sglt1) (Kottra and Daniel, 2007) and thus reducing glucose uptake. Some monomeric flavan-3-ols have also been found to inhibit the response of Sglt1 (Hossain et al., 2002; Kobayashi et al., 2000). Glucose transporter type 2 (Glut2)-mediated glucose uptake is also inhibited by some flavonoids such as quercetin, but not by the procyanidin monomers such as catechin or epicatechin (Kwon et al., 2007). The effects of oligomeric procyanidins, however, have not been explored. These procyanidin mechanisms of reducing blood glycemia should be effective in any condition of glucose homeostasis, but that is not always the case—it seems that this mechanism is not always active, especially in healthy rats. And in long-term treatments of diet-induced insulin-resistance it seems that when procyanidins are administered in a daily bolus they are more effective than when given with food, which indicates the presence of mechanisms of action other than those inhibiting carbohydrate digestion and/or glucose absorption.

These other mechanisms would involve targeting the liver. The liver plays a central role in maintaining glucose homeostasis, and the accumulation of hepatic lipids may be an important factor contributing to insulin resistance. Several flavonoids have been shown to increase the gene expression of Gck and reduce that of G6pc (AePark et al., 2006; Jung et al., 2006; Wolfram et al., 2006). Such effects have also been shown for procyanidins in some conditions, for example, in STZ-treated rats where procyanidins ameliorated glucose homeostasis (Fernandez-Larrea et al., 2007; Pinent et al., 2004). Gck activity has also been increased by grape seed procyanidins in high-fat diet treated rats (Zhang et al., 2009), although not by enough to ameliorate glycemia. And in cafeteria-diet induced diabetic animals the amelioration of glucose homeostasis by 25 mg/kg bw of GSPE was not accompanied by such gene expression changes (Montagut et al., 2009). Therefore, when there is a lack of insulin, procyanidins might act as an insulin-mimetic, acting on the insulin targets Gck and G6pc, but chronically administered in insulin-resistance conditions the effects of procyanidins are not so clear. So, control of glucose input/output by the liver could be one of the mechanisms used by procyanidins to improve glycemia, but would not be enough to fully explain their effects, and depend on the insulinemia status of the organism.

On the other hand, 50 mg/kg bw of GSPE administered to cafeteria-diet fed animals (Montagut et al., 2009) did target the liver, limiting lipogenesis. So, the reduction in hepatic lipogenesis might be a mechanism for improving insulin resistance in models in which the increase of lipid synthesis in the liver is a key factor in the development of diabetes, but might not be so important in models in which insulin resistance is mainly caused by other factors.

The effects of procyanidins might also be mediated peripherally. In vitro studies suggest that procyanidins have insulin-mimetic effects in adipose tissue and muscle (Blay et al., 2003; Li et al., 2007; Montagut et al., 2009; Pinent et al., 2006). We have shown that the oligomeric procyanidins of GSPE activate the insulin receptor by interacting with and inducing its phosphorylation and that this interaction leads to increased glucose uptake mediated by Akt. However, GSPE phosphorylates proteins of the insulin signaling pathway differently than insulin does, as Akt, p44/42 and p38 MAPKs are key points for GSPE-activated signaling mechanisms, suggesting alternative pathways for procyanidins to be effective in insulin-resistant situations (Montagut et al., 2009).

In the Type 1 diabetes model, we found increased mRNA expression of Glut4 in white adipose tissue, which supports the

hypothesis that when there is a lack of insulin, procyanidins might act as insulin-mimetics. The remainder of the studies listed did not analyze the adipose or the muscle tissue after procyanidin treatment; on the other hand, long-term GSPE treatment in insulin-resistant rats down-regulated the gene expression of Glut4, concomitantly with a down-regulation of several adipose cell markers (Montagut et al., 2009). Actually in vitro procyanidins have been shown to inhibit adipogenesis, pointing to the modulation of adipose tissue depots as a mechanism for procyanidins to ameliorate glucose homeostasis (Pinent et al., 2005), though the effect of procyanidins on adipose tissue depots is beyond the scope of this review. Considering the studies that analyze the effects on glucose homeostasis, in the study of grape seed procyanidins and a cafeteria diet, these effects on adipose markers cannot be linked to the amelioration of glucose homeostasis, since similar changes were also found in the model where no amelioration occurred. The key controller of adipogenesis, Pparg, was also down-regulated in epididymal fat in obese Zucker rats treated with GSPE (Montagut et al., 2007), but no changes in insulin resistance were observed. In our opinion, the study periods of this research were not long enough to observe significant changes in adipose tissue depots. Both Yokozawa et al. (2008) and Decorde et al. (2009) found reduced adipose tissue and body weights related to the reduction of glycemia in their studies, although no further analysis of the adipose tissue was performed. Tsai et al. (2008) found increases in Irs1 and Glut4 proteins, which are related to the amelioration of insulin resistance. Therefore, procyanidins might target adipose tissue in a few different ways: acutely, by acting as an insulin-mimetic, and chronically, by exerting changes in adiposity which in the long term would be beneficial against insulin resistance, and by improving glucose uptake by acting at the level of the Glut4 protein, or by means of other mechanisms not fully understood.

Finally, it is also possible that chronic procyanidin treatment targets the pancreas, the organ responsible for insulin secretion after glucose intake. Our group's preliminary results show that the pancreas might be a target tissue for procyanidins. In fact, other flavonoids have been shown to target the pancreas, although their exact role remains unclear (Pinent et al., 2008). This possibility, therefore, deserves further study.

HUMAN STUDIES

Using the same approach as in the animal analyses, Table 5 summarizes the human studies of the effects of procyanidins on different metabolic situations. It is important to note that most of these studies on the effects of procyanidins were conducted with small samples, which diminishes the value of their results as representative in the general population.

The first part of the table shows healthy, normoinsulinemic people. Only 500 mg of cocoa polyphenol intake for two weeks resulted in improvements as measured by an OGTT (Grassi et al., 2005), suggesting improved insulin sensitivity. In contrast with these results, no effects were found in the studies conducted by Baba et al. (2007) and Zern et al. (2005), which used a larger sample size and higher doses of other extracts. Therefore, either these doses were too high or the treatments too long or these results are consistent with the animal results in normoglycemic situations, in which procyanidin treatment was ineffective.

Subsequent studies do not refer to any detailed alterations in glucose homeostasis, but were conducted using subjects that suffer from some pathology. Most of the studies did not find any chronic effect of ingested procyanidins on glycemia or related parameters (glycosylated hemoglobin, insulin). Only Grassi et al. (2005) found a clear improvement in insulin sensitivity parameters, both in hypertensive subjects and hypertensive subjects with IGT (Grassi et al., 2008). All these studies refer to an acute dose of 500 mg of polyphenol in the form of a 100 g chocolate bar for a period of two weeks. A parallel study by Muniyappa et al. (2008) with the same number of subjects (20 hypertensives) also for two weeks, but with two daily doses of cocoa extract (451 mg polyphenols/dose) dissolved in water, did not show any improvement in insulin sensitivity parameters.

The last studies were conducted using samples of subjects with Type 2 diabetes. Two studies by the same author using the same extract (pycnogenol) show positive results. Liu et al. (2004) described a dose-response effect with dosages of up to 200 mg for three weeks. Banini et al. (2006) also found improved glycemic control with 150 mL of muscadine grape products over a period of four weeks. On the other hand, again with a much higher dose (6.8 mg GSPE/kg bw in tablets) for four weeks, no effect on glucose metabolism was documented.

None of the human studies report information about direct mechanisms that would explain the bioactivity of the compounds. Only some studies postulate a relationship between the effects and the clearly identified ability of procyanidins to improve inflammatory response (Grassi et al., 2005; 2005; 2008). So, from all these studies, the clearest conclusion that can be drawn is a rough estimation of the effective dose in humans: around 100–500 mg polyphenols daily, acutely administered for two to three weeks. It is important to bear in mind that this dosage is ten times higher than the estimated normal intake (Grassi et al., 2005; 2005; 2008).

From all the information published in procyanidin studies on glucose homeostasis regulation, we can conclude that procyanidins are more effective when administered in one acute load, and that they act as insulin-mimetics when there is no (or little) insulin present, probably targeting the liver and peripheral tissues. In a glucose-disturbed metabolism, procyanidins are useful because they prevent the induction of damage (fructose-feed models) and/or because they may use alternative targets to exert their insulin-mimetic effects. The validity of these mechanisms in humans cannot be deduced from current research, because such studies are scarce and tend to be conducted with limited sample sizes.

Table 5 Summary of studies on procyanidin effects on human studies

Daily dose	Duration	Beneficial effect	n participants/ treatment	Characteristics of participants	Reference
250 mL/d Sicilian red wine during meals	4 weeks	No changes glucose	24	Healthy people; both sexes	(Avellone et al., 2005)
50 mL Promeganate Juice (PJ)/d (1.5 mmol total polyphenols)	2 weeks	No changes glucose	−13	Healthy, non-smoking men, aged 20–35 years	(Aviram et al., 2000)
The soluble polyphenol content in PJ varies (0.2–1.0%); includes mainly anthocyanins, catechins, ellagic tannins, and gallic and ellagic acids. increasing doses of PJ (20–80 mL/d, equivalent to 0.54–2.16 mmol total polyphenols/d)	10 weeks		−3		
Six pairs foods +/- chocolate (do not specify the amount of chocolate!!)	One dose	Chocolate flavoured version 28% greater insulinemia than the alternate product	10	Healthy people, both sexes	(Brand-Miller et al., 2003)
500 mg polyphenols as 100 g dark chocolate bar	2 weeks	Improved Homa-IR, Quicki, and lower glucose and insulin after OGTT	15	Healthy people, both sexes	(Grassi et al., 2005)
26 g cocoa powder and 12 g sugar/day;twice each day: before noon and during the afternoon. (units/100 g cocoa powder) 377 mg epicatechin, 135 mg catechin, 158 mg procyanidin B2, 96.1 mg procyanidin C1, 2192 mg theobromine, and 470 mg caffeine.	12 weeks	No significant differences	28	Subjects fasted for 12 h, and then blood samples were collected from the intermediate cubital.	(Baba et al., 2007)
36 g of a lyophilized grape powder (LGP) or a placebo. LGP. total phenols, 5.8 g/kg; flavans, 4.1 g/kg; anthocyanins, 0.77 g/kg; quercetin, 102 mol/kg; myricetin, 8 mol/kg; kaempferol, 11 mol/kg; and resveratrol, 7 mol/kg.	4 weeks	Glucose concentrations were not affected	24–20	Pre- and postmenopausal women	(Zern et al., 2005)
100 mL red grape juice (RGJ)/d	2 weeks	Plasma concentrations of glucose, did not change	26 patients/ 15 healthy	Patients receiving hemodialysis and healthy subjects	(Castilla et al., 2006)
2* 180 mg polyphenols	4 weeks	no changes on HbA1c	20	Normotensive with increased cholesterol	(Allen et al., 2008)
1400 mg polyphenols 1.06 g procyanidins (with other polyphenols, total 1.44)	16 weeks	No changes on glucose, nor glycosilated hemoglobin	21/13	Normocholesterolemic/ hypercholesterolemic	(Jiménez et al., 2008)
900 mg flavanols	2 weeks	Improve insulin resistance	20	Essential hypertension	(Muniyappa et al., 2008)
500 mg polyphenols as 100 g dark chocolate bar	2 weeks	Improved Homa-IR, Quicki, and ISI (insulin resistance index)	20	Both sexes, hypertensives	(Grassi et al., 2005)
500 mg polyphenols as 100 g dark chocolate bar	2 weeks	Decreased insulin resistance and increased insulin sensitivity (ISI), and {beta}-cell function	19	Hypertensives with IGT	(Grassi et al., 2008)
190 mL red wine, 13% v/v ethanol, 5 days per week	10 week	No changes on glucose, nor insulin	20	Overweight women (BMI: 29.8 ± 2.2 kg/m^2)	(Cordain et al., 2000)
640 mL/day purple grape juice	8 weeks	No changes glucose, nor insulin	22	Coronary artery disease	(Chou et al., 2001)

0.7±1.2 mL/kg* d of purple grape juice	2 weeks	Baseline: insulin 7.52 ± 6.03 μIU/mL. After GJ: insulin 20.49 ±12.55 μIU/mL ($P = 0.004$).	15	Mixed adults with angiographically documented CAD	(Stein et al., 1999)
100 mg pycnogenol (1.45 mg/kg bw)	12 weeks	Decrease glucose, initial decrase of HbA1	34	T2DM with treatment	(Liu et al., 2004)
50, 100, 200, 300 mg pycnogenol (bw??)	3 weeks each dose, final treatment 12 weeks?	Decrease glucose dose-dependent until 200 mg. decease HbA1c. no changes insulin	40	T2DM	(Liu et al., 2004)
6.8 mg GSPE/kg bw tablets	4 weeks	No improved glucose metabolism. No change HOMA-IR. Decreased frutosamine	32	T2DM with treatment	(Kar et al., 2009)
150 mL grape wine or dealcholized	4 weeks	Improved glycemic control	10	T2DM	(Banini et al., 2006)

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SUPPLEMENTRY DATA

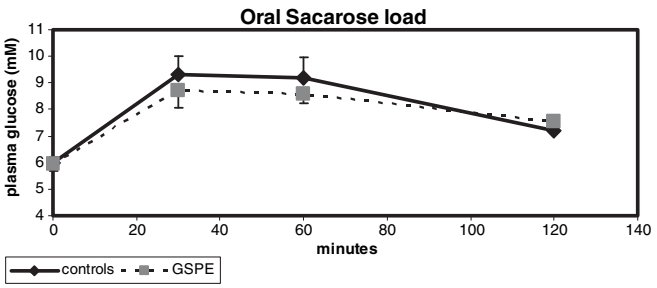

Figure S1 Oral sacarose load on healthy rats, simultaneously to a high GSPE oral acute dose.

3. Posters

GSPE EFFECTS ON PROLIFERATION AND APOPTOSIS MARKERS IN PANCREAS AND MESENTERIC ADIPOSE TISSUE

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INTRODUCTION

Procyanidins are a type of flavonoids present in fruits and vegetables, green tea, chocolate and red wine. They have been reported to have beneficial properties and to act as antioxidants and antiinflammatories. Previous studies done by our research group suggested that grape seed procyanidins extract (GSPE) affects glucose metabolism by modulating plasma insulin levels and peripheral glucose uptake, and pointing out at pancreas and adipose tissue as targets for procyanidins. The aim of the present study is to evaluate the effects of GSPE on proliferation and apoptosis markers in pancreas and mesenteric adipose tissue under different models of rats with metabolic syndrome.

MATERIALS AND METHODS

The GSPE effects were analyzed in 3 experimental models:

a) female Wistar rats under a chow high fat (60%) diet containing GSPE (average intake: 42 mg/kg of body weight) for 19 weeks
b) female Wistar rats fed with a cafeteria diet for 13 weeks and then treated with 25mg/kg bw GSPE for one month
c) male Zucker lean rats treated with chow high fat (35%) diet containing GSPE (average intake: 19,5 mg/kg bw) for 19 weeks.

At the end of the treatments, the animals were sacrificed and tissues were used for RNA isolation. We analyzed Bcl-2 and Cyclin D2 gene expression by Real time-PCR. All statistic analysis was performed using SPSS software.

RESULTS

In pancreas, the expression level of the proliferation marker Cyclin D2 was not modified by any of the tested high fat diets and GSPE treatment did not have any effect.

The expression of the anti-apoptotic marker Bcl-2 was down-regulated by the cafeteria diet but GSPE treatment did not counteract it (Fig 1).

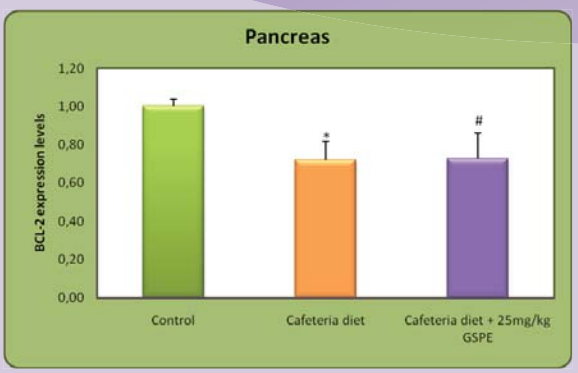

Figure 1: Expression levels of Bcl-2 in cafeteria diet and GSPE treatment in pancreas.
* p < 0,05 vs control
# p < 0,1 vs control (Student's t-test).

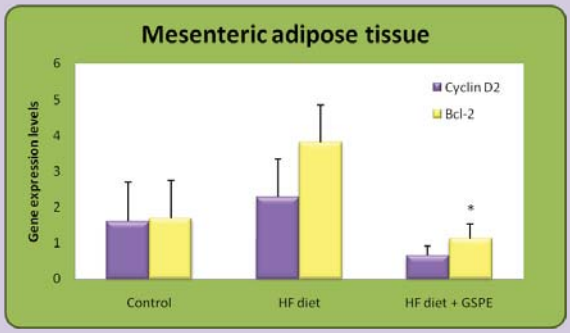

Figure 2: Expression levels of Cyclin D2 and Bcl-2 in 60% high fat diet (HF diet) and GSPE treatment in mesenteric adipose tissue.
•p < 0,05 vs HF diet (Student's t-test).

In mesenteric adipose tissue (Fig 2 and 3), GSPE treatment tended to down-regulate the expression of Cyclin D2 both in the palliative treatment on the cafeteria diet and in the preventive treatment on the 60% high fat diet.

Concerning Bcl-2, GSPE treatment had opposite effects in these experiments: it down-regulated Bcl-2 expression in the preventive treatment, opposing to the effects of the high fat diet, and it tended to up-regulate Bcl-2 expression in the palliative treatment, in which cafeteria diet did not show any effect.

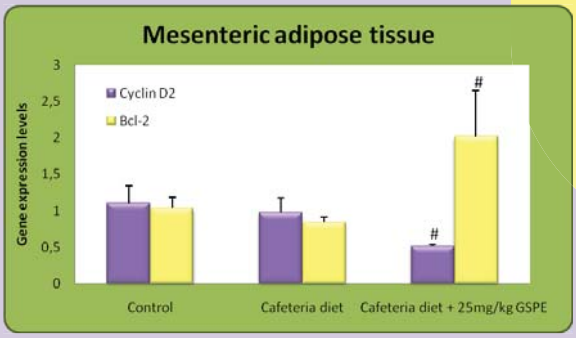

Figure 3: Expression levels of Cyclin D2 and Bcl-2 in cafeteria diet and GSPE treatment in mesenteric adipose tisue.
# p < 0,1 vs control and cafeteria diet in Cyclin D2 and p < 0,1 vs cafeteria diet in Bcl-2 (Student's t-test).

CONCLUSION

While GSPE does not show effects on the tested markers in pancreas, it tends to down-regulate the expression of the proliferation marker Cyclin D2 in mesenteric adipose tissue, pointing out a possible role on limiting adipose mass increase which deserves further study.

This work was supported by Grant AGL2008-01310 from the Spanish Government

GSPE effects on proliferation and apoptosis of pancreatic β-cells in genetically obese rats

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INTRODUCTION

Procyanidins are phenolic compounds from the flavanoids group, widely found in foods and drinks of plant origin such as grapes, cocoa, apples, red wine or chocolate.

They function as antioxidants and anti-inflammatories and studies done in our research group suggested that a grape seed procyanidin extract (GSPE) affects glucose metabolism modulating plasma insulin levels [1], pointing out at pancreas as a possible target for procyanidins. They have also been reported to modulate proliferation and apoptosis in other several tissues [2-4].

The aim of the present work is to evaluate the effect of GSPE on proliferation and apoptosis of pancreatic β-cells in a model of genetically obese rats.

MATERIALS AND METHODS

Female Zucker fa/fa rats were treated with 35mg/kg of GSPE for nearly 10 weeks. We also had a group of Zucker fa/fa rats and Zucker lean rats as control groups.

At the end of the treatment, the animals were sacrificed and the pancreatic islets were extracted. We counted the number of pancreatic islets and we analyzed their levels of apoptosis by ELISA (detection of cytoplasmic DNA-histone fragments); and Bcl-2, Bax, Cyclin D2, Mki67 and Neurogenin 3 gene expression by Real-time PCR.

All statistic analysis was performed using SPSS software.

RESULTS

The number of islets isolated from GSPE-treated rats tended to be higher than that of control fa/fa rats (Fig. 1). We observed that the islets from GSPE treated rats and control fa/fa rats seemed higher than those of lean rats.

However, GSPE tended to down-regulate the proliferation markers Cyclin D2 and Mki67 (Fig. 2), suggesting that the GSPE-treated rats are not in the same stage of proliferation than control fa/fa rats.

Neurogenin 3 was down-regulated in fa/fa rats (0,43±0,12 vs 1,47±0,55 in lean rats), but was not changed by the GSPE treatment (0,31±0,07), pointing out that GSPE does not influence neogenesis.

Fig. 1: Pancreatic islet number p<0.05 (ANOVA test)

Fig. 2: Gene expression of proliferation markers p<0.05 (Student's t-test)

GSPE did not modify the induced apoptosis observed in fa/fa rats (Fig. 3), although it down-regulated the anti-apoptotic marker Bcl-2 (Fig. 4), pointing out at a role of GSPE in modulating some proteins involved in apoptosis regulation.

Fig. 3: Apoptosis levels normalized by islet protein levels p<0.05 (Student's t-test)

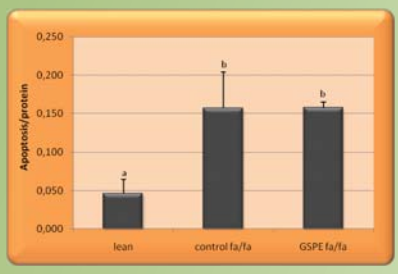
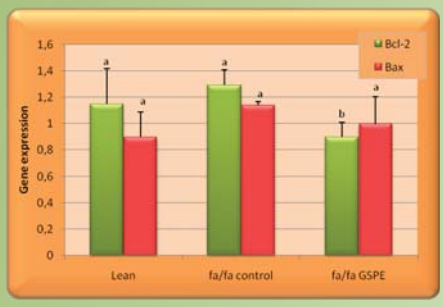


Fig. 4: Gene expression of apoptosis markers p<0.05 (Student's t-test)



To sum up, in this model, pancreas is a target of GSPE at gene expression level but with this duration of the treatment used, GSPE does not modify the pancreas at a physiological level, in agreement with no changes in insulin and glucose plasma levels.

CONCLUSION

In genetically obese rats, GSPE tends to increase the islet number, does not modify the apoptosis of fa/fa rats, and it modulates the gene expression of proteins involved in regulation of proliferation and apoptosis.

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UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF GRAPE SEED PROCYANIDIN EXTRACT ON PROLIFERATION AND APOPTOSIS IN PANCREATIC CELLS
Lídia Cedó Giné
Dipòsit Legal: T 545-2014

GSPE INHIBITS PROLIFERATION OF PANCREATIC β-CELLS

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INTRODUCTION

Procyanidins are the most abundant polyphenols in food as grapes and red wine. Previous studies done in our research group showed that grape seed procyanidin extract (GSPE) affects glucose metabolism modulating plasma insulin levels [1], pointing out at pancreas as a possible target for procyanidins. Procyanidins affect proliferation in cells derived from several tissues [2-4], but their effects in pancreas are not clear. The present work was designed to evaluate the effect of GSPE on proliferation of pancreatic β-cells *in vitro* under conditions that emulate the plasma changes found in the metabolic syndrome and in which proliferation rate is modified [5,6].

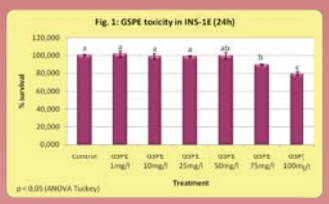
MATERIALS AND METHODS

Pancreatic β-cells INS-1E were treated with different concentrations of GSPE during 24 hours to analyze its toxicity by neutral red assay. The dose 25mg/l was chosen to assay the effects on proliferation, by measuring BrdU incorporation during DNA synthesis (kit Cell Proliferation ELISA BrdU, Roche) and Cyclin D2 gene expression by Real Time PCR. In order to simulate the conditions of metabolic syndrome, we analyzed GSPE effects in 3 different conditions: high concentrations of glucose, insulin and palmitate. All statistic analysis was performed using SPSS software.

RESULTS

· Toxicity

GSPE is toxic to INS-1E at higher doses than 50mg/l in treatment of 24 hours (Fig 1).



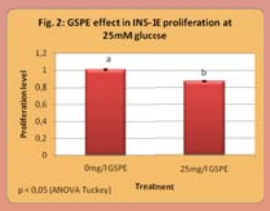
· High concentration of glucose

The treatment of INS-1E cells with 25mM of glucose (after an over night depletion of serum at 0.1% BSA) increases its proliferation level and the expression of Ciclin D2 is up-regulated, in front of the basal level of glucose (11mM) (Table 1).

The treatment of the cells with 25mg/l of GSPE opposed the proliferation-inductors effects, reducing BrdU incorporation (Fig. 2) without changing the expression of Ciclin D2.

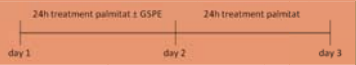
[glc]	Proliferation	Ciclin D2 expression
11mM	1,339 ± 0,02 a	1,01 ± 0,1 a
25mM	1,504 ± 0,04 b	1,42 ± 0,1 b

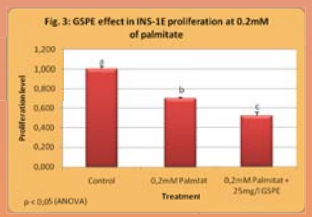
Table 1: Glucose effect (25mM) in INS-1E proliferation level and ciclin D2 expression.



· High concentration of palmitate

When INS-1E cells where treated with 0.2mM of palmitate, in conditions of depletion of serum, the proliferation rate was decreased. The treatment with 25mg/l of GSPE further reduced BrdU incorporation in the cells (Fig. 3).





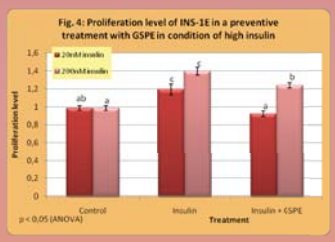
· High concentration of insulin

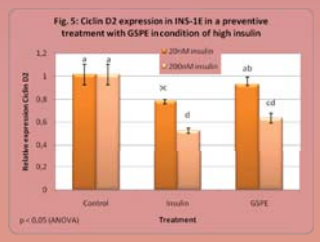
The treatment of INS-1E cells with insulin during 24 hours, in conditions of absence of serum and low glucose (5mM), increased the proliferation rate in a dose-dependent manner. (Table 2)

Treatment	Proliferation
Control	0,302 ± 0,00 a
20nM insulin	0,461 ± 0,02 b
200nM insulin	0,703 ± 0,01 c

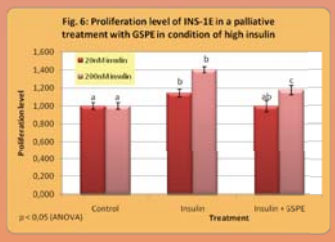
Table 2: Insulin effect in INS-1E proliferation level.

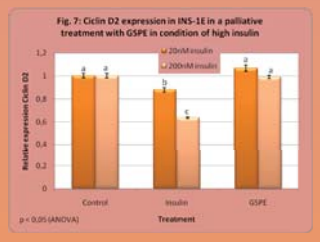
The preventive treatment of INS-1E with GSPE opposed the proliferation-inductor effect of high insulin (Fig. 4) and it also counteract the down-regulation of Ciclin D2 by insulin (Fig. 5).





In a similar way, the palliative treatment of INS-1E with 25mg/l of GSPE also increased the proliferation level of the cells (Fig. 6) and decreased the Ciclin D2 expression (Fig. 7), counteracting the effect of the insulin.





CONCLUSIONS

GSPE treatment decreases proliferation in pancreatic β-cells under conditions that emulate the plasma changes found in the metabolic syndrome.

This work was supported by Grant AGL2008-01310 from the Spanish Government

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GSPE EFFECTS ON APOPTOSIS OF PANCREATIC β-CELLS IN CONDITIONS OF GLUCOTOXICITY

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INTRODUCTION

Hyperglycemia derived from insulin resistance and type 2 diabetes leads to progressive beta cell dysfunction and beta cell loss by apoptosis, known as glucotoxicity. [1,2]

Phenolic compounds, included procyanidins, have been reported to modulate apoptosis in several cell types. [3,4] Procyanidins are the most abundant polyphenols in grapes and red wine and they function as antioxidants and anti-inflammatories. [5]

The aim of the present work is to evaluate the effect of grape seed procyanidin extract (GSPE) on apoptosis of pancreatic β-cells *in vitro* under conditions of high glucose.

MATERIALS AND METHODS

A Pancreatic β-cells INS-1E were treated with different concentrations of glucose and during different periods of time to establish the conditions in which it induces apoptosis in our cells.

B We analyzed the effects of a 24-hour treatment of GSPE, at 25 mM of glucose.

Apoptosis was quantified by measuring:

- Histone-complexed DNA fragments (kit Cell Death Detection ELISA^{PLUS}, Roche)
- Bcl-2 gene expression by Real Time PCR
- Caspase activity (Caspase-Glo® 3/7 Assay, Promega)

All statistic analysis was performed using SPSS software.

RESULTS AND DISCUSSION

A The treatment of INS-1E cells with high glucose concentrations increased the apoptosis at all doses and at all times assayed (Fig. 1), and decreased the anti-apoptotic marker Bcl-2 in most of the treatments (Fig. 2). So the dose 25mM of glucose (24 hours) was chosen to reproduce glucotoxicity in the cells.

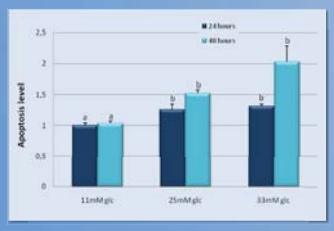

Fig. 1: Apoptosis level measured with the kit of INS-1E treated with high glucose (glc) during 24 or 48 hours.
p < 0.05 (t Student)

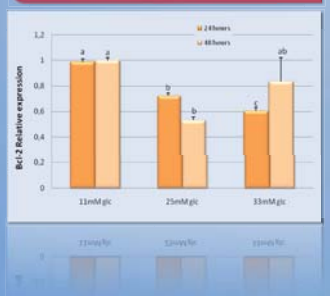

Fig. 2: Gene expression of the anti-apoptotic marker Bcl-2 in INS-1E treated with high glucose during 24 or 48 hours.
p < 0.05 (t Student)

B The co-treatment of the cells with GSPE together with high glucose further increased the apoptosis, reaching statistical significance at 25mg/l (Fig. 3). This was mediated by down-regulation of the anti-apoptotic marker Bcl-2 and the activation of caspases 3/7 (Fig. 4 and 5).

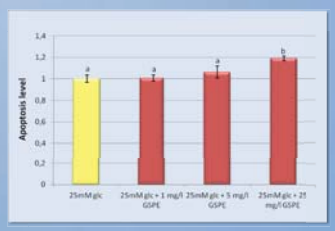

Fig. 3: Effect of GSPE on the apoptosis level of INS-1E cells treated at 25mM of glucose during 24 hours.
p < 0.05 (t Student)

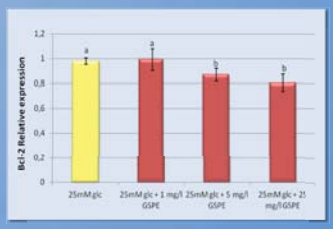

Fig. 4: Effect of GSPE on the expression of Bcl-2 of INS-1E cells treated at 25mM of glucose during 24 hours.
p < 0.05 (t Student)

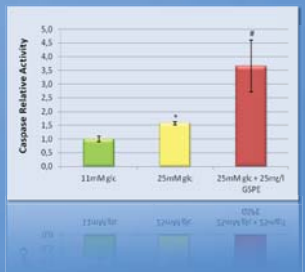

Fig. 5: Caspase 3/7 relative activity in INS-1E cells treated with 25mM of glucose and 25mg/l GSPE during 24h.
* p < 0.05 vs 11mM glc
# p < 0.05 vs 11mM glc and p < 0.07 vs 25mM glc (t Student)

CONCLUSIONS

Under assayed conditions, GSPE does not protect against the apoptosis caused by high glucose in a pancreatic β-cell line.

Some more work needs to be done to better understand the effects and mechanisms of procyanidins on apoptosis induced by high glucose in pancreatic β-cells.

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This work was supported by Grant AGL2008-01310 from the Spanish Government

PANCREATIC ISLET PROTEOME PROFILE IN ZUCKER FATTY RATS CHRONICALLY TREATED WITH A GRAPE SEED PROCYANIDIN EXTRACT

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INTRODUCTION

Procyanidins are the most abundant polyphenols in food as grapes and red wine. Grape seed procyanidin extract (GSPE) has been described to modify glucose metabolism and β-cell functionality through their lipid-lowering effects in a diet-induced obesity model.

OBJECTIVE

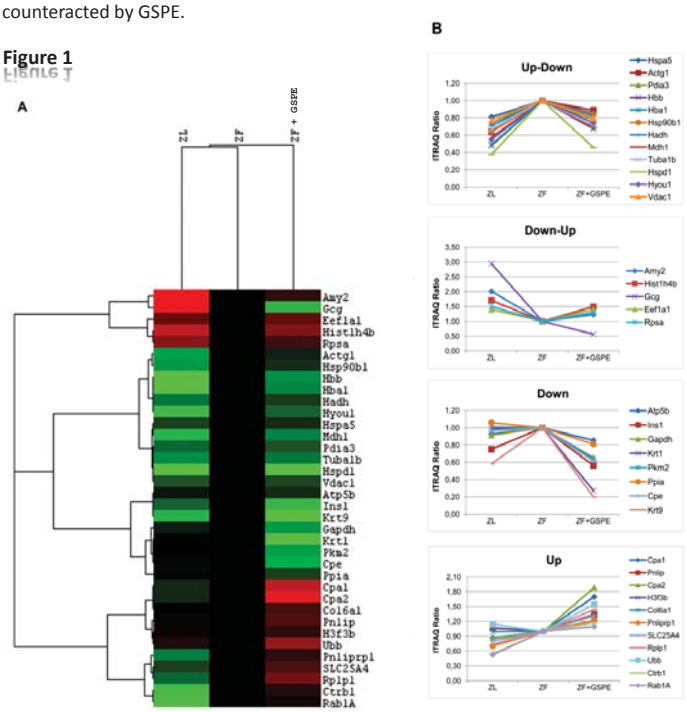
The aim of the present study was to evaluate the effects of chronically administrated GSPE on the proteomic profile of pancreatic islets from Zucker fatty rats.

RESULTS

Female Zucker fatty rats were treated with 35 mg/kg of GSPE for nearly 10 weeks. At the end of the treatment, islets were isolated and an isobaric tag for relative and absolute quantitation (iTRAQ)experiment was conducted. 21 proteins were found to be differentially expressed in Zucker lean rats (ZL) versus Zucker Fatty rats (ZF) rats and 31 proteins in ZF rats treated with GSPE (ZF + GSPE) compared to untreated ZF rats.

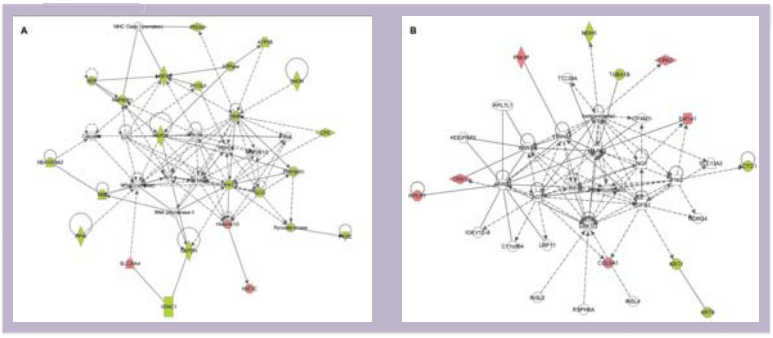
The iTRAQ ratios of ZL/ZF and ZF+GSPE/ZF differentially expressed proteins were hierarchical clustered using Cluster 3.0 software (Figure 1A) and revealed four expression patterns (Figure 1B). Two clusters exhibited a profile in which ZF+GSPE counteracted the effect of ZF: Down-Up in which the expression 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.

Figure 1



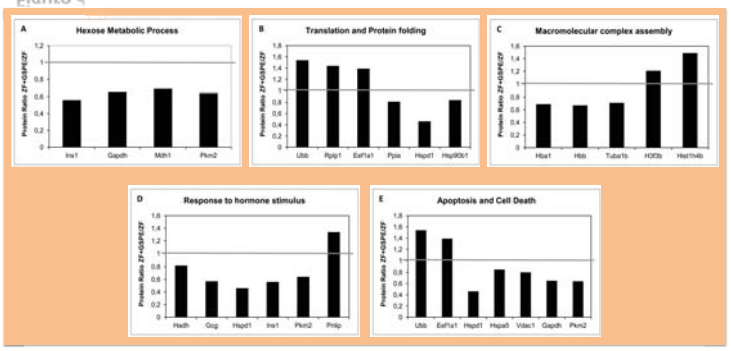
The 31 proteins differentially expressed in ZF+GSPE rats were mapped in two different protein networks in the Ingenuity Pathway Analysis (Figure 2).

Figure 2



The biological process of the proteins differentially expressed was assigned according to DAVID, and five subcategories of biological processes emerged (Figure 3).

Figure 3



On the other hand, plasmatic parameters were determined at the end of the experiment, as well as gene expression by Real Time PCR of some of the proteins detected as differentially expressed in ZF + GSPE in the iTRAQ experiment (Table 1).

Apoptosis was one of the subcategories obtained in the biological process analysis, so, the levels of apoptosis in the islets were determined as well as the gene expression of some markers of apoptosis (Table 1).

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**
Islet gene expression	**ZL**	**ZF**	**ZF + GSPE**
Ins2	1.0 ± 0.1 **a**	3.2 ± 0.2 **b**	1.8 ± 0.4 **a**
Cpe	1.0 ± 0.1 **a**	3.8 ± 0.2 **b**	1.8 ± 0.4 **a**
Vdac1	1.0 ± 0.1 **a**	1.6 ± 0.0 **b**	0.7 ± 0.1 **c**
Bcl2	1.2 ± 0.3 **ab**	1.3 ± 0.1 **a**	0.9 ± 0.1 **b**
Bax	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**
Ccnd2	1.0 ± 0.0 **a**	1.4 ± 0.1 **b**	0.8 ± 0.1 **a**
Mki67	1.0 ± 0.1 **a**	2.2 ± 0.3 **b**	1.6 ± 0.2 **b**
Gcg	1.1 ± 0.2 **a**	0.7 ± 0.1 **ab**	0.5 ± 0.1 **b**
Ppy	1.0 ± 0.1 **a**	0.7 ± 0.2 **ab**	0.5 ± 0.1 **b**
Islet apoptosis	**ZL**	**ZF**	**ZF + GSPE**
AU/µg protein	0.05 ± 0.0 **a**	0.16 ± 0.2 **b**	0.16 ± 0.0 **b**

DISCUSSION AND CONCLUSIONS

Chronically administrated GSPE modulates the proteomic profile of β-pancreatic islets from Zucker Fatty rats. Procyanidins modulate proteins involved in insulin synthesis and secretion and 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.

This work was supported by Grant AGL2011-23879 from the Spanish Government

4. Procediments Normalitzats de Treball de Metodologies

Nº Pàgina

Redactat per: Lídia Cedó Giné	Revisat per: Montse Pinent	Aprovat per:
Data: 29-5-2009	Data: 1-6-2009	Data:

1. Objectius

Extracció i aïllament d'illots pancreàtics de rata.

2. Àmbit d'aplicació

Aquest procediment és d'aplicació a tota persona que hagi de realitzar la metodologia descrita en aquest PNTM en el grup de Nutrigenòmica del Departament de Bioquímica i Biotecnologia.

3. Responsabilitats

- És responsabilitat de la direcció del Sistema de Gestió de Qualitat del grup Nutrigenòmica proposar l'investigador adequat per redactar aquest procediment, així com la seva aprovació.
- És responsabilitat del responsable de Qualitat assignar la codificació del PNTM, així com la seva revisió.

4. Definicions

Illot pancreàtic o de Langerhans: agrupacions de les cèl·lules endocrines del pàncrees: cèl·lules alfa, beta, delta i PP.

5. Normativa aplicable

ISO 9001(2000)

6. Procediment

6.1 Equips

Equip	Ubicació	Nº d'inventari	Nº PNT	Observacions
Bany nüve	114	071639	116	
Centrífuga eppendorf 5804R	115	062679	047	
Lupa Maigi EMT	docència	012796	-	
Lupa Olimpus	docència	026996	-	
NUAIRE DHD Autoflow CO_2 Air-Jacketed Incubator	102 de docència	074914	-	
Balança analítica AND GR-300	114	043139	036	

6.2 Solucions i materials

Nom: Pentobarbital sòdic				
Volum final: 1 ml			**pH :** -	
Manipulació prèvia: -				
Conservació: preparar en fresc				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
Pentobarbital sòdic (1)		Fagron	100mg/ml	100 mg

(1) Pentobarbital sòdic: es troba a l'armari de l'estabulari. Es dissolen 0,1g en 1ml de sèrum fisiològic.

Nom: Medi M-199				
Volum final: 1 litre			**pH :** -	
Manipulació prèvia: -				
Conservació: 4ºC				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
M-199 (1) $NaHCO_3$		M9163/Sigma S6014/Sigma-Aldrich	 0,35 g/l	100 ml 0,35 g

(1) M-199: Guardat a la nevera de cultius. S'ha de diluir 100 ml de medi amb 900 ml d'aigua milliQ.

Nom: Medi M-199 10% FBS				
Volum final: 500 ml			**pH :** -	
Manipulació prèvia: Cal descongelar FBS en un bany a 37ºC				
Conservació: 4ºC				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
FBS (1)		DE14-801F/ Biowhittaker	10% (v/v)	50 ml

(1) FBS: Fetal bovine serum: està aliquotat en falcons de 50ml a -20ºC a la sala de cultius. Es suplementen 450ml del medi M-199 preparat abans amb un 10% de FBS.

Nom: Col·lagenasa				
Volum final: 7ml/rata			**pH :** -	
Manipulació prèvia: -				
Conservació: preparar en fresc i mantenir a 4ºC fins al moment d'injectar-la				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
Collagenase P (1)		11213865001/Roche	1mg/ml	7 mg

(1) Col·lagenasa: es dissol 7mg en 7ml de medi M-199 preparat abans.

Nom: Histopaque				
Volum final: 10 ml/rata			**pH :** -	
Manipulació prèvia: treure de la nevera una estona abans d'utilitzar-lo				
Conservació: 4ºC				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
Histopaque		10771/Sigma		10 ml

Nom: Medi RPMI 1640 suplementat				
Volum final: 500 ml			**pH :** -	
Manipulació prèvia: Es descongelen les solucions en un bany amb aigua a 37ºC				
Conservació: 4ºC				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
RPMI (1)		BE12-167F, Biowhittaker		500 mL
FBS (2)		DE14-801F, Biowhittaker	5 %	27,5 mL
P/S (3)		DE17-602E, Biowhittaker	100 U/ml Penicil·lina/100 μg/ml Estreptomicina	5,5 mL
Glutamina (4)		BE17-605E, Biowhittaker	2 mM	5,5 mL
β-Mercaptoetanol (5)			50 μM	1,92 μL
Piruvat de sodi (6)		BE13-115E, Biowhittaker	1 mM	5,5 mL
HEPES (7)		H3375, Sigma	10 mM	4,4 mL

(1) RPMI: Guardat a l'armari de cultius, a temperatura ambient.
(2) FBS: Fetal Bovine Serum. Està aliquotat en falcons de 50 mL a -20ºC a la sala de cultius
(3) P/S: Penicil·lina/Estreptomicina. Està aliquotada en falcons de 15 mL a -20ºC a la sala de cultius
(4) Glutamina: Està aliquotada en falcons de 15mL a -20ºC a la sala de cultius. Agitar vigorosament el tub fins que es dissolgui abans de pipetejar-la.
(5) β-Mercaptoetanol: Guardat a l'armari de cultius.
(6) Piruvat de sodi: Guardat a la nevera de cultius.
(7) HEPES: Està aliquotat en falcons de 15 mL a -20ºC a la sala de cultius

Per preparar el medi s'afegeixen tots els reactius de la taula en l'envàs del medi.

Material	Capacitat	Tractament Previ	Observacions
Eppendorf	1,5 ml		
Tubs falcon	50 ml		
Cateters			
Xeringues	1 i 10 ml		
Agulles	21 G i 25G		
Material de cirurgia			
Fil i agulla de cosir			
Flascons Cellstar	25 cm^2		
Filtre 800 µm			
Vas precipitats	250 ml		
Pipetes estèrils	10 i 25 ml		
Puntes	100 i 1000 µl		
Pipetes Pasteur			
Plaques Orange	6 pous		

6.3 Precaucions

Cada lot de col·lagenasa és diferent pel que fa a la seva acció i cal comprovar el temps d'incubació i la concentració per tal d'obtenir un bon rendiment a l'hora de fer l'extracció.

Si s'utilitza el medi RPMI 1940 enlloc del M-199 per realitzar la perfusió, el rendiment és més baix.

6.4 Protocol

1. S'anestesia la rata amb una injecció intraperitoneal de pentobarbital sòdic amb una dosi de 50 mg/kg de pes de l'animal.

2. Es realitza una incisió abdominal deixant al descobert la cavitat intraperitonial.

3. Es fa un nus al col·lèdoc a l'alçada de l'intestí amb una agulla enfilada amb fil. Es deixa un altre nus preparat, sense apretar-lo, al col·lèdoc a l'alçada del fetge.

4. S'exsanguina l'animal per l'artèria aorta abdominal i es fa un tall al diafragma per assegurar-se de la mort de l'animal.

5. S'introdueix el catèter al col·lèdoc a l'alçada del fetge abans del nus, un cop dins, s'apreta el nus i s'immobilitza el catèter.

6. Es perfundeixen 7ml de col·lagenasa. Si s'observa que la col·lagenasa se'n va cap a l'intestí prim, cal pinçar-lo a l'alçada del nus al col·lèdoc.

7. S'aïlla el pàncrees inflat començant des de l'última part de l'intestí. Cal anar amb compte de no pinçar el pàncrees. S'agafa utilitzant l'intestí i la melsa, per no perforar el pàncrees.

8. Es posa el pàncrees inflat en un flascó de 25 cm^2 i s'incuba a 37ºC durant 15 minuts agitant el flascó continuament.

9. S'atura el procés de digestió amb l'addició de 40 ml de medi M-199 a 4ºC i es passa el digerit a un tub falcon de 50 ml.

10. Es centrifuga 1 minut a 1500 rpm a 4ºC.

11. Es descarta el sobrenedant fins a 15 ml i s'afegeix medi nou fins a 25 ml. Es vorteja suaument o es desenganxa el pellet per inversió. Aquest pas es realitza dues vegades.

12. Es centrifuga 1 minut a 1500 rpm a 4ºC i es descarta el sobrenedant fins a 10 ml. S'afegeix medi M-199 fins a 25 ml, es vorteja i es filtra l'homogenat per un filtre de 800 µm recollint el filtrat en un vas de precipitats. Es passen 10 ml més de medi pel filtre per tal de passar tot el que queda i es passa el filtrat a un tub falcon de 50ml.

13. Es centrifuga 1 minut a 1500 rpm a 4ºC i es descarta el sobrenedant completament utilitzant una bomba de buit i una pipeta Pasteur.

14. S'afegeixen 10 ml d'Histopaque, es vorteja suaument fins desfer el pellet i s'afegeixen 10 ml de medi M-199 a poc a poc, per les parets del tub, per tal de realitzar un gradient. Els illots han de quedar a la interfase.

15. Es centrifuga 15 minuts a 4ºC i 2300 rpm eliminant el fre de la centrífuga.

16. Es recull el sobrenedant en un tub falcon de 50ml i es centrifuga un minut més a 2300 rpm i 4ºC (tornant a posar el fre) per eliminar el sobrenedant.

17. S'afegeixen 10ml de medi M-199 al 10% FBS. Es vorteja i es centrifuga un minut a 1500 rpm i 4ºC.

18. S'aspira el sobrenedant i s'afegeixen 10ml més de medi M-199 amb 10%FBS.

19. Es deixen precipitar els illots durant 5 minuts en gel o es centrifuga un minut a 1500rpm a 4ºC.

20. Es treu el sobrenedant fins uns 2-3 ml i es dissol el pellet (illots) amb aquest medi. El sobrenedant que es treu es guarda en un altre tub falcon de 50 ml i es guarda en gel.

21. Es posen els illots en una pouet d'una placa de 6 pous i s'observen a la lupa.

22. Es recullen els illots amb una pipeta de 100ml, un per un, i es posen en 2ml de medi RPMI 1640 suplementat nou per tal de netejar-los de possibles restes cel·lulars que hagin quedat. Es tornen a passar a un altre pou amb 2ml de medi RPMI 140 suplementat mentre es conten.

23. Es posen a l'incubador a 37ºC i 5% de CO_2.

6.5 Verificació de la tècnica i interpretació de resultats

Si no s'està segur de quines cèl·lules són els illots, es poden tenyir amb 10 µl de ditizona (50mg de ditizona en 5ml de DMSO) quedant de color vermell. A continuació, cal deixar-los unes hores a l'incubador perquè es destenyeixin.

Si l'extracció va bé, s'obtenen uns 250-300 illots de diferents mides.

6.6. Guia de problemes ("troubleshooting")

Problema	Possible causa	Solució
Mala digestió del pàncrees	Poc temps d'incubació	Incrementar el temps d'incubació
	Col·lagenasa massa diluïda	Utilitzar una dilució de col·lagenasa superior.
La col·lagenasa no entra al pàncrees	Surt per l'intestí o fora de l'òrgan per una mala realització dels nusos o per errors en la perfusió.	Es pot tallar el pàncrees i afegir col·lagenasa al flascó de 25cm^2 i continuar amb el procediment, tot i que el rendiment és més baix.

7. Referències

NO APLICA

8. Control de la documentació

El PNTM una vegada aprovat es guardarà en format electònic (.PDF), aquest s'arxivarà al servidor de qualitat del departament de Bioquímica i Biotecnologia, dins la carpeta *7. Realització del producte/ PNT Metodologies/ NG.*

Els PNTM obsolets en format electrònic s'hauran d'arxivar al servidor de qualitat del departament de Bioquímica i Biotecnologia, dins la carpeta *7. Realització del producte/ PNT Metodologies/ NG/Obsolets.*

Per facilitar la distribució i l'ús als usuaris es guardaran una còpia de cada PNTM en format paper, en un arxiu codificat com: PNTM, al laboratori 113.

9. Annexos

NO APLICA

Nº Pàgina

Redactat per: Anna Castell	Revisat per: Anna Ardévol	Aprovat per: Cinta Bladé
Data: 02-01-2009	Data: 19-01-2009	Data: 02-02-2009

1. Objectius

Cultiu, subcultiu, manteniment, congelació i descongelació de la línea cel·lular INS-1E.

2. Àmbit d'aplicació

Aquest procediment és d'aplicació a tota persona que hagi de realitzar la metodologia descrita en aquest PNTM en el grup de Nutrigenòmica del Departament de Bioquímica i Biotecnologia.

3. Responsabilitats

- És responsabilitat de la direcció del Sistema de Gestió de Qualitat del grup Nutrigenòmica proposar l'investigador adequat per redactar aquest procediment, així com la seva aprovació.
- És responsabilitat del responsable de Qualitat assignar la codificació del PNTM, així com la seva revisió.

4. Definicions

Línia cel·lular d'insulinoma de rata INS-1E aïllades i cedides pel Dr. Claes Wollheim.

5. Normativa aplicable

ISO 9001(2000)

6. Procediment

6.1 Equips

Equip	Ubicació	Nº d'inventari	Nº PNT	Observacions
Cabina flux vertical-telstar Bio-II-A	Cultius	007409	001	
Cabina flux vertical-telestar-av-100-vis	Cultius	067838	010	
Incubador CO_2 SANYO-MCO	Cultius	005584	030	
Bany termostàtic selecta precisterm	Cultius	005480	039	
Centrífuga Hermle Z233MK-2	Lab 115	062679	043	
Congelador -20ºC	Cultius	005536	015	

Liebherr				
Nevera Liebherr	Cultius	005576	069	
Microscopi invertit amb contrast de fase KYOWA	Cultius	007408	058	

6.2 Solucions i materials

Nom: RPMI suplementat				
Volum final: 500 ml			**pH :** -	
Manipulació prèvia: Es descongelen les solucions en un bany amb aigua a 37ºC				
Conservació: 4ºC				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
RPMI (1)		BE12-167F, BIOWHITTAKER		500 mL
FBS (2)		DE14-801F, BIOWHITTAKER	5 %	27,5 mL
P/S (3)		DE17-602E, BIOWHITTAKER	100 U/ml Penicil·lina/100 μg/ml Estreptomicina	5,5 mL
Glutamina (4)		BE17-605E, BIOWHITTAKER	2 mM	5,5 mL
β-Mercapto-etanol (5)			50 μM	1,92 μL
Piruvat de sodi (6)		BE13-115E, BIOWHITTAKER	1 mM	5,5 mL
HEPES (7)		H3375, SIGMA	10 mM	4,4 mL

(1) RPMI: Guardat a l'armari de cultius, a temperatura ambient.
(2) FBS: Fetal Bovine Serum. Està aliquotat en falcons de 50 mL a -20ºC a la sala de cultius
(3) P/S: Penicil·lina/Estreptomicina. Està aliquotada en falcons de 15 mL a -20ºC a la sala de cultius
(4) Glutamina: Està aliquotada en falcons de 15mL a -20ºC a la sala de cultius. Agitar vigorosament el tub fins que es dissolgui abans de pipetejar-la.
(5) β-Mercaptoetanol: Guardat a l'armari de cultius.
(6) Piruvat de sodi: Guardat a la nevera de cultius.
(7) HEPES: Està aliquotat en falcons de 15 mL a -20ºC a la sala de cultius

Per preparar el medi s'afegeixen tots els reactius de la taula en l'envàs del medi.

Nom: PBS 1X				
Volum final:			**pH :** 7,4	
Manipulació prèvia:				
Conservació: Temperatura ambient				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**

Referència protocol sala cultius

Material	Capacitat	Tractament Previ	Observacions
Criotubs	2 mL	Autoclavats	Estèrils
Flascons Falcon BD	25 cm^2		Estèrils
	75 cm^2		Estèrils
Plaques Falcon BD	48 pous		Estèrils
Plaques Orange	12 pous		Estèrils
Eppendorf	1,5 mL	Autoclavats	Estèrils
Tubs falcon	15 i 50 mL		Estèrils
Puntes amb filtre	10, 100 i 1000 μL		Estèrils
Pipetes estèrils	2, 5, 10, 25 mL		Estèrils
Flascó	120 mL		

6.3 Precaucions

- S'han d'individualitzar molt bé les cèl·lules per poder-les sembrar correctament.
- Quan les cèl·lules estan a un passe inferior a 40 i posterior a 100 contenen menys insulina, tot i que mantenen la resposta secretora induïda per glucosa.

6.4 Protocol

Protocol de congelació (Flascó 25 cm^2)

1) Aspirar el medi de cultiu i rentar dues vegades amb 5 mL de PBS (37 ºC) per eliminar les restes de sèrum que contenen inhibidor de tripsina.

2) Aspirar el PBS i afegir 1 mL de Tripsina-EDTA (37ºC). Posar el flascó a l'incubador 2-3 minuts i observar al microscopi que les cèl·lules es desenganxin del fons del flascó.

3) Afegir 4 mL de medi suplementat per inactivar la tripsina i contar les cèl·lules utilitzant la cambra de Neubauer.

4) Centrifugar durant 5 minuts a 900 rpm i temperatura ambient.

4) Aspirar el sobrenedant i resuspendre amb el volum adequat de medi (10% de DMSO) per tal de tenir 1-1,5·10^6 cèl·lules per ml.

5) Es distribueix 1 mL en cada vial de congelació.

6) Es posen els vials en gel durant 15 minuts.

7) Es congelen les cèl·lules a -20ºC per 2 hores.

8) Es posen les cèl·lules al congelador de -80ºC durant 24 hores.

9) Es col·loquen els vials al contenidor (cistella 5) sota atmosfera de N_2 líquid.

Per congelar un flascó de 75 cm^2 es segueix el mateix procediment però s'utilitzen 2,2 ml de tripsina, i s'afegeixen 17,8 ml de medi.

Protocol de descongelació

1) Descongelar el vial agitant-lo suaument en un bany a 37 ºC mantenint la tapa i la zona superior del criotub sobre el nivell de l'aigua per evitar contaminacions. La descongelació ha de ser ràpida (aproximadament 2 minuts).

2) Treure el vial de l'aigua quan s'hagi descongelat el contingut. A partir d'ara es treballa en condicions estèrils.

3) Transferir el contingut del vial (1 mL) a un tub de 15 mL amb 5 mL de medi de cultiu suplementat. Centrifugar a 900 rpm, 5 min a temperatura ambient.

4) Resuspendre el pellet de cèl·lules amb 5 mL de medi de cultiu i sembrar les cèl·lules en flascó de 25 cm^2 (Indicar a la placa el passe, dia, data i nom de l'investigador).

5) Incubar a 37ºC amb 5% CO_2. El medi s'ha de canviar cada 48 hores.

Protocol per subcultiu

Quan les cèl·lules es trobin a un 80% de confluència s'han de fer els subcultius.

Per un flascó de 25 cm^2:

1) Aspirar el medi i rentar dues vegades amb 5 mL de PBS.

2) Afegir 1 mL de Tripsina-EDTA. Posar la placa a l'incubador 1-2 min i observar al microscopi que les cèl·lules es desenganxen del fons de la placa.

3) Afegir 4 ml de medi suplementat i contar les cèl·lules utilitzant la cambra de Neubauer.

4) Sembrar $5 \cdot 10^4$ cèl·lules/pou placa 96 (0,2ml), 10^5 cèl·lules/pou placa 48 (0,5ml), $2{,}5 \cdot 10^5$ cèl·lules/pou placa 24 (0.5ml), $4 \cdot 10^5$ cèl·lules/pou placa 12 (1ml), $8 \cdot 10^5$ cèl·lules/pou placa 6 (2ml), $1 \cdot 10^6$ cèl·lules/flascó 25 cm^2 (10 ml) i $3 \cdot 10^6$/flascó 75 cm^2 (20 ml).

5) Incubar a 37ºC amb 5% CO_2. El medi s'ha de canviar cada 48 hores.

6.5 Verificació de la tècnica i interpretació de resultats

Un cop sembrades les cèl·lules s'han d'observar al microscopi. Per tal d'obtenir un bon creixement s'ha d'observar que les cèl·lules han quedat individualitzades.

6.6 Guia de problemes ("troubleshooting")

Problema	Possible causa	Solució
Creixement cel·lular lent	Tripsinitzar les cèl·lules quan estaven poc confluents	Descongelar un nou passe
Mort cel·lular	Error en la preparació del medi	Prepara medi nou

7. Referències

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

8. Control de la documentació

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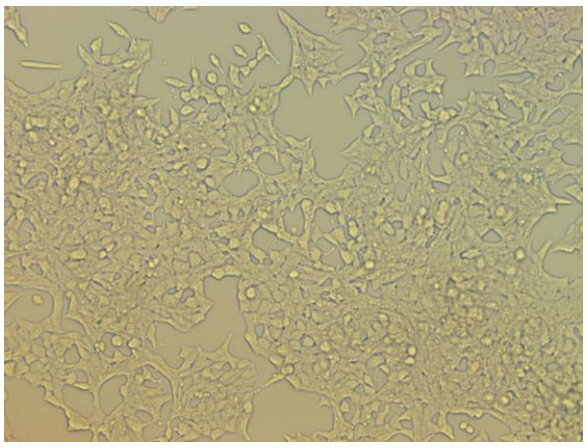
9. Annexos



Nº Pàgina

Redactat per: Lídia Cedó	Revisat per:	Aprovat per:
Data: 24-09-2012	Data:	Data:

1. Objectius

Cultiu, subcultiu, manteniment, congelació i descongelació de la línea cel·lular MIA PaCa-2.

2. Àmbit d'aplicació

Aquest procediment és d'aplicació a tota persona que hagi de realitzar la metodologia descrita en aquest PNTM en el grup de Nutrigenòmica del Departament de Bioquímica i Biotecnologia.

3. Responsabilitats

- És responsabilitat de la direcció del Sistema de Gestió de Qualitat del grup Nutrigenòmica proposar l'investigador adequat per redactar aquest procediment, així com la seva aprovació.
- És responsabilitat del responsable de Qualitat assignar la codificació del PNTM, així com la seva revisió.

4. Definicions

Línia cel·lular d'adenocarcinoma de pàncrees humà MIA PaCa-2, cedides pel Dr. Carlos J. Ciudad de la Universitat de Barcelona.

5. Normativa aplicable

ISO 9001(2000)

6. Procediment

6.1 Equips

Equip	Ubicació	Nº d'inventari	Nº PNT	Observacions
Cabina flux vertical-telstar Bio-II-A	Cultius	007409	001	
Cabina flux vertical-telestar-av-100-vis	Cultius	067838	010	
Incubador CO_2 SANYO-MCO	Cultius	005584	030	
Bany termostàtic selecta precisterm	Cultius	005480	039	
Centrífuga Hermle Z233MK-2	Lab 115	062679	043	
Congelador -20ºC Liebherr	Cultius	005536	015	
Nevera Liebherr	Cultius	005576	069	
Microscopi invertit amb contrast de fase KYOWA	Cultius	007408	058	

6.2 Solucions i materials

Nom: DMEM suplementat				
Volum final: 570 ml			**pH :** -	
Manipulació prèvia: Es descongelen o s'atemperen les solucions en un bany amb aigua a 37ºC				
Conservació: 4ºC				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
DMEM (1)		BE12-614, BIOWHITTAKER		500 mL
FBS (2)		DE14-801F, BIOWHITTAKER	10 %	57 mL
P/S (3)		DE17-602E, BIOWHITTAKER	100 U/ml Penicil·lina/100 μg/ml Estreptomicina	5,7 mL
Glutamina (4)		BE17-605E, BIOWHITTAKER	2 mM	5,7 mL

(1) DMEM: Guardat a la nevera de cultius, a 4ºC.
(2) FBS: Fetal Bovine Serum. Està aliquotat en falcons de 50 mL a -20ºC a la sala de cultius
(3) P/S: Penicil·lina/Estreptomicina. Està aliquotada en falcons de 15 mL a -20ºC a la sala de cultius
(4) Glutamina: Està aliquotada en falcons de 15mL a -20ºC a la sala de cultius. Agitar vigorosament el tub fins que es dissolgui abans de pipetejar-la.

Per preparar el medi s'afegeixen tots els reactius de la taula en l'envàs del medi.

Nom: PBS 1X				
Volum final:			**pH :** 7,4	
Manipulació prèvia:				
Conservació: Temperatura ambient				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**

Referència protocol sala cultius

Material	Capacitat	Tractament Previ	Observacions
Criotubs	2 mL	Autoclavats	Estèrils
Flascons Orange	25 cm^2		Estèrils
	75 cm^2		Estèrils
Plaques Orange	96, 48, 12, 6 pous		Estèrils
Eppendorf	1,5 mL		Estèrils
Tubs falcon	15 i 50 mL	Autoclavats	Estèrils
Puntes amb filtre	10, 100 i 1000 μL		Estèrils
Pipetes estèrils	2, 5, 10, 25 mL		Estèrils

6.3 Precaucions

- S'han d'individualitzar molt bé les cèl·lules per poder-les sembrar correctament.

6.4 Protocol

Protocol de congelació (Flascó 25 cm^2)

1) Aspirar el medi de cultiu i rentar dues vegades amb 5 mL de PBS (37 ºC) per eliminar les restes de sèrum que contenen inhibidor de tripsina.

2) Aspirar el PBS i afegir 0.5 mL de Tripsina-EDTA (37ºC). Posar el flascó a l'incubador 2-3 minuts i observar al microscopi que les cèl·lules es desenganxin del fons del flascó.

3) Afegir 4.5 mL de medi suplementat per inactivar la tripsina i contar les cèl·lules utilitzant la cambra de Neubauer.

4) Centrifugar durant 5 minuts a 900 rpm i temperatura ambient.

4) Aspirar el sobrenedant i resuspendre amb el volum adequat de medi (10% de DMSO) per tal de tenir $2 \cdot 10^6$ cèl·lules per ml.

5) Es distribueix 1 mL en cada vial de congelació.

6) Es posen els vials en gel durant 15 minuts.

7) Es congelen les cèl·lules a -20ºC per 2 hores.

8) Es posen les cèl·lules al congelador de -80ºC durant 24 hores.

9) Es col·loquen els vials al contenidor (cistella 6 del contenidor nou) sota atmosfera de N_2 líquid.

Per congelar un flascó de 75 cm^2 es segueix el mateix procediment però s'utilitzen 2 ml de tripsina, i s'afegeixen 8 ml de medi.

Protocol de descongelació

1) Descongelar el vial agitant-lo suaument en un bany a 37 ºC mantenint la tapa i la zona superior del criotub sobre el nivell de l'aigua per evitar contaminacions. La descongelació ha de ser ràpida (aproximadament 2 minuts).

2) Treure el vial de l'aigua quan s'hagi descongelat el contingut. A partir d'ara es treballa en condicions estèrils.

3) Transferir el contingut del vial (1 mL) a un tub de 15 mL amb 4 mL de medi de cultiu suplementat i sembrar les cèl·lules en flascó de 25 cm^2 (Indicar a la placa el passe, dia, data i nom de l'investigador).

4) Incubar a 37ºC amb 5% CO_2. El medi s'ha de canviar l'endemà per tal d'eliminar el DMSO, tòxic per les cèl·lules.

Protocol per subcultiu

Quan les cèl·lules es trobin a un 80% de confluència s'han de fer els subcultius.

Per un flascó de 25 cm^2:

1) Aspirar el medi i rentar dues vegades amb 5 mL de PBS.

2) Afegir 0.5 mL de Tripsina-EDTA. Posar la placa a l'incubador 1-2 min i observar al microscopi que les cèl·lules es desenganxen del fons de la placa.

3) Afegir 4.5 ml de medi suplementat i contar les cèl·lules utilitzant la cambra de Neubauer.

4) Sembrar $6 \cdot 10^3$ cèl·lules/pou placa 96 (0,1ml), $2 \cdot 10^4$ cèl·lules/pou placa 48 (0,5ml), $5 \cdot 10^4$ cèl·lules/pou placa 24 (0.5ml), 10^5 cèl·lules/pou placa 12 (1ml), $2 \cdot 10^5$ cèl·lules/pou placa 6 (2ml), $4 \cdot 10^5$ cèl·lules/flascó 25 cm^2 (5 ml).

5) Incubar a 37ºC amb 5% CO_2. El medi es canvia cada 48 hores.

Observacions: el cultiu en flascons de 25 cm^2 a la densitat descrita es manté de tal manera que s'ha de tripsinitzar dos cops a la setmana (dilluns i divendres). Les densitats descrites per a les plaques permeten fer el tractament a les 48 hores d'haver sembrat. Les cèl·lules tenen un creixement ràpid, de manera que si s'han de sembrar el divendres per fer tractament el dilluns, cal sembrar-les més diluïdes.

6.5 Verificació de la tècnica i interpretació de resultats

Un cop sembrades les cèl·lules s'han d'observar al microscopi. Per tal d'obtenir un bon creixement s'ha d'observar que les cèl·lules han quedat individualitzades.

6.6 Guia de problemes ("troubleshooting")

Problema	Possible causa	Solució
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NO APLICA

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