MODULATION OF ACTIVE GLUCAGON-LIKE PEPTIDE-1 (GLP-1) LEVELS BY GRAPE-SEED PROCYANIDINS

Noemi González Abuin

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Modulation of active glucagon-like peptide-1 (GLP-1) levels by grape-seed procyanidins

DOCTORAL THESIS

codirected by Dr. Anna Ardévol Grau

and Dr. Montserrat Pinent Armengol

Departament de Bioquímica i Biotecnologia

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FEM CONSTAR que aquest treball, titulat **"Modulation of active glucagon-like peptide-1 (GLP-1) levels by grape-seed procyanidins"**, que presenta Noemi González Abuín per a l'obtenció del títol de Doctora, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que acompleix els requeriments per poder optar a Menció Europea.

Tarragona, 6 de setembre de 2013

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"¡No seas impaciente! No tengas prisa de llegar inmediatamente. Permite al tiempo madurar los frutos, para poder cosecharlos a tiempo. Camina seguro y perseverante, porque todo se nos dará a la hora oportuna y exacta"

Carlos Torres Pastorino

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A mi familia

y a José

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ABBREVIATION LIST

bHLH	Basic helix-loop-helix
cAMP	Cyclic AMP
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CCK	Cholecystokinin
CCK	Cholecystokinin
CgA	Chromogranin A
COMT	Catechol-O-methyltransferase
DAG	Diacylglycerol
DiBAC$_4$(3)	Bis-(1,3-dibutylbarbituric acid) trimethine oxonol
DPP4	Dipeptidyl-peptidase 4
Epac2	Exchange protein directly activated by cAMP 2
FFA	Free fatty acid
FFAR	Free fatty acid receptor
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GLP-1R	Glucagon-like peptide-1 receptor
GLP-2	Glucagon-like peptide-2
GLUT	Glucose transporter
GPCR	G-protein couple receptor
GRPP	Glicentin-related polypeptide
GSPE	Grape-seed procyanidin extract
HFD	High-fat diet
IP-1 and 2	Intervening peptide-1 and 2
IP3	Inositol triphosphate
IPGTT	Intraperitoneal glucose tolerance test
K_{ATP} channel	ATP-sensitive K^+ channel
LA	Linoleic acid
L-Pro	L-Proline
MPFG	Major proglucagon fragment
NEP 24.11	Neutral endopeptidase 24.11
OGTT	Oral glucose tolerance test
OLETF	Otsuka Long–Evans Tokushima fatty
PC	Hexin/subtilisin-like prohormone convertase
PDX-1	Pancreatic and duodenal homeobox gene 1
PIP_2	Phosphatidylinositol 4,5-biphosphate

PKA	Protein kinase A
PLCβ2	Phospholipase Cβ2
PYY	Peptide YY
SGLT1	Sodium-glucose cotransporter 1
SNAT2	Sodium-couple neutral aminoacid transporter 2
STZ	Streptozotocin
STZ	Streptozotocin
SULT	Sulfotranferases
T2D	Type 2 Diabetes Mellitus
TEER	Trans-epithelium electrical resistance
UGT	Uridine 5'-diphosphate glucuronosyltransferase
VGCC	Voltage-gated calcium channel
αMG	Methyl-α-glucopyranoside
$\Delta\Psi_{cell}$	Cellular membrane potential
$\Delta\Psi_{m}$	Mitochondrial membrane potential

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RESUM

Les procianidines son els polifenols més abundants a la dieta degut a la seva presència en gran diversitat d'aliments com son el cacau, el raïm, el vi negre, les pomes, les prunes, les cireres, els cereals i les llegums. Diversos estudis demostren que les procianidines del pinyol de raïm tenen efectes beneficiosos sobre la salut, ja que poden actuar com agents antiinflamatoris, antigenotòxics i antioxidants, i s'ha vist que tenen efectes moduladors sobre el metabolisme dels lípids i la glucosa.

En estudis previs realitzats al nostre grup de recerca (grup de Nutrigenòmica) es va demostrar que un extracte de procianidines de pinyol de raïm (GSPE) pot millorar un estat de resistència a la insulina induït per una dieta de cafeteria. En els animals tractats amb GSPE es va trobar una disminució de l'índex HOMA-IR i de la insulinèmia en condicions de dejuni, que es van atribuir als efectes insulino-mimètics de les procianidines al teixit adipós. Aquests resultats també suggerien una afectació del GSPE a la funcionalitat del pàncrees, que posteriorment van ser ratificats, evidenciant que les procianidines modulen la producció i la secreció d' insulina, així com la proliferació de les cèl·lules β.

Donat que els mecanismes que actuen sobre les cèl·lules endocrines són força comuns entre els diferents tipus de cèl·lules productores d'hormones, les cèl·lules enteroendocrines es van plantejar com una possible diana de les procianidines, en concret, la secreció i/o producció de l'hormona "glucagon-like peptide-1" (GLP-1), incretina que incrementa la producció de insulina i millora la sensibilitat a la insulina en els teixits perifèrics. Aquestes accions, incrementen la rellevància de aquesta incretina com a potencial via de tractament per a situacions de desregulació del metabolisme de la glucosa. Un altre fet rellevant és la ràpida eliminació de GLP-1 que té lloc immediatament després de la seva secreció degut a l'activitat de l'enzim dipeptidil-peptidasa 4 (DPP4), que és una serinexopeptidasa altament susceptible a la inhibició per part de les procianidines degut a la seva elevada afinitat per seqüències riques en prolina.

L'efecte de les procianidines a nivell de secreció, producció i/o degradació de GLP-1 encara no ha estat descrit. Per tant, aquesta tesi doctoral es va centrar en l'estudi del efectes de les procianidines sobre la secreció, producció i degradació de la hormona incretina GLP-1. Per assolir els nostres objectius es van utilitzar models *in vivo* de rates sanes i amb resistència a la insulina induïda per la dieta o per factors genètics; i models

in vitro, pels que es van utilitzar las línies cel·lulars CaCo-2, STC-1 i HUVEC, que son cèl·lules absortives del epiteli intestinal, enteroendocrines i endotelials, respectivament.

La secreció de GLP-1 és induïda pel consum oral de nutrients principalment la glucosa, per tant, l'efecte *in vivo* d'un tractament agut de GSPE sobre el nivells de GLP-1 activa es va avaluar simultàniament amb una administració oral de glucosa i es va observar que el GSPE era capaç d'augmentar el nivells de GLP-1 activa induïts per glucosa. Aquest increment podia venir donat per l'acció de GSPE sobre la producció, secreció i/o també sobre l'eliminació d'aquesta hormona.

Pel què fa als efectes sobre la secreció de GLP-1, es va estudiar la secreció per part de cèl·lules enteroendocrines en presència de GSPE amb o sense l'estímul dels nutrients. El tractament amb dosis baixes de GSPE va produir una tendència a augmentar la secreció de GLP-1 en absència de nutrients, mentre que dosis elevades van inhibir aquesta secreció. En presència de diferents tipus de nutrients (glucosa, àcids grassos o aminoàcids) només les dosis elevades de GSPE van afectar a la secreció de GLP-1, inhibint-la. A més a més, es va observar una modulació del potencial de membrana cel·lular en paral·lel als efectes sobre secreció. Per tant, la modulació produïda pel GSPE sobre el potencial de membrana cel·lular condiciona la secreció de GLP-1 per part de las cèl·lules enteroendocrines, sent un efecte dosi-depenent.

Respecte l'eliminació de GLP1, frenar la ràpida eliminació a través de la inhibició de l'enzim DPP4, és una de les aproximacions que actualment s'està treballant en alguns dels nous tractaments antidiabètics. Quan avaluem el potencial inhibitori de GSPE, s'observa que GSPE inhibeix DPP4 a nivell intestinal. En concret, després de desenvolupar un model *in vitro* que ens permetés simular l'absorció intestinal dels components de l'extracte de procianidines, vam demostrar que la catequina, la epicatequina, la procianidina B2 i l'àcid gàlic, presents als extractes, actuen simultàniament inhibint la activitat de la DPP4 present al endoteli del capil·lars. Aquesta inhibició pot retardar la inactivació de GLP-1, fet que explicaria l'augment dels nivells de GLP-1 activa en plasma trobats després del tractament agut amb GSPE.

L'efecte inhibitori del GSPE, a nivell intestinal, també es va observar en tractaments crònics en cèl·lules CaCo-2 i en rates sanes. A més a més, es va avaluar l'efecte del GSPE en diferents models de resistència a la insulina. En el cas de una inducció per dieta de cafeteria durant 13 setmanes, aquesta no va provocar cap canvi a la DPP4 intestinal i el tractament correctiu amb GSPE durant 30 dies va inhibir la seva activitat. En el cas de un tractament preventiu, simultani amb la inducció amb dieta de cafeteria durant 12 setmanes, el GSPE no va inhibir l'activitat de la DPP4 intestinal, però es va observar una prevenció de l'efecte inhibitori produït per la dieta. En un model d'obesitat induïda per

factors genètics, rates *Zucker Fatty,* que no presentaven alteració de la DPP4 intestinal, el tractament correctiu amb GSPE no va produir cap efecte. Amb aquests resultats es va concloure que l'efecte del GSPE depèn del tipus de tractament i el grau d'obesitat assolit en els diferents models.

Finalment, vam estudiar l'efecte de la dieta de cafeteria sobre la producció de GLP-1 i l'efecte d'un tractament simultani (preventiu) amb GSPE. La dieta de cafeteria va produir un decrement en la producció de GLP-1 a nivell intestinal, efecte que va ser parcialment contrarestat pel tractament simultani amb GSPE. A més a més, es va observar que aquests canvis en la producció de GLP-1 estaven associats amb una modulació del nombre de cèl·lules enteroendocrines. Per una altra banda, la producció de GLP-1 al hipotàlem es va veure augmentada pel tractament amb GSPE, incrementant l'efecte de la dieta de cafeteria; mentre que l'expressió gènica del seu receptor es va veure disminuïda.

En conclusió, una part de l'efecte del GSPE modulant el metabolisme de la glucosa, passa per la seva acció sobre la producció i secreció de GLP-1, a nivell intestinal; així com per la seva acció sobre la producció i activitat de DPP4, l'enzim encarregat d'inactivar GLP-1.

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I. INTRODUCTION

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A. INCRETINS

1. DEFINITION OF INCRETINS

Following the intake of a meal, a complex set of physiological responses is activated, providing neural and endocrine signals that regulate the ingestion, absorption, and assimilation of the ingested nutrients [1]. Among these signals are the incretins, which were initially identified in several studies that reported on the induction of a higher insulin response by the administration of oral glucose compared to an equivalent intravenous infusion (reviewed in [2]) (**figure 1**). This observation confirmed the connection between the intestinal tract and the endocrine pancreas, an organ that is highly involved in nutrient homeostasis. This was defined as the incretin effect, and the hormones responsible are called incretins. Specific criteria have to be met for an agent to be considered an incretin: it must be released in response to an oral nutrient ingestion, and reach physiological concentrations in vivo to provoke insulin release [3]. Currently, the following two gut hormones are considered incretins: glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP).

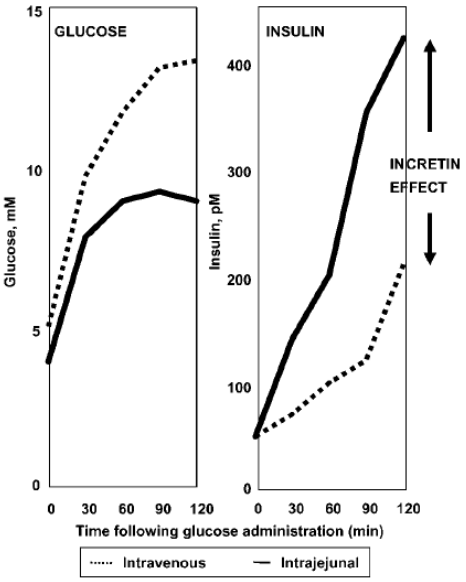

Figure 1. The incretin effect [3]

GLP-1 and GIP reach their target cells and bind to specific G protein-coupled receptors (GPCRs) to activate their respective signaling pathways. The GIP receptor is expressed in pancreatic β-cells, adipose tissue, bones and the brain, whereas the GLP-1 receptor is expressed in pancreatic α- and β-cells, the central and peripheral nervous system, heart, kidney, lung and the gastrointestinal tract [4]. This wide distribution of receptors results in a wide range of incretin-induced biological activities (**table 1**). Both hormones promote glucose-dependent insulin secretion and biosynthesis, enhance proliferation and inhibit the apoptosis of pancreatic β-cells. In addition to its insulinotropic effects, GLP-1 inhibits gastric emptying and reduces food intake and the rate of endogenous glucose production (reviewed in [3,5,6]). Furthermore, GIP also regulates fat metabolism in adipocytes, enhancing the insulin-stimulated incorporation of fatty acids into

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triglycerides, increasing lipoprotein lipase activity and stimulating fatty acid synthesis ([7].

Table 1. GLP-1 and GIP effects [2]

Action	GLP-1	GIP
Pancreatic β cell	↑insulin release (glucose-dependent) ↑β-cell proliferation and ↓apoptosis	↑insulin release (glucose-dependent) ↑β-cell proliferation and ↓ apoptosis
Pancreatic α and δ cell	↓ glucagon (glucose-dependent) ↑somatostatin	↔
Gastric emptying	↓ gastric emptying	↔
Appetite and weight	↓decrease appetite and promote weight loss	↔
Neuroprotection	Evidence in preclinical studies	Evidence in preclinical studies
Cardiac effects	Improved CV risk factors: BP, lipids, inflammatory markers; evidence of cardioprotection in preclinical and early clinical trials	↔
Bone effects	↑bone formation ↓bone resorption	↑bone formation ↓ bone resorption
Adipose tissue	Likely no direct effect	↑lipogenesis

BP, blood pressure; CV, cardiovascular

2. PRODUCTION AND SECRETION

2.1 Enteroendocrine cells

Incretins are predominantly synthesized and secreted by intestinal enteroendocrine cells. The intestinal surface exhibits a unique architecture that consists of villus and crypt structures. Gut stem cells reside within the crypts and are triggered to differentiate into absorptive enterocytes, bactericidal Paneth cells, mucus-producing goblet cells and hormone-secreting enteroendocrine cells by Notch-mediated signaling. The enteroendocrine cells are fully differentiated cells that, together with the goblet and Paneth cells, constitute the secretory cell lineages of the intestine, which comprise 10% of the epithelium [8] (**figure 2**).

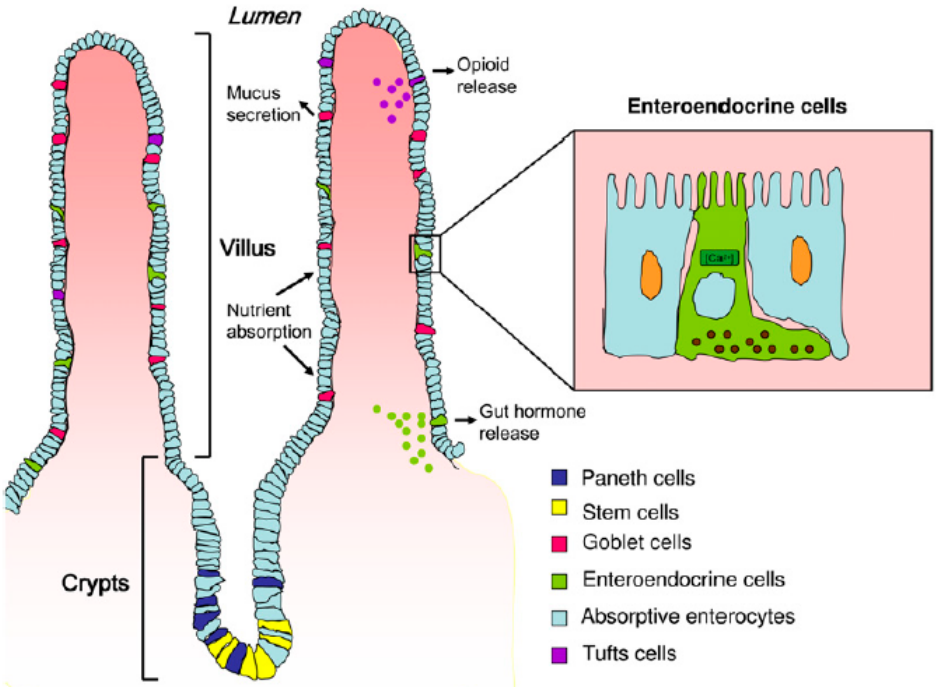

Figure 2. Intestine morphology and distribution of epithelial cell lines [8]

Enterocytes, goblet cells and enteroendocrine cells migrate up the crypt-villus axis and turn over every 3-5 days. Enteroendocrine cells actively self-renew and differentiate throughout the life of an animal from a large reservoir of stem cells. As mature enteroendocrine cells, they migrate to the tip of the villi, where they undergo apoptosis and are extruded into the lumen. These cells are individually scattered throughout the mucosa, and although they comprise only approximately 1% of total intestinal luminal cells, they are nonetheless considered the largest endocrine organ in the body [9].

The different enteroendocrine cell types have been classified according to their epithelial localization as follows: the “closed cells” that do not reach the intestinal lumen and the “open cells” that project a tuft of apical microvilli into the intestinal lumen and that extend into the basal lamina (lamina propria) [10]. Open types are considered the primary chemoreceptors that respond to luminal nutrients by releasing secretory products, which activate neuronal pathways, nearby cells or in distant targets. Closed cells can be regulated by luminal content indirectly through neural and humoral mechanisms [11].

Enteroendocrine cells have also been classified into at least 10 types based on their morphology, principal hormone product(s) and distribution along the intestinal tract [12] (the types are summarized in **table 2**). The most studied enteroendocrine cells are I,

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L-, and K-cells due to their secreted products, including cholecystokinin (CCK), glucagon-like peptides, and GIP, respectively [8].

Table 2. Summary of the different subset of enteroendocrine cells, localization and hormone(s) secreted

Cell type	Highest density	Peptide released
G-cells	Stomach	Gastrin
X-cells	Stomach	Ghrelin
ECL-cells	Stomach	Histamin
Unamed cells	Stomach and duodenum	Gastrin Releasing Peptide (GRP)
S-cells	Duodenum and jejunum	Secretin
I-cells	Duodenum and jejunum	Cholecystokinin (CCK)
K-cells	Duodenum and jejunum	Glucose-dependent insulinotropic polypeptide (GIP)
N-cells	Ileum	Neurotensin
L-cells	Ileum and colon	Glucagon-like peptides (GLP's), Peptide YY (PYY)
D-cells	Entire GI tract	Somatostatin
EC-cells	Entire GI tract	5-hydroxytryptamin (5-HT, Serotonin)

EC-cells, enterochromaffin cells; ECL-cells, enterochromaffin-like-cells; glucagon-like peptides (GLP's) include GLP-1, GLP-2, oxyntomodulin, glicentin.

The L-cell is an open–type intestinal cell that makes direct contact with the lumen, sensing nutrients, and represents the predominant GLP-1-secreting cell [8]. These cells are scattered along the gastrointestinal tract, predominantly within the ileum and colon, but they are anatomically distinct. Mature ileal L-cells exhibit a classic L-shape, whereas mature colonic cells exhibit a spindle- or sigmoidal-like contour. In the ileum, the apical populations maintain minimal contact with the intestinal lumen, whereas in the colon is more prominent, but L-cells are in contact with the lumen and the lamina propria in both intestinal regions [13].

2.2 GLP-1 synthesis in enteroendocrine cells

GLP-1 is a product of the proglucagon gene, which is located on the long arm of the chromosome 2 and also encodes glucagon, GLP-2 and other proglucagon-derived peptides [5]. Although only a single gene encodes for proglucagon in intestinal L-cells and pancreatic α-cells, producing identical mRNAs in both tissue types, distinct gene products are synthesized in these tissues due to tissue-specific posttranslational processing [14,15]. The differential posttranslational processing of proglugacon results in the production of glucagon, glicentin-related polypeptide (GRPP), intervening peptide-1 (IP-1) and a major proglucagon fragment (MPFG) in the pancreas, whereas

glicentin, IP-2 and glucagon-like peptides-1 and 2 (GLP-1 and GLP-2) are produced in the gut [16] (**figure 3**).

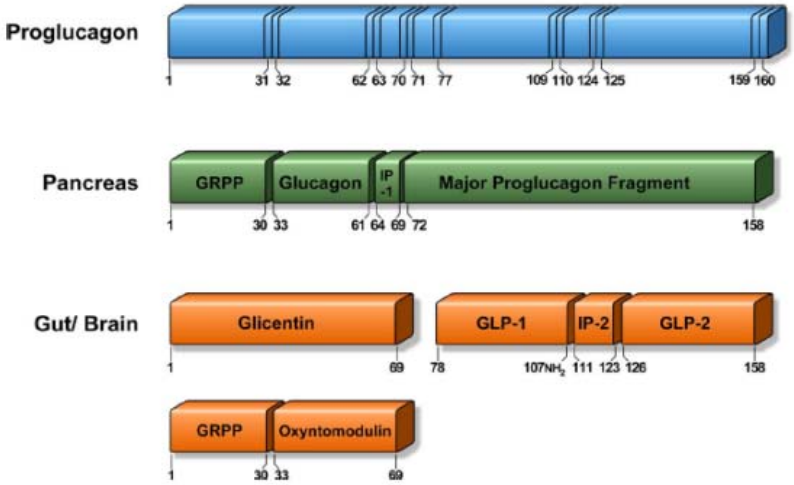

Figure 3. Differential posttranslational processing of proglucagon in the pancreas and in the gut and brain [16]

Two proteases of the six-member kexin/subtilisin-like prohormoneconvertase (PC) family are responsible for the tissue-specific processing of proglucagon. PC2 is highly expressed in pancreatic α-cells, whereas its expression is significantly reduced in intestinal L-cells. In contrast, PC1/3 is highly expressed in intestinal L-cells, whereas their expression is significantly reduced in pancreatic α-cells. PC2 has been demonstrated to process proglucagon into glucagon, whereas PC1/3 cleaves proglucagon into glicentin, GLP-1 and GLP-2 [17-19].

GLP-1 is produced as an inactive 37-amino acid peptide (GLP-1 (1-37)), the C-terminus of which contains glycine. Its active form is produced by the posttranslational cleavage of six amino acids from the N-terminus (GLP-1 (7-37)). This truncated form of GLP-1 can also be amidated at the C-terminal glycine residue (GLP-1 (7-36) amide), which represents the major circulating form (**figure 4**). Both GLP-1 (7-37) and GLP-1 (7-36) amide represent equipotent insulinotropic peptides [3].

The proglucagon gene is also expressed within the central nervous system, predominantly in the brainstem and hypothalamus [20,21]. Its posttranslational processing has been reported to be identical to that found in intestinal cells, and immunostaining techniques have demonstrated the presence of GLP-1 within the brainstem and several regions of the hypothalamus [22].

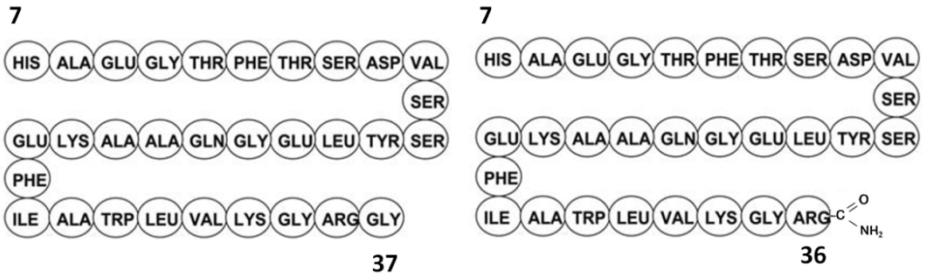

Figure 4. Amino acid sequences of GLP-1_{7-37} and GLP-1_{7-36} amide (adapted from [3])

2.3 GLP-1 secretion by enteroendocrine cells

GLP-1 secretion by the intestinal L-cells after the intake of a meal is biphasic, with an early phase beginning within minutes and persisting for 30-60 minutes, and an overlapping second phase that is characterized by prolonged secretion between 60 and 120 minutes after the ingestion of a meal. The mechanisms involved in the regulation of GLP-1 secretion have been suggested to be distinct between these two phases. Given the predominant distribution of L-cells in the distal intestine, several authors have suggested that the first phase is indirectly mediated through neuroendocrine pathways, whereas the direct contact with luminal nutrients induces the subsequent peak of secretion (reviewed in [23]). Conversely, Theodorakis et al. reported that the number of L-cells present in the duodenum is sufficiently high to account for the early phase secretion of GLP-1, although the neuroendocrine pathway might also contribute [24].

Several *in vivo* and *in vitro* studies have shown that GLP-1 secretion is stimulated by nutrients such as monosaccharides, fats (the efficacy of the stimulation is directly proportional to the length of unsaturated acyl chains), proteins, and amino acids. However, other signals including hormones and neurotransmitters can trigger GLP-1 release (reviewed in [16]). *In vivo* experiments have demonstrated that the caloric value and the nature of ingested nutrients represent determinant factors for the GLP-1 levels secreted into the plasma or portal vein. *In vitro* studies have exhibited different effectiveness depending on the nutrient concentration.

2.3.1 Nutrient regulation

Carbohydrates

Carbohydrates—glucose in particular—are the most investigated nutrients in the context of stimulating incretin release, and there is a consensus that glucose is a potential GLP-1 secretagogue [25]. Moreover, oral glucose has been reported to be more effective at stimulating GLP-1-release than other monosaccharides, such as

fructose or galactose, [26,27] and several mechanisms have been proposed to explain how intestinal L-cells sense glucose.

ATP-sensitive K^+ channels (K_{ATP} channels) are well characterized as glucose sensors in pancreatic β-cells and were also found in other glucose-sensitive tissues, such as cardiac muscle, brain, and other islet cell types. K_{ATP} channels are open during low glucose levels, and an acute increase in the extracellular glucose induces a dose-dependent increase in the ATP/ADP ratio due to increased glucose metabolism. The increase in intracellular ATP levels promotes the closure of the K_{ATP} channels, consequently inducing membrane depolarization, which leads to the opening of L-type voltage-gated calcium channels (VGCC) and Ca^{2+} influx, inducing the exocytosis of insulin-vesicles [28] (**figure 5**). Studies on GLUTag cells (a murine enteroendocrine cell line) have shown that glucose triggers membrane depolarization, Ca^{2+} influx and GLP-1 secretion. In addition, the K_{ATP} channel blocker tolbutamide has been shown to elicit the same effects, suggesting the involvement of K_{ATP} channel-closure on the glucose-induced GLP-1 release of intestinal L-cells [29]. However, studies in humans have not demonstrated changes in GLP-1 levels between healthy and diabetic subjects presenting a mutation in the gene encoding the K_{ATP} channel subunit Kir6.2 [30]. El-Ouaghlid et al. also observed that the treatment with glibenclamide, a K_{ATP} channel blocker, induces insulin release but not GLP-1, as indicated by an oral glucose tolerance test [31]. Taken together, K_{ATP} channel blockage has therefore been excluded as the predominant mechanism underlying glucose-sensing by intestinal L-cells.

The evidence precluding K_{ATP} channel closure in response to glucose as the central mechanism for GLP-1 secretion has been further supported by the reported stimulation of GLP-1 secretion by non-metabolizable sugars, such as 3-O-methylglucose or 2-deoxyglucose [26], suggesting that other mechanisms may also be involved in glucose-dependent GLP-1 release. These non-metabolizable sugar analogues are substrates for the sodium/glucose co-transporter 1 (SGLT1), which is expressed in intestinal L-cells. Gribble et al. reported that high concentrations of the non-metabolizable glucose analogue, methyl-α-glucopyranoside (αMG) can stimulate GLP-1 secretion and membrane depolarization in the GLUTag cell line. The effects elicited by glucose or its non-metabolizable analogue were strongly reduced by the SGLT inhibitor phloridzin, suggesting that sugar metabolism is dispensable for GLP-1 secretion [32]. Studies in primary L-cells demonstrated that reduced glucose levels and αMG trigger GLP-1 release, supporting the previous observations in GLUTag cells [33]. Moreover, Gorboulev et al. previously reported no changes in plasma GLP-1 levels after the administration of oral glucose in *Sglt1*$^{-/-}$ mice, further supporting the role of SGLT1 on glucose-dependent stimulation of GLP-1 release [34]. Thus, this proposed mechanism to stimulate GLP-1 secretion in the absence of glucose metabolism involves glucose transport via SGLT-1,

which requires the co-transportation of two Na^+ ions per glucose molecule to induce a sufficient inward gradient to trigger cell membrane depolarization and the release of GLP-1 [25] (**figure 5**).

A third glucose-sensing mechanism involving sweet taste receptors that does not require sugar metabolism has been proposed. The sweet taste is detected by a small family of GPCRs-T1R1, T1R2 and T1R3. A common intracellular signal transduction pathway is activated by T1R2 and T1R3 stimuli, involving activation of α-Gustducin. This taste-specific G-protein activates a signal transduction cascade that involves phospholipase Cβ2 (PLCβ2) and inositol triphosphate (IP_3), which promote the release of Ca^{2+} from intracellular stores and the gating of the cation channel TRPM5, leading to a Na^+ influx and membrane depolarization (**figure 5**) (reviewed in [35]). The presence of sweet taste receptors and α-gustducin in L-cells has been reported *in vivo* and in enteroendocrine cell line models [36-38]. The impairment of GLP-1 secretion has been reported *in vivo* in α-gustducin and T1R3 knock-out mice and *in vitro* in the NCI-H716 cell line that exhibits decreased α-gustducin expression [37,39], supporting the hypothesis of an involvement of sweet taste receptors in glucose-sensing by L-cells.

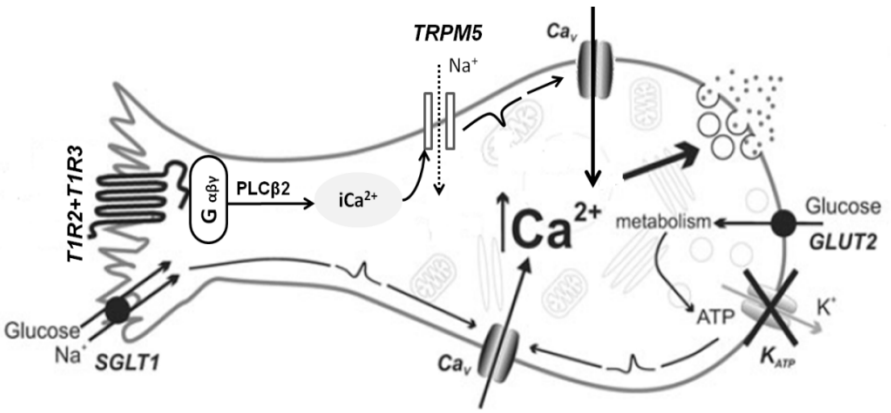

Figure 5. Model of intestinal glucose-sensing (adapted from [180])

Lipids

Fat is a potent stimulator of GLP-1 secretion, and the ileal perfusion of lipids has been previously reported to elicit similar effects on GLP-1 compared to an ileal carbohydrate [40]. Thomsen et al. demonstrated that olive oil, which is rich in monounsaturated fatty acids, is a more potent stimulator of GLP-1 secretion than butter, which is predominantly composed of saturated fatty acids [41], suggesting that the response to fat stimulation is significantly dependent on the degree of fatty acid saturation. Several authors have also reported that ingested triglycerides need to be

hydrolyzed to long-chain free fatty acids to stimulate the release of GLP-1 (reviewed in [42].

Free fatty acids (FFAs) have been found to be ligands for GPCRs, which function on the cell surface and play significant roles in nutrition regulation [43]. GPR40 or free fatty acid receptor 1 (FFAR1) is activated by medium- and long-chain FFAs. GPR41 (FFAR3) and GPR43 (FFAR2) are activated by short-chain FFAs, whereas GPR120 is activated by medium- to long-chain unsaturated FFAs, and GPR119 is activated by lysophosphatidylcholine. All of these receptors are expressed in enteroendocrine L-cells (reviewed in [43,44]).

Stimulation of GPR120 by FFAs and selective receptor agonists has been reported to stimulate GLP-1 secretion and the mobilization of intracellular Ca^{+2} in STC-1, a murine enteroendocrine cell line [45]. FFA-triggered GLP-1 secretion has also been shown to be markedly reduced in GPR120 knock-out mice [46]. The stimulation of GPR120 by long-chain FFAs has been suggested to be coupled to the G-protein subunit $G_{\alpha q/11}$, which in turn activates the phospholipase-C-mediated hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP3), activating the mobilization of Ca^{2+} from endoplasmic reticulum Ca^{2+} stores. Ca^{2+} activates the monovalent cation channel TRPM5, leading to Na^{+} influx and subsequent membrane depolarization, which results in the opening of VGCCs and increased intracellular Ca^{2+} levels [44,47] (**figure 6**).

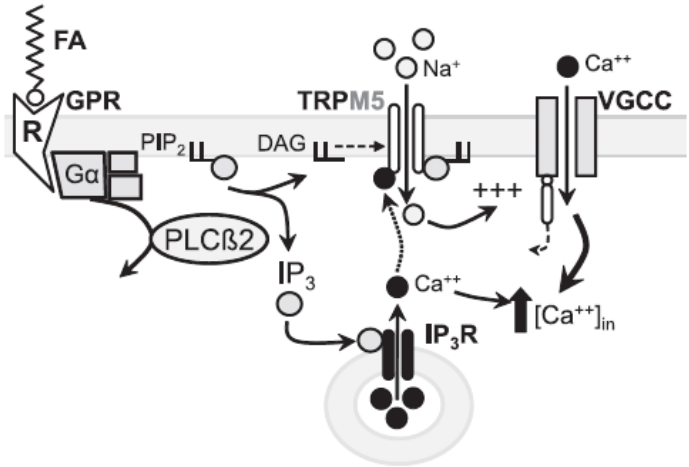

Figure 6. Model of stimulated GPRC120, GPR40 and FFAR2 transduction pathway [47]

GPR119 stimulation with oleoylethanolamide and PSN632408, a specific GPR119 agonist, has been reported to trigger GLP-1 secretion from mouse and human intestinal

L-cells. The direct intraluminal infusion of oleoylethanolamide in rats increases bioactive GLP-1 levels. Moreover, *in vitro* studies have shown that oleoylethanolamide stimulation increases intracellular cAMP levels, whereas the silencing of GPR119 significantly decreases cAMP levels and GLP-1 secretion, demonstrating the role of GPR119 in FFAs-stimulated GLP-1 release [48]. GRP119 activation is coupled to the G-protein subunit $G_{\alpha s}$, causing the activation of adenylyl cyclase and the production of intracellular cAMP [49].

Lastly, GPR43 (or FFAR2) activation by short-chain FFAs has also been shown to increase intracellular Ca^{2+} levels in L-cells and to enhance GLP-1 release from primary colonic cultures. Moreover, reduced GLP-1 levels were detected in FFAR2 knock-out mice. The FFAR2 signaling pathway was found to be coupled by G-protein subunits $G_{\alpha q/11}$, such as GPR120 [50].

Proteins and amino acids

Proteins are generally considered the least effective of the macronutrients in terms of stimulating incretin secretion [8,42]. However, studies in humans, animals and enteroendocrine cell lines have shown the stimulation of GLP-1 secretion by proteins, protein hydrolysates and amino acids.

Cordier-Bussat et al. demonstrated that protein hydrolysates stimulate GLP-1 release from native L-cells in isolated vascularly perfused rat jejuno-ileum, as well as in the murine enteroendocrine cell line STC-1. Studies in humans showed that amino acids and a protein amount equivalent to a daily intake led to an increase in GLP-1 secretion after oral ingestion [51]. Greenfield et al. have found that oral glutamine increases circulating GLP-1 in healthy, obese and diabetic men [52]. Other studies in GLUTag cells and in *in situ* intestinal loop experiments have also shown that hydrolysates prepared from corn zein stimulate GLP-1 release [53]. Several studies in GLUTag and STC-1 have found that intact proteins and a broad range of amino acids directly stimulate GLP-1 secretion [54-56]. Concerning the mechanisms involved, glutamine stimulation on GLUTag induces GLP-1 secretion, which was found to be triggered by a membrane depolarization and an increase in intracellular Ca^{2+} levels [54]. Young et al. also reported that L-proline, L-serine, L-alanine, L-glycine, L-histidine, L-cysteine and L-methionine triggered an increase in intracellular Ca^{2+} levels in STC-1 cells, with L-proline being the major stimulant. Moreover, they found evidence to suggest that the sodium-coupled neutral amino acid transporter 2 (SNAT2), which leads to membrane depolarization, activation of L-type VGCCs that mediate Ca^{2+} influx leading to an increase in the intracellular Ca^{2+}, is involved in the amino acid-sensing mechanism [56].

2.3.2 Neural/endocrine regulation

A proximal-distal signaling loop was demonstrated to also stimulate GLP-1 secretion, relaying information about nutrients, predominantly fat, from the proximal duodenum to the distal L-cells. This loop involves both endocrine and neural factors, with the vagus nerve as the essential component [57].

GIP is one of the mediators of this loop. The arrival of the chyme at the duodenum stimulates K-cells to secret GIP, which stimulates the afferent vagus nerve of the central nervous system, thereby activating the efferent vagus nerve to the ileum. It acts via nicotinic synapse stimulating the secretion of the neuropeptide gastrin-releasing peptide (GRP) by intrinsic neurons, which induces acetylcholine release from cholinergic neurons in the enteric nervous system, which activates the muscarinic receptors on the L-cells. The activation of the subtype 1 muscarinic receptor (M1) activates the PLC/Ca^{2+} pathway, which stimulates GLP-1 secretion from L-cells (**figure 7**) [58].

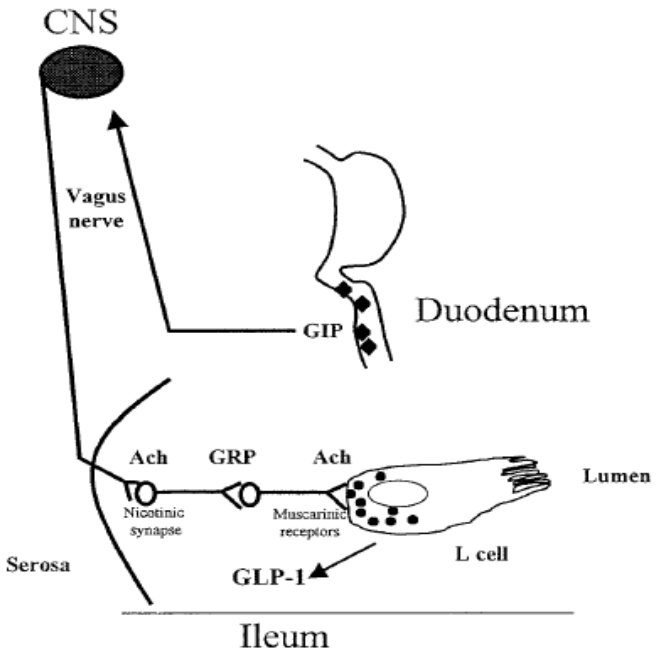

Figure 7. Model of the indirect mechanism underlying GIP-induced GLP-1 secretion after a meal pathway [58]

Other neurotransmitters, such as glycine, GABA or epinephrine, and the adrenergic activation of enteroendocrine L-cells have also been reported to stimulate GLP-1 [59,60].

Moreover, several hormones, such as leptin and insulin, have been shown to enhance GLP-1 secretion [6,61]. In contrast, somatostatin, which is produced by the intestinal enteroendocrine D-cells, was reported to inhibit GLP-1 secretion by activating the PKC/PKA pathways. The proximity of D- to L-cells suggests the existence of a negative local feedback loop within the gut [62].

2.4 GIP synthesis and secretion by enteroendocrine cells

GIP is synthesized by the intestinal enteroendocrine K-cells, which are found all along the small intestine but in increased amounts in the duodenum and jejunum [63]. This hormone is the product of the GIP gene, which is located on chromosome 17 [6].

The GIP gene encodes a 153-amino acid polypeptide in humans and a 144-amino acid polypeptide in rats, which are GIP precursors (proGIPs) [7]. ProGIP consists of three domains, of which the middle domain corresponds to a 42-amino acid sequence that is GIP. GIP is thought to be the only biologically active peptide derived from the precursor. Ugleholdt et al. have shown that the PC1/3 is essential for the cleavage of proGIP to GIP [64]. Moreover, the amino acid sequence of GIP has been demonstrated to exhibit structural homology with other gastrointestinal regulatory peptides, including secretin, glucagon, GLP-1 and GLP-2 [7].

Concerning nutrient stimulation, glucose and fat are characterized as the more potent stimulators for GIP secretion in humans, provoking a rapid release by intestinal K-cells that peaks at 15-30 minutes after the oral ingestion of glucose and 30-45 minutes after the oral ingestion of fat. Active GIP plasma levels remain elevated for at least 2 hours after the ingestion of a meal (reviewed in [65]).

GIP levels released from K-cells have been reported to be proportional to the calories ingested, and the rate of carbohydrate absorption [65,66]. K-cells have been shown to express SGLT1, K_{ATP} channels and glucokinase, and the finding that GIP release is three-fold greater after glucose than αMG stimulation indicates that glucose metabolism is needed for a complete secretory response [67]. Margolskee et al. found that the sweet taste receptors T1R2 and T1R3 sense luminal glucose and increase GIP release from enteroendocrine cells [38]. However, other authors have not found compelling evidence of sweetener-induced GIP secretion, suggesting that these receptors do not play a major role in the carbohydrate stimulation of GIP [37].

Lastly, dietary fat has been found to be a major stimulus for GIP secretion in humans [7]. Thomsen et al. reported that increased GIP secretion is produced after the oral ingestion of olive oil compared to butter, suggesting that, similar with GLP-1 secretion, the fatty

acid nature of the stimulus is relevant and that monounsaturated FFAs appear to be more potent stimuli for GIP release than saturated FFAs [41]. Moreover, triglyceride hydrolysis has been shown to be crucial for the K-cell secretory response [68]. These enteroendocrine cells express the G protein-coupled receptors GPR40, GPR119 and GPR120, and exhibit significant GIP secretion after stimulus with potent agonists, such as linoleic acid and AR231453 [67].

3. GLP-1 CLEAVEAGE BY ENZYMATIC DEGRADATION: DIPEPTIDYL-PEPTIDASE 4

Secreted GLP-1 is active in its GLP-1 (7-37) and GLP-1 (7-36) amide forms, but they are rapidly truncated by the dipeptidyl-petidase 4 (DPP4) enzyme. DPP4, also known as the T-cell antigen CD26, is a serine exopeptidase that belongs to the S9B protein family and that cleaves X-proline or -alanine dipeptides from the N-terminus of polypeptides, such as chemokines, neuropeptides, and peptide hormones [69]. This enzyme is widely distributed among almost all of the tissues in the body. DPP4 is also in close proximity with the circulating hormones in the blood due to its presence in the blood plasma as a soluble enzyme and its location on the endothelial cells of the blood vessels. Moreover, DPP4 is strongly expressed in the capillary epithelia of endocrine organs (reviewed in [70]).

Mentlein et al. reported that incretin hormones (GIP and GLP-1) are targets for DPP4 and they demonstrated that this enzyme is the predominantly responsible for the cleavage of the GLP-1 (7-36) amide in plasma [71], generating a new form of the peptide, GLP-1(9-36). The C-terminus of the peptide has been reported to be important for receptor binding, whereas the N-terminus is required for signal transduction and its biological action [72]. Thus, the DPP4-mediated cleavage inactivates the GLP-1(7-36) amide. Improved active GLP-1 levels have been observed in *in vivo* experiments performed in animals with a disrupted DPP4/CD26 gene [73] or in animals that were treated with different DPP4 inhibitors [74,75], strongly supporting the potential involvement of DPP4 on incretin metabolism.

Intact GLP-1 has been shown to be rapidly degraded by DPP4 after reaching the plasma, with a half-life of 1-2 minutes [76,77]. Hansen et al. reported that DPP4 is present in the endothelium of the capillaries in the intestine and demonstrated that approximately half of the newly secreted GLP-1 is already inactive by the time it reaches the level of systemic circulation. This early cleavage was attributed to the DPP4 present on the endothelium of the capillary bed that is in close proximity to the GLP-1-secreting L-cells [78], confirming the previous notion of a tissue-specific metabolism of GLP-1 [79]. The

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liver also plays an important role in GLP-1 inactivation due to the increased concentrations of DPP4 present on hepatocytes, which facilitates the degradation of a significant proportion of active GLP-1. This fact is of particular physiological relevance because GLP-1 that is released from intestinal L-cells must pass through the liver prior to distribution to the rest of the body (**figure 8**). Moreover, active GLP-1 degradation by DPP4 has also been detected in the kidneys, hind-limb, portal bed, and the lungs (reviewed in [79]). Kidneys are the major site of extraction of GLP-1 metabolites (GLP-1 (9-36) amide), which involves glomerular filtration and tubular uptake and catabolism, although these mechanisms are less significant in terms of GLP-1 degradation by DPP4 [77].

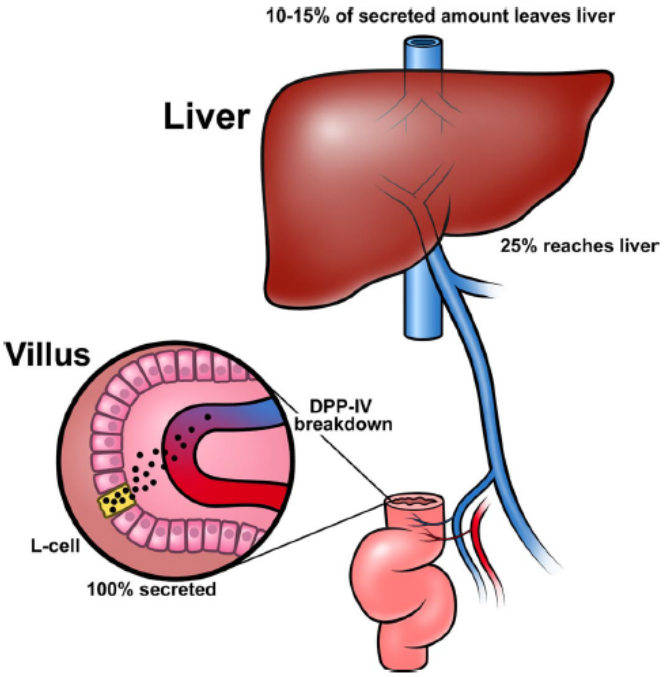

Figure 8. GLP-1 degradation by DPP4 after secretion [16]

Lastly, it should be mentioned that in addition to DPP4, limited *in vitro* studies have suggested that another enzyme, neutral endopeptidase 24.11 (NEP 24.11), is involved in the cleavage of GLP-1 [80,81]. NEP 24.11 cleaves peptides at the N-terminal side of the aromatic or hydrophobic amino acids and is found at high concentration within the kidneys. Six potential NEP 24.11 cleavage sites have been identified within the GLP-1 polypeptide (in the central and C-terminal positions) [82]. However, its relevance to GLP-1 metabolism *in vivo* has not been fully elucidated.

4. GLP-1 SIGNALLING IN TARGET CELLS

Active GLP-1 elicits several effects on peripheral tissues prior to clearance by DPP4. Its effects are mediated through the activation of the GLP-1 receptor (GLP-1R), which is a 463-amino acid G protein-coupled receptor [83] that is widely expressed in pancreatic islets, kidney, lung, heart, muscle, adipocytes, liver, brain, stomach, duodenum, and in multiple regions of the peripheral and central nervous system (reviewed in [4,5,23]).

In addition to the endocrine function of active GLP-1, the involvement of a neural pathway has been suggested based on the finding that active GLP-1 is extensively degraded by DPP4 before reaching systemic circulation. GLP-1, which is secreted by intestinal L-cells, can bind to and activate the GLP-1Rs that are present at the terminals of the afferent sensory nerve fibers. The activity of these fibers provides sensory input via the nodose ganglia to the solitary tract nucleus [84]. The same neuronal pathway might be activated by sensory neurons in the hepatoportal region and in the liver. Ascending fibers from the solitary tract neurons generate reflexes within the hypothalamus, and descending impulses activate vagal motor neurons that send stimulatory or inhibitory impulses to the peripheral tissues [85] (**figure 9**).

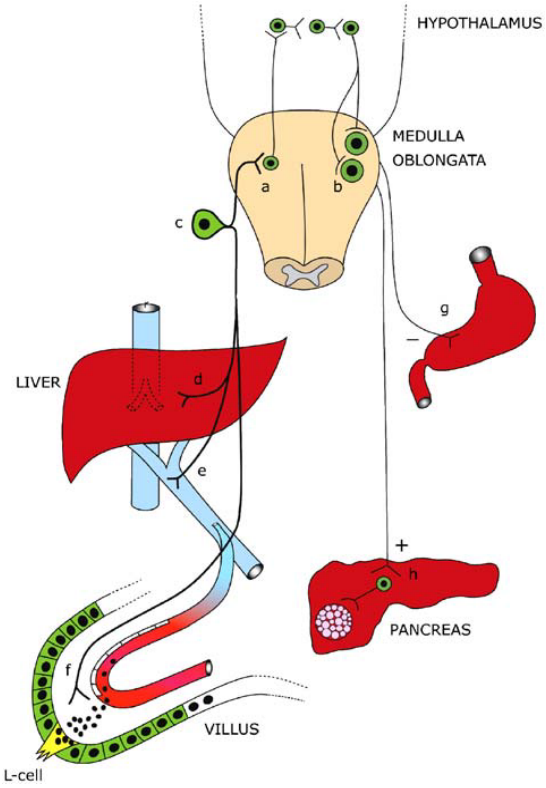

Figure 9. Schematic diagram of the neural pathway for the action of GLP-1 [85]

GLP-1 targets a wide range of tissues due to the wide distribution of its receptor and the activation of neuronal pathways. In the pancreas, GLP-1 enhances insulin secretion and biosynthesis and stimulates the expansion of the β-cell mass (reviewed in [1,16]). GLP-1 also promotes the proliferation and neogenesis of β-cells and decreases apoptosis [86]. Glucose uptake has been shown to be enhanced by GLP-1 in several tissues such as liver and muscle [87,88] as well as in adipocytes [89]. GLP-1 was also found to inhibit appetite and food intake [90], which was linked to its previously reported effect on delaying

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gastric emptying [91]. Moreover, GLP-1 has been reported to be involved in the regulation of vital brainstem functions, such as body temperature, blood pressure, heart rate and water balance, and the enhancement of memory and learning behavior [3].

4.1 Insulinotropic actions of GLP-1

GLP-1 enhances the responsiveness of the β-cells to glucose, and a synergistic interaction was proposed based upon the finding that GLP-1 does not affect insulin release in the absence of glucose [92]. Within islets, the GLP-1R is predominantly located in β-cells, although it is also expressed on α- and δ-cells [1]. The activation of GLP-1R leads to the stimulation of adenylate cyclase and the accumulation of intracellular cAMP [93]. Several mechanisms for GLP-1-enhanced insulin-release have been proposed (**figure 10**):

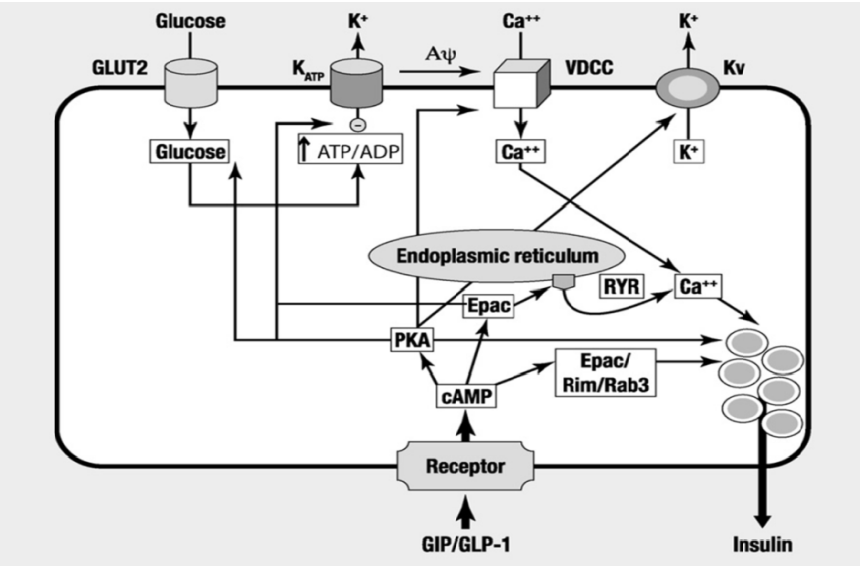

Figure 10. GLP-1 receptor signaling and regulation of glucose-induced insulin secretion [4]

1) cAMP activates protein kinase A (PKA), which phosphorylates K_{ATP} channels, inducing their closure and subsequent membrane depolarization [92]. This change in membrane potential leads to activation of VGCCs, which triggers Ca^{2+} influx. PKA can also phosphorylate VGCCs, thereby increasing Ca^{2+} influx [94,95], and activate Ca^{2+} mobilization from intracellular stores (predominantly from the endoplasmic reticulum) [96].

2) cAMP also activates the exchange protein directly activated by cAMP 2 (Epac2), which binds to K_{ATP} channels and induces their closure and the subsequent depolarization and Ca^{2+} influx. Epac2 has also been reported to sensitize ryanodin and IP_3 receptors on endoplasmic reticulum, triggering Ca^{2+} release.

The increased intracellular Ca^{2+}levels induce fusion of the insulin-containing granules to the plasma membrane of the β-cell. PKA has also been reported to recruit insulin-containing vesicles from a reserve pool to a readily releasable pool and to facilitate Ca^{2+}-dependent fusion of secretory granules with the plasma membrane. Moreover, Epac2 has been found to directly induce insulin release by interacting with insulin granule-associated proteins, such as Rim2 (reviewed in [97]).

The activation of GLP-1R has also been found to up-regulate gene transcription and protein expression of the transcription factor PDX-1 (pancreatic and duodenal homeobox gene 1), which activates insulin gene expression [98]. GLP-1 also up-regulates the gene expression of GLUT2 (glucose transporter 2) and glucokinase (that together with the insulin are the main gene transcripts that define the physiology of the β-cells), so the differentiation of pancreatic ductal epithelial cells into β-cells is also promoted by GLP-1 [99]. Moreover, GLP-1 increases cell survival by up-regulating the gene expression of the anti-apoptotic proteins *bcl-2* and *bcl-xL* and by down-regulating the gene expression and intracellular levels of the pro-apoptotic protein caspase-3 [100]. Therefore, GLP-1 not only enhances insulin release and synthesis but also improves β-cell mass by enhancing proliferation, inhibiting apoptosis and by increasing β-cell differentiation [86].

5. INCRETINS ON INSULIN RESISTANCE AND OBESITY

A decrease in the capacity of adipose tissue to store lipids and trap fatty acids was previously found in obesity, resulting in an increase of circulating fatty acids that leads to the ectopic storage of fat in non-adipose tissues such as muscle, liver, and pancreas, and possibly other organs. This increase in visceral fat results in several metabolic derangements that are associated with the deterioration of glucose tolerance and insulin resistance, leading to increased insulin secretion to maintain glucose homeostasis [101,102].

The important role of GLP-1 on glucose homeostasis suggests that incretin impairment might be involved in the pathogenesis of obesity. However, few studies have reported on this topic, although the incretin effect was found to be decreased in insulin resistant

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obese subjects compared to non-insulin resistant lean subjects [103,104]. Several authors have linked this impaired incretin effect to a decrease in GLP-1 secretion by L-cells [105-110], and Dao et al. also reported a decreased intestinal GLP-1 production in rats fed a high-fat diet [109]. However, other authors linked this reduced incretin effect to a decreased incretin sensitivity by pancreatic β-cells [104,111], which has been suggested to represent a very early stage in the pathophysiology of type 2 diabetes mellitus (T2D) [112]. In T2D subjects, an attenuated incretin effect was found [104], which is associated with decreased GLP-1 secretion [106,113]. Furthermore, the decrease in the incretin effect and GLP-1 secretion have been found to be proportionally correlated with the degree of obesity [103,114], suggesting that incretin dysfunction might be involved in the development of T2D, but whether the decrease in GLP-1 secretion is a cause or consequence of these metabolic deregulations remains to be elucidated.

Increased DPP4 activity has also been linked to a reduced incretin effect, which is suggestive of increased incretin inactivation. Accordingly, several studies have reported the increased activity of circulating DPP4 in obese and T2D subjects [115-119]. However, limited studies have found decreased activity of plasma DPP4 in diabetic subjects [120] or unchanged activity in obese, diabetic and impaired-glucose tolerance subjects [116,121]. Taken together, these data indicate that a severe degree of hyperglycemia is required to increase the activity of circulating DPP4, whereas mild hyperglycemia is insufficient to induce such an increase, suggesting that decreased levels of active GLP-1 during the early phases of T2D might be attributable to decreased hormone secretion [116,121].

B. FLAVONOIDS AND THE INCRETIN SYSTEM

1. FLAVONOIDS

Plant-derived foods contain many minor components with the capacity to alter enzymatic and chemical reactions and to exert biological responses in mammalian systems, thereby both positively and negatively impacting health. These components were identified as phytochemicals, phytonutrients and non-traditional nutrients. In general, `phytochemicals´ are classified by structure or a unique molecular content, such as carotenoids, polyphenols, sulfides and thiols. One of the largest groups of `phytochemicals´ that may elicit beneficial effects on health, is a subclass of polyphenols called flavonoids [122,123].

Over 4,000 structurally unique flavonoids have been identified from plant sources. They are widely distributed in fruits and vegetables, as well as in tea and red wine [124], which are regularly consumed in the human diet.

The structure of flavonoids is based on two aromatic rings (A and B) bound together by three carbon atoms that form an oxygenated heterocycle (C) (**figure 11**) [125]. This basic structure allows for a multitude of substitution patterns within the A, B, and C rings, resulting in several subgroups. Depending on the oxidation levels of the C-ring, flavonoids are classified into anthocyanidins, flavanols or flavan-3-ols (catechins), flavones, flavonols, flavanones and isoflavones (**table 3**). The basic structure of the flavonoid nucleus allows for a multitude of substitutions, including glycosilation, hydrogenation, hydroxylation, malonylation, methylation, and sulfation [126]. Moreover, the degree of polymerization increases structural diversity, resulting in thousands of structures with different chemical, physical, and biological properties [127].

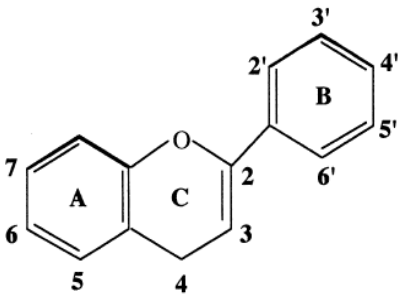
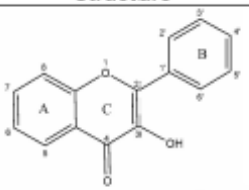
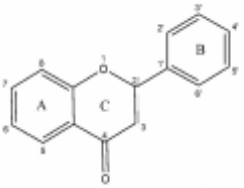
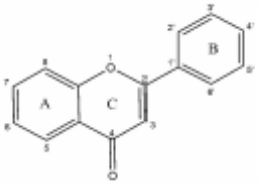
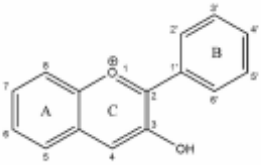
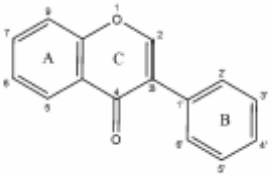
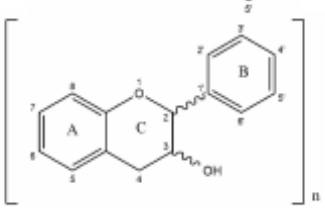


Figure 11. Flavonoid nucleus [126]

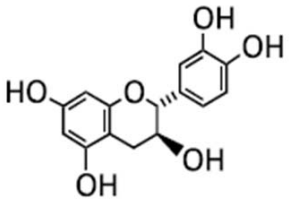
Table 3. Classification, representative structure, and food source of main flavonoids [127]

Flavonoids	Structure	Food source
Flavonols (for example Quercetin)		Onion, cherry tomato, and leeke
Flavanones (for example Naringenin)		Orange, grapefruit, and lemon juice
Flavones (for example Apigenin)		Celery, parsley, and pepper
Anthocyanidins (for example Pelargonidin)		Aubergine, black grape, and black currant
Isoflavones (for example Genistein)		Soy beans and soy products
Flavanol monomer, dimers, trimers,etc. (for example EGCG)		Tea, chocolate, and red wine

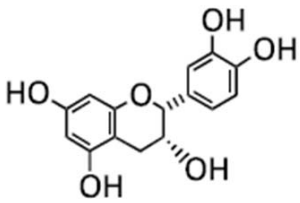
1.1 Structure and classification of proanthocyanidins

Flavan-3-ols are widely dispersed in the human diet and represent one of the most abundant dietary flavonoids in red wine, tea, berries, apples and chocolate. This is a complex subclass of flavonoids, which includes the simple monomers, (+)-catechin and its isomer (-)-epicatechin, and the oligomeric and polymeric proanthocyanidins (also

known as condensed tannins) [128]. Proanthocyanidins are dimers, oligomers, and polymers of catechins that are bound by links between C4 and C8 or C4 and C6 [125]. Dimers with these types of linkages are classified as B-type, whereas trimers are classified as C-type. The A-type is another group of proanthocyanidin dimers that are more rigid than the B-type due to the incorporation of the two following interflavan linkages: one C→C and the other C→O [129]. The proanthocyanidins, which exclusively consist of (epi)catechin units, are designated as procyanidins and represent the most abundant form found in plants [130] (**figure 12**).



(+)-Catechin



(–)-Epicatechin

Trivial name	
Procyanidin B1	Epicatechin-(4β → 8)-catechin
Procyanidin B2	Epicatechin-(4β → 8)-epicatechin
Procyanidin B3	Catechin-(4α → 8)-catechin
Procyanidin B4	Catechin-(4α → 8)-epicatechin
Procyanidin B5	Epicatechin-(4β → 6)-epicatechin
Procyanidin B6	Catechin-(4α → 6)-catechin
Procyanidin B7	Epicatechin-(4β → 6)-catechin
Procyanidin B8	Catechin-(4α → 6)-epicatechin
Procyanidin C1 (Cinnamtannin A1)	Epicatechin-(4β → 8)-epicatechin-(4α → 8)-epicatechin
Procyanidin C2	Catechin-(4α → 8)-catechin-(4β → 8)-catechin
Procyanidin A1	Epicatechin-(4β → 8, 2β → O → 7)-catechin
Procyanidin A2	Epicatechin-(4β → 8, 2β → O → 7)-epicatechin
Procyanidin A4	Epicatechin-(4β → 8, 2β → O → 7)-ent-epicatechin

Figure 12. Catechins and procyanidins structures (adapted from [128,130])

1.2 Intake, bioavailability and metabolism

Proanthocyanidins are among the most abundant polyphenols in our diet. Several studies have reported their presence in berries and many fruits such apples, plums and cherries. Proanthocyanidins are found at high concentrations in some nuts, cereals and legumes, cacao and in several beverages such as wine, beer or fruit juices (reviewed in [131-133]) (**table 4**).

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Table 4. Proanthocyanidins content in different foodstuffs and beverages [132]

	Content[a]	*Analytical procedure*	*Proanthocyanidin type*[b]
Cereals and pulses (mg per 100 g)			
Lentil	316–1040 (dry weight)	Vanillin assay	PC, PD
	tr–2.4	HPLC	
Faba bean	nd–740	Vanillin assay	PC
Sorghum	nd–3900	Vanillin assay	PC
Barley	64–126	HPLC	PC, PD
Fruits and berries (mg per 100 g)			
Apple	17–50 (dry weight)	Vanillin assay	PC
	tr–15	HPLC	
Pear	0.7–12	HPLC	PC
Grape	1–160	HPLC	PC, PD, gall
Sweet cherry	10–23	HPLC	PC, PD
Blueberry	1–7	HPLC	PC, PD
Red raspberry	2–48	HPLC	PC, PD
Strawberry	2–50	HPLC	PC, PD, gall
Blackberry	9–11	HPLC	PC
Juices and drinks ($mg\,l^{-1}$)			
Apple juice	nd–298	HPLC	PC
Apple purée	16–43	HPLC	PC
Peach purée	9.5–24	HPLC	PC
Pear juice	11–74	HPLC	PC
Grape juice	3.5–46	HPLC	PC
Cider	2290–3710	Photometry at 280 nm	PC
Red wine	nd–500	HPLC	PC, PD
White wine	tr–7	HPLC	PC
Rosé wine	tr–43	HPLC	PC
Sherry wine	45–54	HPLC	PC
Wine vinegar	4–414	Vanillin assay	PC
Beer	3.5–19.5	DMACA assay	PC, PD
Others (mg per 100 g)			
Cacao bean	260–1200	Not given	PC

[a] Expressed as catechin equivalent. HPLC values only include oligomers (DP = 2–3).
[b] PC, procyanidins; PD, prodelphinidins; gall, galloylated derivatives.

Wang et al. estimated that the total proanthocyanidin intake in US population is 95 mg/day, with tea and legumes as the predominant food sources, followed by red wine. Tea is a major source of proanthocyanidin monomer and dimer intake, whereas legumes contribute to oligomeric and polymeric proanthocyanidin intake [134]. Moreover, studies in the Spanish population have reported elevated flavonoid consumption

(313.26 mg/day), with proanthocyanidins as the most significant contributor (60.1%). Apples and red wine were found as the major sources of proanthocyanidins [135].

The biological properties of polyphenols depend on their bioavailability and indirect evidence of their absorption through the intestinal barrier increase in the antioxidant capacity of the plasma after the consumption of polyphenol-rich foods. Confirmation of the presence of flavan-3-ol low-molecular weight metabolites in the urine and feces of rats, chickens and sheep, indicates that polymeric proanthocyanidins might not be absorbed through the intestinal barrier without being degraded into low-molecular weight metabolites (reviewed in [129,136]). The chemical structure of polyphenols determines their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma [137,138]. Moreover, the degree of polymerization and galloylation of flavan-3-ols affects their bioavailability [139].

Monomeric flavan-3-ols are absorbed in the small intestine, where they are extensively metabolized into glucuronide conjugates. The glucuronidation occurs within the luminal portion of the endoplasmic reticulum of the enterocytes via the superfamily of uridine 5'-diphosphate glucuronosyltransferases (UGTs). UGLT1 is predominantly responsible for flavonoid glucuronidation. Flavan-3-ols can also accumulate in the liver, where they are mainly sulfated and methylated through cytosol sulfotranferases (SULT) and catechol-O-methyltransferase (COMT) [139]. Next, conjugated flavanols go to the systemic circulation or can be returned to the intestinal lumen via bile (entero-hepatic circulation) [140].

The 90-95% of the total consumed polyphenols cannot be absorbed by the small intestine, so they pass into the colon, and together with the compounds returned by the entero-hepatic circulation, they are metabolized by the colonic microflora [141]. The colon contains diverse microbial populations of obligate anaerobes and facultative anaerobes, which degrade the non-digested food matrix and transform components into microbial metabolites [140]. First, the colonic microflora catalyzes the breakdown of the polymeric flavan-3-ols into monomers [136] and facilitates the formation of phenolic acids and phenylvalerolactones by opening the C-ring. Next, the phenylvalerolactones are slowly degraded into phenylvaleric acids, which are rapidly converted into 3-(3-hydroxyphenyl)propionic acid [142]. Finally, dehydroxylation reactions produce mono-hydroxylated and non-hydroxylated phenolic acids [139,143].

The microbial metabolites are absorbed by the colonocytes and arrive at the liver, where they are subjected to glucoronidation, methylation and sulphatation. Then, they go to the systemic circulation or travel to the kidneys, where they are excreted in urine [139] (**figure 13**). Moreover, some flavonoids have been found within the following

tissues: stomach, small intestine, colon, liver, pancreas, spleen, kidney, muscle, heart, endothelium, lung, brain, thyroid bone, skin, bladder, prostate, testes, vagina, uterus, ovary, mammary gland, fat, adrenal gland, esophagus, eyes, lymph nodes, and pituitary gland (reviewed in [127]).

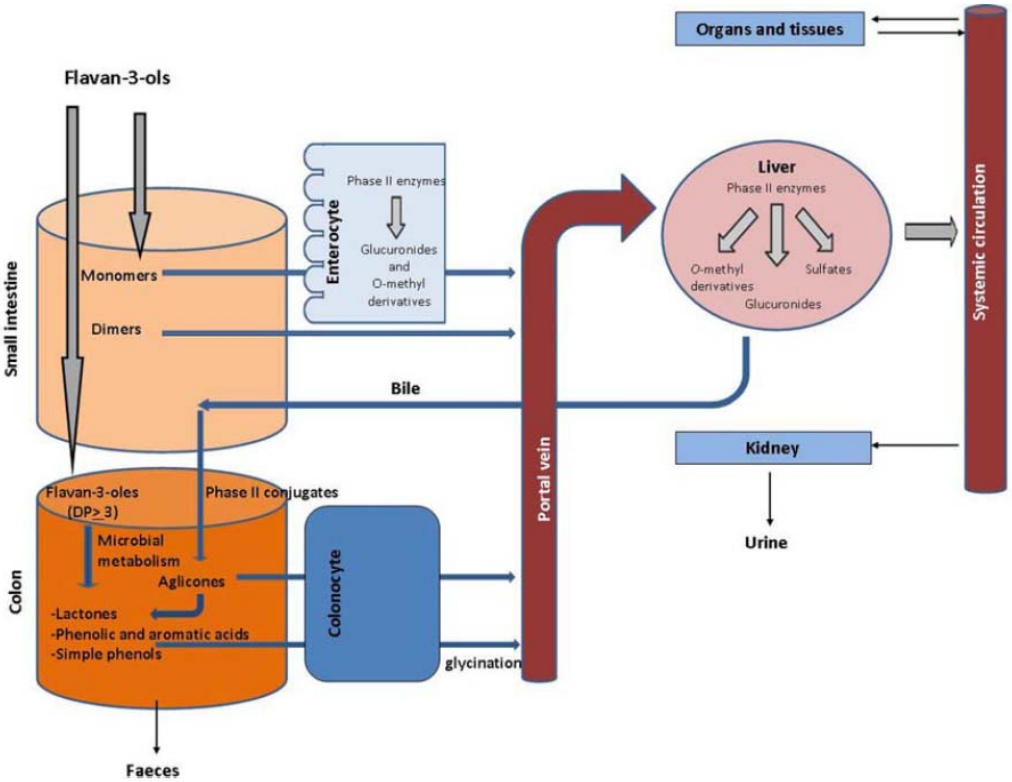

Figure 13. Schematic diagram of organs, reactions and agents involved in flavan-3-ols metabolism [139]

2. PROCYANIDINS ACTION ON GLUCOSE HOMEOSTASIS

The beneficial effects of procyanidins have been widely studied, and procyanidins were identified as potential cardioprotectants (reviewed in [144]). Procyanidins also act as hypolipidemic [145], antioxidant [146], antigenotoxic [147], antimicrobial [148], anti-inflammatory [149], and anti-cancer/anti-proliferative [150] agents. Moreover, procyanidins have been reported to improve glucose homeostasis, but the results from different glucose disrupted-homeostasis models are controversial (reviewed in [151]).

2.1 Effects on glucose homeostasis

Procyanidins have been suggested to act as antihyperglycemic agents, and several studies in healthy and glucose homeostasis-disrupted situations have reported different effects. These different effects might depend on the specific molecular origin of the glucose homeostasis disruption [151].

Diabetes mellitus type 1 is characterized by a progressive destruction of pancreatic β-cells, which produces an absolute insulin deficiency that leads to hyperglycemia [152]. This type of hyperglycemia can be reproduced in animals with streptozotocin (STZ), which induces the destruction of the pancreas. Several studies have reported that procyanidin treatment elicits antihyperglycemic effects in STZ rats, which might be attributed to its insulin-mimetic effect on insulin-sensitive tissues [153-156].

Hyperglycemia is also produced during insulin resistance conditions, which initially causes insulin hypersecretion (hyperinsulinemia) to compensate for the lack of effect and to normalize blood glucose levels. Next, the pancreas becomes dysfunctional, leading to diabetes mellitus type 2 [157], in which decreased beta cell mass also plays an important role. This type of glucose homeostasis-disruption can be achieved in animals by the induction of insulin resistance using different diets, such as high-fructose [158,159], high-fat (HF) [160] or cafeteria (high-fat, high-sucrose) diets [161], and by direct genetic disruption [162]. Pinent et al. reported in a review that procyanidins might ameliorate metabolic disruption but their effects are highly dependent on the amount of procyanidins, the vehicle used for administration and the different molecular origins of the metabolic disturbance [151].

During a non-pathological (normoinsulinemic) situation, Al-Awwadi et al. reported that procyanidins can reduce glycemia [153]. Conversely, several reports have found no glycemic changes in healthy animals, which suggest that procyanidins act as insulin-mimetic agents only during disrupted conditions [151,162,163].

Although the molecular mechanisms underlying the effects elicited by procyanidins remain unclear, several mechanisms have been proposed (reviewed in [151]). Indeed, procyanidins of different origins have been found to inhibit enzymes involved in intestinal carbohydrate digestion, such as α-amylase and α-glucosidase [164-166], and some monomeric flavan-3-ols have been shown to inhibit the glucose transporter SGLT-1 [167]. Procyanidins have been suggested to elicit insulin-mimetic effects on insulin-sensitive cell lines, such as adipocytes and muscle [154,168]. Moreover, a recent study on insulin-resistant rats showed that procyanidins might target the pancreas,

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modulating insulin production and secretion and lipid accumulation, thereby ameliorating insulin-resistance [169].

2.2 Effects on GLP-1

The key role of incretins on glucose homeostasis due to their insulinotropic action, suggests that they might represent potential therapeutic targets to improve glucose homeostasis. There are limited studies suggesting that procyanidins might modulate GLP-1. Berry purees, which are rich in proanthocyanidins, when administered simultaneously with sucrose in healthy humans, increased plasma levels of active GLP-1 [170]. Cinnamtannin A2, a tetramericprocyanidin, has also been reported to increase the plasma levels of active GLP-1 and insulin in fasted animals [171]. In regards to other flavonoids, resveratrol, which is present in red grapes, increases plasma levels of active GLP-1 and enhances GLP-1 production in the colon, which was linked to the improvement of glycemia in HF diet-fed rats [109].

Increased GLP-1 levels induced by bioactive compounds can be attributed to several mechanisms. They might be due to the direct stimulation of GLP-1 secretion in intestinal L-cells. Although the direct effect of procyanidins on GLP-1-secreting cells has not yet been reported, studies have reported that such an effect is elicited by other phenolic compounds. *In vitro* assays have shown that genistein and daidzen isoflavonoids, chlorogenic acid, and curcumin can increase GLP-1 secretion from enteroendocrine NCI-H716, STC-1 and GLUTag cell lines, respectively [172-174]. Moreover, the increased colonic GLP-1 production induced by resveratrol *in vivo* suggests a direct stimulus of this polyphenol on intestinal L-cells [109].

Another possible mechanism might be the inhibition of the incretin degrading enzyme DPP4. Indeed, the intraperitoneal administration of a flavonoid-rich fraction of *Pilea microphylla* was shown to modulate glucose homeostasis *in vivo* and to inhibit the activity of plasma DPP4 [155], suggesting the involvement of DPP4 inhibition on the antidiabetic effect of flavonoids. In addition, Tebib et al. also reported that grape-seed tannins are able to inhibit the activity of several intestinal enzymes including DPP4 [175]. In fact, procyanidins have been found to exhibit an affinity for salivary and plasma enzymes [176-178], and an epicatechin derivative found in *Vitis vinifera* has been predicted *in silico* to be a DPP4 inhibitor [179].

In conclusion, a few studies have shown that flavonoids might improve glycemia by increasing GLP-1 secretion and production or decreasing the DPP4 activity, the enzyme that cleaves GLP-1. However, the modulation of active GLP-1 levels by procyanidins as an underlying mechanism of their antihyperglycemic effects remains unstudied.

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II. HYPOTHESIS AND OBJECTIVES
HIPÒTESI I OBJECTIUS

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Previous studies performed in our research group (Nutrigenomics group) reported that procyanidins, which are the most abundant polyphenols in diet due to its presence in a wide range of food such as fruits and their derived drinks, can improve a situation of insulin resistance induced by a cafeteria diet. A decrease on the HOMA-IR index and fasting insulinemia has been found in animals treated with GSPE (grape-seed procyanidin extract), which was linked to the procyanidins insulin-like effect in adipose tissue. These results also suggested an action on pancreas functionality, which was subsequently confirmed, evidencing that procyanidins modulate insulin production and secretion, as well as β-cell proliferation.

Since the mechanisms acting on endocrine cells are very similar between the different types of hormone-producing cells, enteroendocrine cells have been suggested to be a target for procyanidins, specifically, secretion and/or production of GLP-1, which is an incretin that increases insulin production and improves insulin sensitivity in peripheral tissues. These effects highlight the relevance of targeting this incretin as a potential treatment for glucose metabolism disrupted situations. However, procyanidin effects on GLP-1 secretion, production and/or cleavage have not been reported yet. Another relevant fact is that GLP-1 is rapidly degraded by the enzyme DPP4, a serinexopeptidase that is highly susceptible to inhibition by procyanidins due to their high affinity to prolin-rich sequences.

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

The glucose homeostasis improvement caused by GSPE could be, in part, explained by its interaction at intestinal level modulating active GLP-1 levels.

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

1. To prove that GSPE modulates active GLP-1 levels *in vivo*.
2. To evaluate whether GSPE modulate active GLP-1 levels through affecting its cleavage.
3. To study whether GSPE modulates GLP-1 secretion from enteroendocrine cells and the mechanisms involved.
4. To evaluate whether GSPE affects GLP-1 secretion and production in a preventive manner in a model of insulin resistance induced by a cafeteria diet.

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II. Hypothesis and objectives

The work presented in this doctoral thesis was performed in the Nutrigenomics group at the Universitat Rovira i Virgili, together with a 4 month stay at the Institute of Global Food Security (before, Institute of Agri-Food and Land Use) at the Queen's University of Belfast (Northern Ireland, UK). The funding of this doctoral thesis came from a FPI fellowship from the Ministerio de Ciencia e Innovación of the Spanish Government.

En estudis previs realitzats al nostre grup de recerca (grup de Nutrigenòmica) es va demostrar que les procianidines, que són els polifenols més abundants a la dieta degut a la seva presència en gran diversitat d'aliments com són fruites i begudes derivades, poden millorar un estat de resistència a la insulina induït per una dieta de cafeteria. En els animals tractats amb GSPE (extracte de procianidines del pinyol de raïm) es va trobar una disminució de l'índex HOMA-IR i de la insulinèmia en condicions de dejuni, que es van atribuir als efectes insulino-mimètics de les procianidines al teixit adipós. Aquests resultats també suggerien una afectació del GSPE a la funcionalitat del pàncrees, que posteriorment van ser ratificats, evidenciant que les procianidines modulen la producció i la secreció d' insulina, així com la proliferació de les cèl·lules β.

Donat que els mecanismes que actuen sobre les cèl·lules endocrines són força comuns entre els diferents tipus de cèl·lules productores d'hormones, les cèl·lules enteroendocrines es van plantejar com una possible diana de les procianidines, en concret, la secreció i/o producció de GLP-1, incretina que incrementa la producció de insulina i millora la sensibilitat a la insulina en els teixits perifèrics. Aquestes accions, incrementen la rellevància de aquesta incretina com a potencial via de tractament per a situacions de desregulació del metabolisme de la glucosa. No obstant, el efecte de les procianidines a nivell de secreció, producció i/o degradació de GLP-1 encara no ha estat descrit. Un altre fet rellevant és la ràpida eliminació de GLP-1 que té lloc degut a l'activitat de l'enzim DPP4, una serinexopeptidasa altament susceptible a la inhibició per part de les procianidines degut a la seva elevada afinitat per seqüències riques en prolina.

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

La millora de la homeòstasi de la glucosa que exerceix GSPE es podria explicar, en part, per la seva interacció a nivell intestinal modulant els nivells de GLP-1 activa

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

1. Demostrar que GSPE modifica els nivells de GLP-1 activa *in vivo.*
2. Avaluar si GSPE modula els nivells de GLP-1 activa degut a efectes sobre la seva degradació.
3. Estudiar si GSPE modula la secreció de GLP-1 per part de las cèl·lules enteroendocrines i per quins mecanismes.

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4. Avaluar si GSPE té efectes en la secreció i producció de GLP-1 de manera preventiva en un model de resistència a la insulina induït per una dieta de cafeteria.

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 4 mesos al *Institute of Global Food Security* (abans, *Institute of Agri-Food and Land Use*) a la Queen's University of Belfast (Irlanda del Norte, UK). El finançament de la tesi doctoral va provenir de una beca FPI del Ministeri de Ciència i Innovació del Govern de Espanya.

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III. RESULTS

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CHAPTER 1

Grape seed-derived procyanidins inhibit intestinal DPP4 activity and increases plasma glucagon-like peptide-1

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To achieve the first objective of this thesis, that was to evaluate whether GSPE modulates active GLP-1 levels *in vivo,* healthy rats were orally administered with a high dose of GSPE for 1 hour, simultaneously to an oral challenge of glucose, the main stimulus for GLP-1 secretion [1], and GLP-1 levels were assessed.

Since one of the described mechanisms to improve active GLP-1 levels is the inhibition of its degrading enzyme, DPP4 [2,3], our second objective was to evaluate whether the modulation of active GLP-1 levels produced by GSPE is due to an inhibition of DPP4. Thus, we assayed its activity in plasma and intestine

The effect of GSPE on intestinal DPP4 was also studied *in vitro* to determine if it occurs in the inner intestine, such as in the epithelium of capillaries next to the GLP-1-producing cells, and to define which molecules are the responsible of such effect. Therefore, we developed an indirect co-culture system to mimic the intestinal environment.

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Grape seed-derived procyanidins inhibit intestinal dipeptidyl-peptidase 4 activity and increases plasma glucagon-like peptide-1

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Abstract

We previously reported that a grape-seed procyanidin extract (GSPE) improves glycemia and inhibits dipeptidyl-peptidase 4 (DPP4) activity. Because DPP4 regulates active incretin levels, in the present study we evaluated whether GSPE modulates active glucagon-like peptide-1 (GLP-1) levels and attempted to determine the mechanisms involved. An acute GSPE treatment in healthy rats increased plasma active GLP-1 after an oral glucose load, simultaneous with an increase in plasma insulin and a decrease on glucose levels and intestinal DPP4 activity. We also found that the compounds absorbed by intestinal CaCo-2 cells after an acute treatment with GSPE inhibit DPP4 activity in endothelial HUVEC cells. These compounds were identified and catechin, epicatechin, B2 dimer, and gallic acid were found to directly inhibit endothelial DPP4 in an additive way. In conclusion, GSPE improves glycemia through inhibiting inner intestinal DPP4 activity, leading to an increase on GLP-1 levels, which, in turn, may affect insulin release.

Keywords: GSPE/DPP4/GLP-1/glycemia/insulin

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Introduction

The high prevalence of diabetes mellitus type 2 highlights the need to identify effective therapies [1], including the use of natural products that could help to avoid the development of this pathology. One approach could be to improve the ability of some foods to increase the life time of glucagon-like peptide-1 (GLP-1), an incretin hormone produced by the L-cells in the distal ileum and colon that is secreted after oral consumption of nutrients, mainly glucose. This hormone has a wide range of biological activities, including increased insulin secretion and biosynthesis, inhibition of glucagon secretion, enhancement of β cell mass proliferation and neogenesis, reduction in food intake, and enhancement of satiety (reviewed in [2] and [3]).

Certain natural compounds have been reported to promote incretin secretion. Chlorogenic acid, a major phenolic compound in coffee, was shown to increase GLP-1 production and secretion by L-cells and improve glycemia in mice after oral glucose [4,5]. Similarly, an *Ilex paraguariensis* leaf extract, which is rich in the phenolic compound 3,5-*O*-dicaffeoyl-*D*-quinic acid, was shown to improve glycemia and insulinemia in mice fed a high-fat diet (HFD), a result that could be related to higher active plasma GLP-1 levels [6,7]. Berberine, a major active constituent of *Rhizoma coptidis*, was also reported to improve glycemia, insulinemia and GLP-1 levels after oral glucose challenge [8]. Resveratrol, a polyphenolic compound presented in such plant as red grapes, exhibits an anti-hyperglycemic effect, which was been attributed to increases in GLP-1 levels [9]. Berries, a rich source of polyphenols, have also been shown to enhance GLP-1 levels and improve insulin concentrations after sucrose consumption [10]. Altogether, it appears that some phenolic compounds have the ability to enhance glucose-stimulated GLP-1, though the mechanisms remain unclear.

The GLP-1 half-life is less than two minutes due to its rapid cleavage by the enzyme dipeptidyl-peptidase 4 (DPP4) [11]. In fact, synthetic DPP4 inhibitors are currently used for the treatment of type 2 diabetes mellitus because of their capacity to improve glycemic control by avoiding rapid incretin cleavage [12,13]. We have previously reported that a grape seed procyanidin extract inhibits intestinal DPP4 activity and increases the plasma insulin/glucose ratio in rats in response to orally administered glucose, suggesting an incretin effect [14], which could help to explain the antihyperglycemic effect of procyanidins [15-17].

Therefore, the aim of the present study was to prove that grape seed procyanidins modulate glucose-stimulated incretin level, and to elucidate the mechanism by which the effect is produced.

Research Design and Methods

Reagents

The grape seed procyanidin extract (GSPE) was obtained from *Les Dérivés Résiniques et Terpéniques* (Dax, France) and was previously fully characterized in our research group [18]. Briefly, the main phenolic compounds present in GSPE are monomers (catechin and epicatechin) and dimers.

Cell culture reagents were obtained from BioWhittaker (Veviers, Belgium) and EmbryoMax 0.1% Gelatin Solution was purchased from Millipore (Madrid, Spain).

For the HPLC-MS/MS analysis, pure molecules ((+)-catechin, (-)-epicatechin, gallic acid epigallocatechin gallate, and procyanidin B2) and the internal standard (IS) pyrochatecol were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Acetone (HPLC analytical grade), methanol (HPLC analytical grade) and phosphoric acid were purchased from Sigma-Aldrich (Madrid, Spain). Ultrapure water was obtained using a Milli-Q Advantage A10 system from Millipore (Madrid, Spain), and glacial acetic acid was purchased from Panreac (Barcelona, Spain).

Animal experimental procedures

Female Wistar rats were purchased from Charles River Laboratories (Barcelona, Spain), housed in animal quarters at 22ºC with a 12-h light/12-h dark cycle and maintained for 1 week in quarantine.

Food was withdrawn at 8 p.m. on the day before sacrifice. At 8 a.m. on the sacrifice day, the rats were divided into three groups (6 animals/group): (i) control group, rats treated with the vehicle (tap water), (ii) GSPE group, rats treated with 1 g of GSPE/kg of body weight (bw); and (iii) positive control group, rats treated with 1 mg of vildagliptin/kg of bw (Axon Medchem, Groningen, The Netherlands). The treatment was administered by oral gavage. Tail blood samples were collected before treatment administration. After 40 minutes of GSPE/vildagliptin treatment, an oral glucose load (2 g of glucose/kg of bw) was administered; 20 minutes later, the animals were anesthetized with 50 mg of pentobarbital/kg of bw and sacrificed by bleeding. A schematic diagram of the animal experiment is shown in **Scheme 1**. The blood was collected and treated with commercial DPP4 inhibitor (Millipore, Madrid, Spain) and serine protease inhibitor, Pefabloc SC, (Roche, Barcelona, Spain) to avoid active GLP-1 and ghrelin inactivation, respectively. The animal tissues were immediately frozen in liquid nitrogen and stored at -80ºC until analysis.

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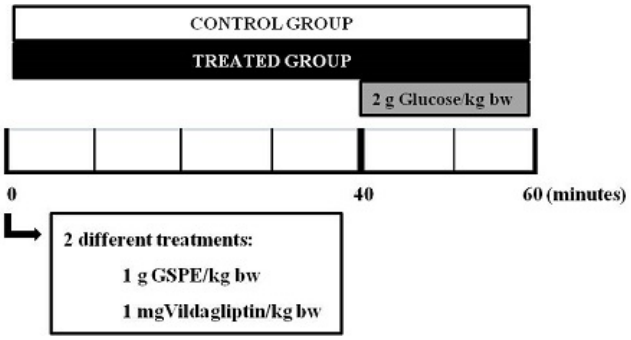
All the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (permission number: 4250).



Scheme 1. Schematic diagram of the animal experiment

Plasma parameter measurements

The glucose plasma concentrations were assayed using an enzymatic colorimetric kit (GOD-PAP method from QCA, Tarragona, Spain). The insulin and GLP-1 plasma concentrations were determined using Rat Insulin ELISA/Ultrasensitive Rat Insulin ELISA (Mercodia, Uppsala, Sweden) and GLP-1 (active) ELISA (Millipore, Madrid, Spain) kits, respectively, following the manufacturer's instructions.

Cell lines and indirect co-culture

Caco-2 cells were obtained from American Tissue Culture Collection (ATCC) (LGC Standards S.L.U., Barcelona, Spain) and cultured in DMEM supplemented as previously described [19]. HUVEC cells were obtained from Cascade Biologics^{TM} (Carlsbad, CA, USA), cultured in 0.1% gelatin-coated flasks and maintained in Endothelial Growth Medium-2 (EGM-2).

Caco-2 cells were seeded onto Millicell hanging cell culture inserts (Millipore) for 6-well plates at a cell density of $1.3*10^4$ cells/cm^2. The volume of the culture medium was 1 mL on the apical side and 2 mL on the basolateral side. The trans-epithelium electrical resistance (TEER) of the Caco-2 cells was monitored daily using the Millicell-ERS System (Millipore); when a confluent monolayer was formed (21 days of culture), the cells were treated with 250 mg GSPE/L. The basolateral media were collected after 1 hour of GSPE treatment and stored at -20ºC.

HUVEC cells were seeded in 6-well plates coated with gelatin 0.1% at a cell density of 8-10.5 x 10^4 cells/cm^2. When cells reached the 80-90% of confluence, they were treated with the collected basolateral media for 1 hour or with 25 mg of GSPE/L for 2 hours.

Measurement of DPP4 activity

DPP4 was extracted from rat intestine and HUVEC cells as previously described [14] and [20] respectively). Briefly, the intestines were homogenized with lysis buffer (PBS containing 100 KIU/mL aprotinin and 1% Triton X-100), centrifuged at 1000 x g at 4ºC for 10 minutes to eliminate the cellular debris, and centrifuged twice at 20000 x g at 4ºC for 10 minutes. The HUVEC cells were washed twice with 1X PBS, harvested in 100 mM Tris-HCl, and centrifuged. The supernatant was collected; the pellet was dissolved in the same buffer supplemented with 2% Triton X-100 and, after centrifugation, the supernatant was added to the first supernatant and stored at -80º C until analysis.

The DPP4 activity in the cell and intestinal lysates and rat plasma, was measured following the manufacturer's instructions provided by the *DPP4 Drug Discovery Kit-AK499* (Enzo Life Sciences International, Inc.), with a few modifications based on the volume of the DPP4-containing sample, adjusting the final volume with Tris-HCl buffer. The protein level in all samples was determined as previously reported [21] and used to normalize the DPP4 activity values.

In vitro *direct inhibition of DPP4 activity by pure compounds*

DPP4 was isolated from non-treated HUVEC cells as described above and incubated with 100 µg/L of catechin, epicatechin, procyanidin B2 or gallic acid, either alone or together, for 1 hour. Then, DPP4 activity was determined as described above.

DPP4 gene expression

The total RNA from HUVEC cells was extracted using RNAeasy Kit (Qiagen, Hilden, Germany) and the total RNA from intestine was extracted using the TRIzol reagent following the manufacturer's instructions. cDNA was generated using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). Quantitative PCR amplification and detection for DPP4 was performed using the following TaqMan assay-on-demand probes (Applied Biosystems, Madrid, Spain): Rn00562910_m1 and Hs00175210_m1 for DPP4 for rat and HUVEC cells, respectively. The results were referenced to β-actin Rn00667869_m1 for rat and cyclophilin Hs99999904_m1 (Ppia) for HUVEC cells. The relative mRNA expression levels were calculated using the ΔΔCt method.

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Chromatographic analysis of procyanidins and their metabolites

Standard preparation: A 200 mg/L stock standard mixture in methanol of (+)-catechin, (-)-epicatechin, gallic acid and epigallocatechin gallate and 100 mg/L of procyanidin B2 were prepared weekly and stored in a dark flask at -20ºC. This stock standard solution was diluted daily to the desired concentration using an acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution and stored under the same conditions until the chromatographic analysis.

Micro-solid phase extraction: Prior to the chromatographic analysis, cell medium samples were pre-treated by off-line µ-SPE using OASIS® HLB µ-Elution Plates 30 µm (Waters, Barcelona, Spain) following the methodology for biological samples as previously described [22],

Instrument conditions: The eluted solution was directly analyzed using a 1200 LC Series coupled to a 6410 QqQ-MS/MS (Agilent Technologies, Palo Alto, U.S.A.). The chromatographic method used for both the apical and basolateral media was performed using a Zorbax C18 column (100 mm x 2.1 mm i.d., 1.8 µm particle size, Agilent Technologies). The procyanidins and their metabolites were analyzed with ESI as the ionization technique and in negative mode, as previously was described [23].

Data analyses

The results are expressed as the mean ± SEM. The effects were assessed by ANOVA or Student's t-test. All calculations were performed using SPSS software (SPSS, Chicago, USA).

Results

GSPE enhances glucose-induced incretin release

To prove that GSPE modulates incretin levels, active GLP-1 levels in plasma were analyzed after an oral glucose load (2 g of glucose/kg of bw). As shown in **figure 1a,** the active GLP-1 levels are higher in rats treated with GSPE for 1 hour than in the control group.

The increase in GLP-1 levels was accompanied by a reduction in plasma glucose levels in the rats treated with GSPE compared to the controls (**figure 1b**). As shown in **figure 1c,**

there was also a higher insulin/glucose ratio in the GSPE-treated rats compared to the control group.

The GSPE effects greatly resemble those obtained for the positive control group treated with vildagliptin, a well-described DPP4 inhibitor (**figure 1**).

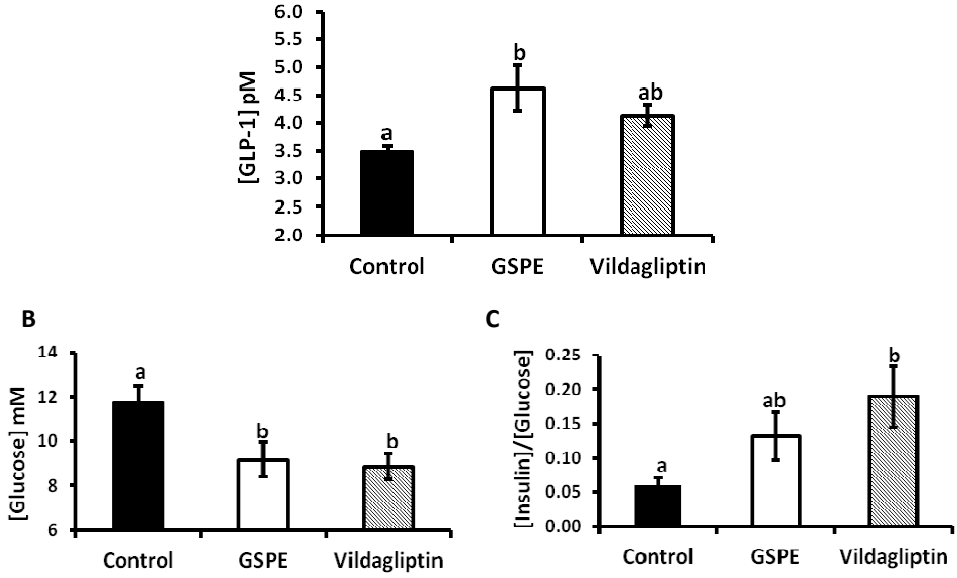

Figure 1. GSPE effect on plasma active GLP-1, glucose and insulin levels. An oral load of 2 g glucose/kg of bw was performed in rats treated with 1 g GSPE/kg of bw or 1 mg vildagliptin/kg of bw. Effect on the plasma active GLP-1 levels (**A**), glucose levels (**B**), and insulin concentration per plasma glucose (**C**). The data are displayed as the mean ± *SEM*. **a** and **b**, statistically significant differences at $P < 0.05$.

GSPE inhibits intestinal DPP4 activity

To analyze whether an acute dose of procyanidins exert the incretin effect through its previously described effects as a DPP4 inhibitor, we assayed the DPP4 activity in plasma and intestine. The DPP4 plasma activity was not modified after 1 hour of GSPE treatment (**figure 2a**), whereas GSPE decreased the intestinal DPP4 activity by approximately 28%, as shown in **figure 2b**.

Vildagliptin was found to inhibit plasma DPP4 activity, causing up to 29% inhibition; furthermore, vildagliptin also reduced the intestinal DPP4 activity by approximately 37%, which is a newly described effect.

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Both the GSPE and vildagliptin treatments significantly up-regulated intestinal DPP4 gene expression (**figure 2c**).

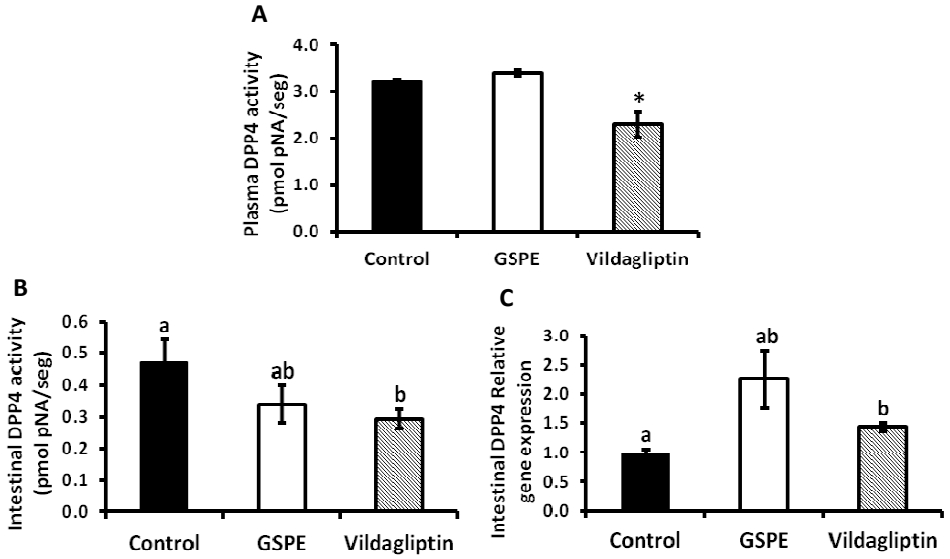

Figure 2. Effect of GSPE on DPP4. Effect on plasma DPP4 activity (**A**), and intestinal DPP4 activity (**B**) and gene expression (**C**) in rats treated with 1 g GSPE/kg of bw for or 1 mg vildagliptin/kg of bw for 1 hour. The data are displayed as the mean ± *SEM*. *significant differences *vs.* Control group at $P < 0.05$.

GSPE inhibits endothelial DPP4

Because *in vivo* measurements are obtained using whole intestine, we used an *in vitro* approach to show that GSPE inhibits the DPP4 located in the capillaries, as GLP-1 is secreted into intestinal capillaries. First, HUVEC cells were directly treated with 25 mg of GSPE/L, a non-toxic dose, and an inhibition on DPP4 activity of 39% ± *4* ($P = 0.026$) was observed. Nevertheless, GSPE is absorbed and metabolized by the intestine before reaching the endothelium of the capillaries, thus indirect co-culture to mimic the intestinal barrier were also performed. To this end, HUVEC cells were cultured for 1 hour with the basolateral medium of CaCo-2 cells, grown in Millicell hanging cell culture inserts, and treated on the apical side with 250 mg of GSPE/L for 1 hour. As shown in **figure 3**, the GSPE compounds absorbed by the CaCo-2 cells inhibited the DPP4 activity in the HUVEC cells by approximately 32% (**figure 3a**). No significant changes were observed in DPP4 gene expression (**figure 3b**).

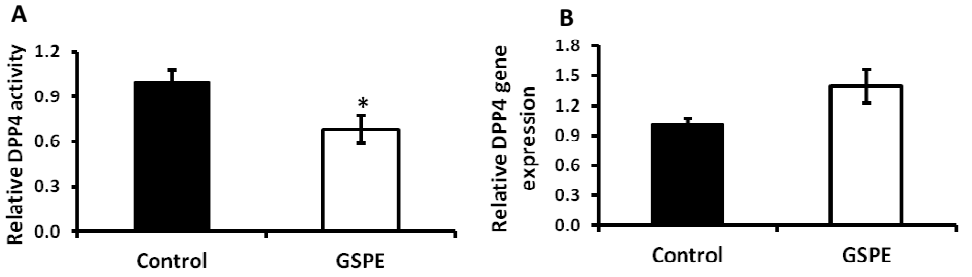

Figure 3. Effect of GSPE absorbed molecules and/or metabolites on endothelial DPP4. Effect on DPP4 activity (**A**) and expression (**B**) in HUVEC cells cultured with the basolateral medium of Caco2 cells treated, apically, with 250 mg GSPE/L for 1 hour (n = 4). The data are displayed as the mean ± *SEM*. *significant differences at $P < 0.05$.

Pure molecules found in the GSPE directly inhibit DPP4 activity

To identify which molecule(s) might be responsible for the observed inhibition, a chromatographic analysis by HPLC-MS/MS of the apical and basolateral medium of GSPE-treated Caco-2 cells was performed. As shown in **table 1**, the basolateral medium contained catechin, epicatechin, gallic acid, and procyanidin dimer. Sulfate forms of catechin and epicatechin were also found. Other forms present in GSPE (shown in **table 1** of Supplemental Data), such as epicatechin gallate and procyanidin trimer, and other products of intestinal absorption, such as glucoronide forms, were also detected in the basolateral medium but their levels were too low to be quantified.

To assess whether DPP4 is directly inhibited by the absorbed molecules found in the basolateral medium, the *in vitro* DPP4 activity was assayed in the presence of the molecules. DPP4 isolated from HUVEC cells was incubated for 1 hour with 100 µg/L of each pure molecule, a concentration similar to that found in the basolateral medium. Catechin, procyanidin B2, and gallic acid significantly inhibited the DPP4 activity, showing between 4 and 8% inhibition (**figure 4**); epicatechin also showed a slight (approximately 3%) but not statistically significant inhibition. The sum of the inhibitions obtained by the four compounds was 21.19% ± 5.74. DPP4 was also incubated with a mixture of 100 µg/L of each pure molecule, and approximately a 26% inhibition was found, as shown in **figure 4**. The sum of each separate inhibition was not significantly different from the inhibition achieved by the mixture, and it was also not different than the inhibition produced by the basolateral medium on the indirect co-cultures ($P > 0.05$).

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Table 1. Flavanols and their metabolites in basolateral media of CaCo2 inserts treated with 250 mg of GSPE/L.

Compound	**Total amount (µM)**	**Total amount (µg/L)**
Catechin	0.21±0.06	60.09±16.54
Epicatechin	0.28±0.06	79.86±18.39
Procyanidin Dimer [(3)]	0.12±0.03	67.05±15.26
Gallic acid	0.29±0.02	49.68±2.70
Epicatechin gallate[(4)]	n.q.	n.q.
Dimer gallate [(3)]	n.q.	n.q.
Trimer[(3)]	n.q.	n.q.
EGCG	n.q.	n.q.
Procyanidin dimer gallate[(3)]	n.q.	n.q.
Metabolite		
Catchin-glucuronide[(1)]	n.q.	n.q.
Epicatechin-glucuronide[(2)]	n.q.	n.q.
Methyl-catechin-glucuronide[(1)]	n.q.	n.q.
Methyl-epicatechin-glucuronide[(2)]	n.q.	n.q.
Catechin-sulfate[(1)]	0.12±0.01	44.38±3.77
Epicatechin-sulfate[(2)]	0.16±0.01	58.87±4.63
3-o-methyl-epicatechin[(2)]	n.q.	n.q.
4-o-methyl-epicatechin[(2)]	n.q.	n.q.
Methyl-catechin-o-sulfate[(1)]	n.q.	n.q.
Methyl-epicatechin-o-sulfate[(2)]	0.21±0.02	78.48±8.11
Epicatechin gallate glucuronide[(2)]	n.d.	n.d.

n.d.= not detected. n.q.= not quantified. EGCG= Epigallocatechin Gallate

[1] Quantified as Catechin

[2] Quantified as Epicatechin

[3] Quantified as Dimmer B2

[4] Quantified as EGCG

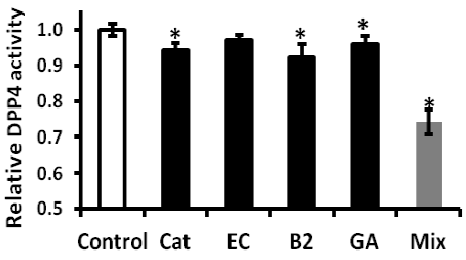

Figure 4. ***In vitro*** **DPP4 inhibition by pure molecules.** Effect of 100 µg/L catechin (Cat), epicatechin (EC), procyanidin B2 (B2), or gallic acid (GA) and a mixture of 100 µg/L each (Mix) on DPP4 activity extracted from HUVEC cells (n = 4). The data are displayed as the relative DPP4 activity ± *SEM*. *significant differences *vs*. control group at P < 0.05.

Discussion

Although the antihyperglycemic effect of procyanidins has been previously reported [24] and their incretin-like effect has been suggested [14], their actual effect on active GLP-1 levels had not been yet described. The current study shows that an extract of procyanidins from grape seed increases the glucose-induced active GLP-1 levels in plasma. This effect could be explained, at least in part, due to the inhibition provoked by the absorbed polyphenols on DPP4 activity.

A few studies have previously reported the effect of polyphenols on incretin levels [8-10]. Resveratrol, a polyphenolic compound present in red grapes, was shown to increase GLP-1 plasma levels in HFD-induced diabetic mice, which was linked to an amelioration of the glucose profile and an increase in insulin in response to OGTT [9]. The authors show that these effects were dependent on the GLP-1 receptor, but the exact mechanisms responsible for such effects were not further elucidated. In our case, we also found an amelioration of the insulin/glucose plasma profile as a possible consequence of increased active GLP-1. Although our current experiment is an acute treatment, GSPE has been shown to ameliorate the HOMA-IR index in cafeteria-fed rats [15,25]; thus, the chronic effects on incretin levels deserve further study.

With regard to the mechanism leading to GLP-1 increase, we had previously shown that GSPE inhibits DPP4 [14]. Indeed, inhibition of the DPP4 enzyme is an emerging strategy to improve glycemia, and several DPP4 inhibitors are currently used in the pharmacological treatment of type 2 diabetes [26,27]. Previous [14] and current experiments report no in vivo effects of GSPE on plasma DPP4 activity. However, we did find an inhibition of intestinal DPP4 activity due to an acute (1 hour) GSPE treatment concomitant with an oral glucose load. These results are in agreement with previous results that also show reduced intestinal DPP4 activity due to acute and chronic treatments in healthy and cafeteria-fed rats [14]. The positive control vildagliptin, a commercial DPP4 inhibitor, significantly inhibited the plasma DPP4 activity, as previously published [28]. Commercial DPP4 inhibitors, such as vildagliptin, have been commonly reported to exert their incretin-like effect via the inhibition of plasma DPP4 activity, thereby delaying the incretin cleavage in plasma (reviewed in [29]). However, it was also shown in healthy humans that the increase in plasma GLP-1 levels is not proportional to plasma DPP4 inhibition, suggesting that routes other than plasma DPP4 inhibition are involved [30]. Moreover, Hansen et al. [31] reported that half of the newly secreted GLP-1 is N-terminally degraded prior to reaching systemic circulation, suggesting cleavage soon after its secretion by the DPP4 present in the endothelium of the capillaries adjacent to the GLP-1-secreting cells. Moreover, a recent study reported that low active doses of siltagliptin, another DPP4 inhibitor, cannot exert the same effect on

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glucose homeostasis when administered intravenously versus when orally administered. It was also shown that the oral administration of siltagliptin selectively inhibits intestinal DPP4 activity [32]. The present study shows that vildagliptin can also inhibit intestinal DPP4 activity, an effect that has not been reported to date. All these findings support the relevance of the DPP4 enzyme located in tissues close to site of the incretin production. Thus, we hypothesize that the inhibition of inner intestinal DPP4 by the absorbed/metabolized forms of GSPE could be, at least in part, responsible for the increased GLP-1 plasma levels.

In our experiments, DPP4 activity was determined using lysates of whole intestine; thus, the GSPE-reduced DPP4 activity could also be due to a direct inhibition of the enzyme present on the brush border membrane of the absorptive cells. Therefore, we used an *in vitro* approach to test our hypothesis: we analyzed whether the molecules contained in GSPE and/or their metabolites absorbed by intestinal cells (Caco-2 cells cultured on hanging inserts to simulate the intestinal barrier) are able to decrease the activity of the DPP4 present in the endothelium of the capillaries (HUVEC cells). To this end, we used indirect co-cultures due to the low viability of HUVEC cells cultured directly under the Caco-2 monolayer (results not shown). Our results show that the collected basolateral medium of the Caco-2 cells treated with GSPE significantly decreased the activity of endothelial DPP4. This result supports the hypothesis that GSPE might act on the DPP4 present in the endothelium of the capillaries, potentially leading to increases in active GLP-1.

We previously showed that GSPE can directly inhibit DPP4 activity but also modulate DPP4 gene expression [14]. Because we did not find reduced DPP4 gene expression either *in vivo* or *in vitro*, we hypothesize that the decrease in DPP4 activity could be due to a direct effect of the GSPE molecules and/or their metabolites. However, there is little information regarding the modulation of DPP4 activity by phenolic compounds. In agreement with our results, Tebib et al. also showed inhibition of DPP4 by grape seed procyanidins [33]; quercetin and a flavonoid-rich extract from *Pilea microphylla* have also previously been shown to inhibit DPP4 [34]. However, other flavonoids, such as curcumin [35] and flavone-8-acetic acid [36], do not inhibit DPP4 activity, showing that the effect on enzyme modulation depends on the type of phenolic compound. As GSPE is a mixture of phenolic compounds, the identification of the exact molecules of the extract that are responsible for the inhibition of DPP4 is important to better understand the mechanisms that might occur *in vivo*. Accordingly, the molecules absorbed by the Caco-2 monolayer were identified by a chromatographic analysis of the basolateral medium, and the major pure molecules found (catechin, epicatechin, procyanidin dimer and gallic acid) were used for *in vitro* experiments. A low but significant decrease in DPP4 activity was found after incubation of the enzyme with catechin, procyanidin B2,

or gallic acid, and a higher decrease was found when all the molecules were tested together at concentrations similar to these found in the basolateral medium. The inhibition observed was not significantly different from the sum of the inhibitions produced by each pure molecule alone, which suggests an additive effect of the compounds absorbed. In addition, no significant differences were found between the inhibition achieved by the mixture of molecules and the inhibition produced by the basolateral medium, proving that the GSPE compounds absorbed by the intestine can directly inhibit endothelial DPP4. Moreover, DPP4 was significantly inhibited when GSPE was directly added to the HUVEC cells which indicates that the major inhibition role is not due to the metabolites found in the basolateral medium but to the original compounds. Therefore, we describe for the first time the inhibition of DPP4 by pure compounds: catechin, gallic acid, and B2 dimer, molecules that can be absorbed in the intestine [37].

In conclusion, this study indicates that an acute treatment with GSPE is able to enhance insulin release after oral glucose consumption, a result that could be explained, in part, by increased active GLP-1 levels in plasma. Our results suggest that the inhibition of the intestinal DPP4 located in the endothelium of the capillaries could delay GLP-1 inactivation soon after secretion, explaining the higher levels on plasma active GLP-1. We also characterized the compounds responsible for DPP4 inhibition. These findings help us to understand one of the mechanisms by which GSPE exerts its antihyperglycemic effect.

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The authors declare that they have no conflict of interest.

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Supplemental Data (Online Appendix)

Table 1. Initial composition of apical cell culture media with 250 mg of GSPE/L.

Compound	Total amount (µM)	Total amount (mg/g)
Catechin	60.47	70.21
Epicatechin	62.53	72.61
Procyanidin Dimer [3]	32.93	76.20
Gallic acid	5.71	3.88
Epicatechin gallate[4]	5.49	9.71
Epigallocatechin[4]	n.q.	n.q.
Procyanidin Trimer[3]	3.24	11.22
Procyanidin Tetramer[3]	n.q.	n.q.
Procyanidin Pentamer[3]	n.d.	n.d.
EGCG	n.q.	n.q.
Procyanidin dimer gallate[3]	n.q.	n.q.

n.d.= not detected. n.q.= not quantified. EGCG = Epigallocatechin Gallate

[1] Quantified as Catechin

[2] Quantified as Epicatechin

[3] Quantified as Dimer B2

[4] Quantified as EGCG

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CHAPTER 2

Grape seed-derived procyanidins decrease dipeptidyl-peptidase 4 activity and gene expression

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To further understand the mechanisms used by GSPE to exert its inhibitory effect on DPP4 activity, and complete the second objective of this thesis, several *in vitro* and *in vivo* approaches were used.

To assess whether GSPE inhibits DPP4 activity in a direct way, *in vitro* direct inhibition assays where performed using DPP4 from different sources. Moreover, intestinal absorptive CaCo-2 cells, which significantly express DPP4 after differentiation [1], were treated with GSPE to evaluate whether procyanidins inhibit DPP4 activity by modulating its gene expression.

Because previous experience in our group showed different effects of procyanidins depending on the dose and the animal model [2], DPP4 activity and gene expression were also determined in different situations *in vivo*, including animals with normal-weight and obesity (induced by diet or by genetic factors).

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Grape Seed-Derived Procyanidins Decrease Dipeptidyl-peptidase 4 Activity and Expression

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Supporting Information

ABSTRACT: Dipeptidyl-peptidase 4 (DPP4) inhibitors are among the newest treatments against type 2 diabetes. Since some flavonoids modulate DPP4 activity, we evaluated whether grape seed-derived procyanidins (GSPEs), which are antihyperglycemic, modulate DPP4 activity and/or expression. In vitro inhibition assays showed that GSPEs inhibit pure DPP4. Chronic GSPE treatments in intestinal human cells (Caco-2) showed a decrease of DPP4 activity and gene expression. GSPE was also assayed in vivo. Intestinal but not plasmatic DPP4 activity and gene expression were decreased by GSPE in healthy and diet-induced obese animals. Healthy rats also showed glycemia improvement after oral glucose consumption but not after an intraperitoneal glucose challenge. In genetically obese rats, only DPP4 gene expression was down-regulated. Thus, procyanidin inhibition of intestinal DPP4 activity, either directly and/or via gene expression down-regulation, could be responsible for some of their effects in glucose homeostasis.

KEYWORDS: *diabetes, DPP4 inhibitors, intestinal DPP4, procyanidins*

INTRODUCTION

The high prevalence in developed societies of obesity and the related-associated pathologies, such as insulin resistance and diabetes, highlights the relevance of finding bioactive food components capable of ameliorating these situations. Dipeptidyl-peptidase IV (DPP4) inhibitors are among the newest treatments against type 2 diabetes.[1] DPP4 is a prolyl peptidase that cleaves proteins and peptides with proline or alanine as the penultimate residue and is widely distributed in almost all human tissues and fluids.[2] The gut-derived peptide hormones GLP-1 and GIP, also known as incretins, are among DPP4's targets. Incretins are released in response to glucose ingestion, enhancing insulin secretion by the pancreas. Evidence suggests that incretins might also act to increase β-cell mass and to protect the pancreas from apoptosis (reviewed in refs 3 and 4). Cleavage of incretins by DPP4 starts almost immediately after their secretion due to the presence of DPP4 at their site of production, which results in a short half-life that is less than two minutes.[5] Preventing the degradation of endogenous incretin hormones by inhibiting DPP4 therefore has emerged as a strategy for the control of glucose homeostasis.

Procyanidins are among the bioactive compounds that have been shown to modulate glucose homeostasis. Procyanidins are phenolic structures, abundant in fruits and vegetables, with a wide variety of beneficial effects, and they can act as cardioprotectants, antioxidants, and hypolipidemic agents (reviewed in refs 6 and 7). To exert their effects, procyanidins might use different mechanisms, such as directly interacting and modulating the activity of signaling proteins and/or prevent oxidation.[8] Procyanidins can also interact with transcription factors[9] and enzymes.[10] Several studies have shown an antihyperglycemic effect of procyanidins extracted from grape seed in insulin resistant animals.[11−13] Given the emerging role of DPP4 as a target for glucose-homeostasis regulation, it could be hypothesized that the effects of procyanidins might also be mediated by the modulation of DPP4. There are only a few studies on the effects of phenolic compounds on DPP4 activity, and published results show differential effects. The potential beneficial effects of the phenolic compounds curcumin have been studied to determine if they resulted from the inhibition of DPP4; however, no such inhibitory effect was observed.[14] Similarly, synthetic derivatives of flavone-8-acetic acid, which act as inhibitors of APN/CD13 ectopeptidase, were shown to be unable to inhibit DPP4 peptidase.[15] However, a recent paper has shown that a plant (*P. microphylla*) extract enriched in monomeric flavonoids can modulate in vivo glucose homeostasis and inhibit plasma DPP4 activity when administered intraperitoneally.[16] One of the monomers that this DPP4-inhibitory extract contains is apigenin-7-*O*-glucoside, but its nonglycosylated form, apigenin (as well as genistein but not kaempferol), up-regulates cell-surface CD26 (another name for DPP4) and increases DPP4 activity in human colorectal cancer cells (HT-29) after a 2-day treatment.[17] Finally, a 3-day treatment with grape seed procyanidins in both healthy rats and rats with acute renal failure showed an increase in DPP4 activity in the kidney.[18] Thus, particular flavonoids can modulate the DPP4 activity, although these effects are highly dependent on the flavonoid structure, the experimental conditions, and models used. The present study was therefore developed in order to evaluate whether grape seed-derived procyanidins modulate DPP4 activity.

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Table 1. In Vitro DPP4 Inhibition[a]

		mg GSPE/L				
		0	10	50	100	200
human	recombinant commercial DPP4	0 ± *0*	13.20 ± *4.1*	37.16 ± *0.0**	54.67 ± *0.7**	69.45 ± *0.7**
	intestinal CaCo2 cells	0 ± *0*	11.68 ± *5.6*		7.45 ± *1.5**	17.33 ± *8.6*
	saliva	0 ± *0*	11.18 ± *4.5**		12.35 ± *4.0**	21.25 ± *6.4**
rat	gut	0 ± *0*		19.68 ± *0.1**	36.88 ± *0.1**	39.60 ± *0.1**
	plasma	0 ± *0*		2.89 ± *0.1**	12.99 ± *0.0**	14.96 ± *0.1**

[a]DPP4 extracted from different sources was incubated with different GSPE concentrations. The data are displayed as % inhibition of the DPP4 activity mean ± SEM. *, significant differences vs 0 mg GSPE/L at $P < 0.05$.

MATERIALS AND METHODS

Materials. The grape seed procyanidin extract (GSPE) was purchased from Les Dérivés Résiniques et Terpéniques (Dax, France). The extract contains essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5−13 U; 31.7%) procyanidins.[19,20]

Cell culture reagents were obtained from BioWhittaker (Vervier, Belgium), and the Bradford reagent was from Sigma-Aldrich (Madrid, Spain).

Glucose plasma concentrations were assayed using an enzymatic colorimetric kit (GOD-PAP method from QCA, Tarragona, Spain). Insulin plasma concentrations were determined using Rat Insulin ELISA and Ultrasensitive Rat Insulin ELISA (Mercodia, Uppsala, Sweden). DPP4 activity was measured using the DPP4 Drug Discovery Kit-AK499 (Enzo Life Sciences International, Inc.).

Animal Experimental Procedures. Several groups of animals (detailed below) were purchased from Charles River Laboratories (Barcelona, Spain), housed in animal quarters at 22 °C with a 12-h light/12-h dark cycle, maintained for 1 week in quarantine, and then used for the experiments described below (and summarized in Table 1, Supporting Information). Females were used for the experiments due to their higher sensitivity to the cafeteria diet.[21] After the different GSPE treatments, the animals (except those of the cafeteria treatment) were anesthetized with 50 mg of pentobarbital/kg body weight (bw) and sacrificed by bleeding. The blood was collected, and animal tissues were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. All of the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (permission number: 4250).

Acute GSPE Treatment on Healthy Rats. Overnight-fasted Female Wistar rats were divided into two groups (6 animals/group): (i) the control group, treated with the vehicle (water), and (ii) the GSPE group, treated with 1 g GSPE/kg of bw dissolved in water. Treatment of both groups was administered by an oral gavage after overnight fasting. Tail blood samples were taken at 0 and 60 min of treatment, after which the animals were sacrificed.

Chronic GSPE Treatment on Healthy Rats. Wistar female rats were divided into two groups (11 animals/group): (i) the control group, treated with the vehicle (sweetened condensed milk, diluted 1:10 with water), and (ii) the GSPE group, treated with 25 mg GSPE/kg bw per day. Food was withdrawn daily at 8 a.m. and at 8 p.m., at which time the rats were fed either the vehicle or GSPE dissolved in the vehicle by controlled oral intake with a syringe, and immediately afterward, the food was renewed. At day 19, the rats were fasted overnight, and two glucose tolerance tests were carried out: (i) an oral glucose tolerance test (OGTT), 2 g glucose/kg bw dissolved in water (6 animals/group), and (ii) and an intraperitoneal glucose tolerance test (IPGTT), 1 g glucose/kg bw dissolved in water (the remaining 5 animals/group). Tail blood samples were taken at 0, 15, 30, and 120 min after the glucose load, and plasma glucose and insulin were measured. After 45 days of GSPE treatment, the animals were sacrificed after an overnight fast.

Chronic GSPE Treatment on Cafeteria-Fed Rats. To study the effects of a chronic GSPE treatment on obese animals, obesity was induced by a cafeteria diet (bacon, sweets, biscuits with pâté, cheese, muffins, carrots, and milk with sugar), as previously described.[11] Briefly, female Wistar rats were divided into two groups: a control group (6 animals) fed with standard diet and a cafeteria group (12 animals) fed with standard diet plus a cafeteria diet. After 13 weeks, the cafeteria group was divided into two subgroups (6 animals/group): a control cafeteria group, treated with the vehicle (sweetened condensed milk), and a cafeteria + 25 group, treated daily with 25 mg of GSPE/kg bw (dissolved in the same vehicle). The diet and GSPE dose were daily administered at 9.a.m. After 30 days of GSPE treatment, rats were sacrificed by beheading after three hours of fasting.

Chronic GSPE Treatment on Zucker fa/fa Rats. To work with a genetic model of obesity, we used Zucker animals. This strain has a genetic defect in the leptin receptor causing obesity under homozygosis. Female Zucker rats were divided into three groups (10 animals/group): (i) the control group (Lean), (ii) the fa/fa group, treated with the vehicle (sweetened condensed milk, diluted 1:6 with water), and (iii) the fa/fa + GSPE group, treated daily with 35 mg GSPE/kg bw. Animals were fasted at 9 a.m. every day; at 4 p.m., vehicle or GSPE dissolved in the vehicle was administered by controlled oral intake with a syringe, and 1 h later the food was renewed. At day 60 from the beginning of the GSPE treatment, the animals were sacrificed after an overnight fast.

Cell Culture and Treatments. Caco-2 cells were obtained from the ATCC (American Tissue Culture Collection) and cultured as previously described.[22] The cells were seeded into 12-well culture plates at a cell density of $3.5–4 \times 10^4$ cells/cm^2 and were used for the experiments after 10 days once the confluent monolayer had formed (3−4 days after seeding). To study the effect of GSPE on DPP4, Caco-2 cells were incubated at 500 mg of GSPE/L for 3 h, 750 mg of GSPE/L for 24 h, and 1, 10, or 100 mg of GSPE/L for 3 days in culture medium. GSPE was diluted in 100% ethanol to a 0.5% final concentration of ethanol on culture medium. Treated cells were used for DPP4 analysis, and cells incubated with the vehicle were designated as the control. At least three independent experiments were performed for each treatment.

Measurement of DPP4 Activity and in Vitro Inhibition Assay. DPP4 was extracted from the Caco-2 cells monolayer and rat intestine as previously described with some changes.[23,24] Caco-2 cells were washed twice with PBS and incubated for 5 min in lysis buffer (PBS containing 100 KIU/mL aprotinin and 1% Triton X-100); intestines were homogenized with the same lysis buffer. The obtained samples were first centrifuged at 1000*g* at 4 °C for 10 min to eliminate cellular remainders and then centrifuged twice at 20000*g* at 4 °C for 10 min. Supernatants were stored at −80 °C until analyses.

DPP4 activity from cell and intestine lysates, as well as from rat plasma, was measured following the manufacturer's instructions provided by DPP4 Drug Discovery Kit-AK499 with a few modifications based on the volume of the DPP4-containing sample, adjusting the final volume with Tris-HCl buffer. Protein levels from intestine and Caco-2 cell extracts were determined as previously reported.[25] DPP4 Drug Discovery Kit-AK499 was also used to test the in vitro effects of different concentrations of GSPE on a commercial recombinant human enzyme (provided by the kit). Each assay was performed 3−6 times.

Quantitative RT-PCR. The total RNA from Caco-2 cells was extracted using an RNeasy kit (Qiagen, Hilden, Germany), and the total RNA from intestine was extracted using the TRIzol reactive

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following the manufacturer's instructions. cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR amplification and detection were done using TaqMan assay-on-demand probes (Applied Biosystems): Rn00562910_m1 and Hs00175210_m1 for DPP4 and rat and Caco-2 cells, respectively. The results were referenced to β-actin Rn00667869_m1 for rat and cyclophiline Hs99999904_m1 (Ppia) for Caco-2 cells.

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

RESULTS

GSPE Inhibits DPP4 Activity. To assess whether procyanidins inhibit DPP4 activity, we incubated a commercial recombinant human enzyme with different GSPE concentrations. As shown in Table 1, GSPE is able to inhibit DPP4 activity, achieving around 70% inhibition at 200 mg/L of GSPE.

To check if the same inhibition occurs when DPP4 is derived from other sources, we reproduced the same assay using human salivary and intestinal (from Caco-2 cells) DPP4, as well as rat plasmatic and intestinal DPP4. The results showed that the inhibitory activity varies depending on the DPP4 source, always being lower than in the assay with the commercial enzyme. At 200 mg of GSPE/L, the inhibition of the human Caco-2 enzyme and human salivary enzyme is around 20% and that of the rat intestinal DPP4 is around 40%, but there is only around 15% inhibition in rat plasmatic DPP4 (Table 1).

Finally, we compared the human and rat DPP4 protein sequences to evaluate if they are structurally different enough to explain the observed differential GSPE effects. The two sequences show an 84.8% similarity, and only one amino acid of the active center is different (Figure 1, Supporting Information).

GSPE Inhibits DPP4 Activity and Expression in Human Intestinal Caco-2 Cells after a Chronic Treatment. We next assessed whether procyanidins exert inhibitory effects in living cells. To do so, we performed several GSPE treatments in human intestinal Caco2 cells. We found that an acute treatment of 3 h with a high concentration of GSPE (500 mg/L) did not inhibit DPP4 activity (1.00 ± *0.1* and 1.10 ± *0.1*, control and GSPE-treated, respectively). A 24-h treatment with a higher dose of GSPE (750 mg/L) also did not modify DPP4 activity (1.00 ± *0.1* and 1.01 ± *0.1*, control and GSPE-treated, respectively). However, a longer (3-day) treatment with 10 or 100 mg of GSPE/L led to an inhibition of DPP4 activity of around 20%, as shown in Figure 1A. These results suggested that the DPP4 inhibition could be due to a decrease in the DPP4 gene expression; therefore, mRNA levels were studied. As shown in Figure 1B, the higher dose of GSPE (100 mg/L) down-regulates DPP4 gene expression, while 10 mg of GSPE/L does not. All of the doses assayed were nontoxic (Figure 2, Supporting Information).

Oral Intake of GSPE Modulates DPP4 Activity and Gene Expression in Rats. We next assessed whether the DPP4 inhibitory effects of GSPE could also be found in vivo. To do so, we used several animal models. An acute (1 h) treatment with 1 g of GSPE/kg bw resulted in a 34.3% decrease in the intestinal DPP4 activity ($P < 0.05$) and tended to down-regulate its gene expression 21.93% ($P < 0.1$). A slight inhibition (10%) of DPP4 activity was also observed in healthy rats after a chronic (45 days) treatment of 25 mg of GSPE/kg bw. In this case, a stronger effect was observed at the gene expression level, which was down-regulated around 40%

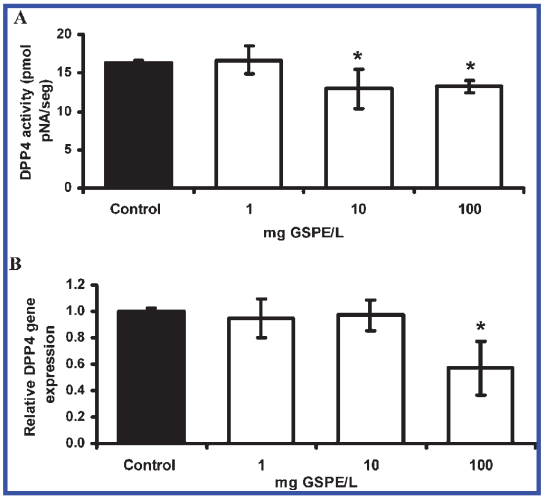

Figure 1. Dose–response effect on DPP4 activity (A) and gene expression (B) in Caco-2 cells treated with GSPE during 3 days. Cells treated with 0 mg GSPE/L were used as the control. The data are displayed as the mean ± SEM. *, significant differences vs the control group at $P < 0.05$.

(Figure 2A and B). DPP4 did not modify plasma DPP4 activity in any of these experiments (Table 2).

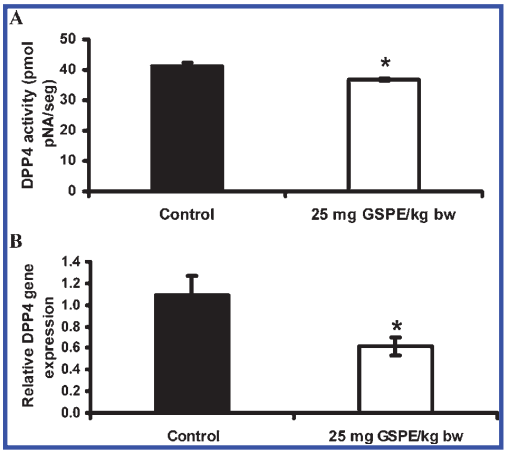

Figure 2. Effect on intestinal DPP4 activity (A) and gene expression (B) on healthy animals treated for 45 days with 25 mg of GSPE/kg bw. The data are displayed as the mean ± SEM. *, significant differences vs the control group at $P < 0.05$.

To test whether procyanidins have similar effects under glucose metabolism deregulation, we also tested the GSPE effects on two pathological animal models: one with diet-induced metabolic syndrome and another model of genetically obese rats. Diet-induced metabolic syndrome was achieved by feeding rats with a cafeteria diet. Afterward, these animals were treated with 25 mg of GSPE/kg bw for 30 days. DPP4 plasma activity was not modified due to either the diet or the procyanidins (Table 2). As shown in Figure 3, procyanidin treatment decreased intestinal DPP4 activity by around 40%

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Table 2. In Vivo Plasmatic DPP4 Inhibition[a]

	treatment	plasmatic DPP4 activity (pmol pNA/seg)
healthy rats, acute treatment	control	1.54 ± *0.14*
	1 g/kg bw, 1 h	1.48 ± *0.10*
healthy rats, chronic treatment	control	1.53 ± *0.13*
	25 mg GSPE/kg bw, 45 days	1.54 ± *0.09*
Zucker rats	lean	1.54 ± *0.22*
	fa/fa	1.20 ± *0.15*
	fa/fa +35 mg GSPE/kg bw, 60 days	1.13 ± *0.10***
cafeteria rats	control	1.66 ± *0.08*
	cafeteria	1.28 ± *0.28*
	cafeteria +25 mg/kg bw, 30 days	1.52 ± *0.10*

[a]Plasmatic DPP4 activity was determined in different animal models treated with GSPE. The data are displayed as DPP4 activity, mean ± SEM. **, significant differences vs the control group at $P < 0.1$.

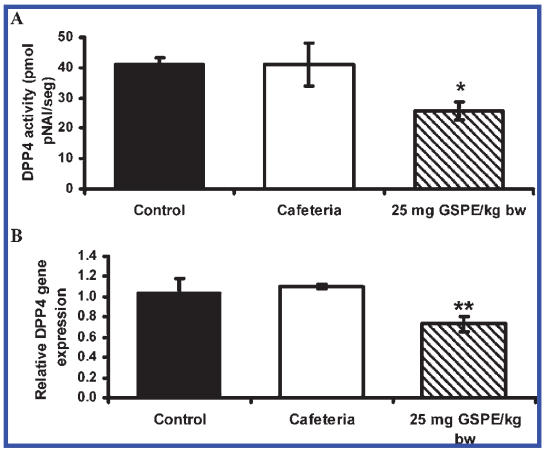

Figure 3. Effect on intestinal DPP4 activity (A) and gene expression (B) on cafeteria rats treated for 30 days with 25 mg of GSPE/kg bw. The data are displayed as the mean ± SEM. *, significant differences vs the control group at $P < 0.05$; **, significant differences vs the cafeteria group at $P < 0.1$.

and repressed gene expression by around 30%. The diet itself had no effect on these parameters when compared with with that of normally fed rats (Figure 3). Zucker fa/fa rats, which have genetic obesity, were treated with 35 mg of GSPE/kg bw for 60 days. In this case, only intestinal DPP4 expression was decreased by GSPE; no changes were observed in intestinal (Figure 4) or plasmatic (Table 2) DPP4 activity.

Effects of Chronic GSPE Treatment on the Plasma Insulin/Glucose Ratio after Glucose Tolerance Tests. Finally, we tested whether GSPE had differential effects on insulin secretion after an oral or intraperitoneal glucose tolerance test, which could be linked to a modulation of the incretin effect. To do so, an oral glucose tolerance test (OGTT) and an intraperitoneal glucose tolerance test (IPGTT) were performed in healthy rats that had been treated with 25 mg of GSPE/kg bw for 19 days. The glucose area under the curve (AUC) after an OGTT was lower in the GSPE treated animals than in the controls, an effect not found in the IPGTT (Figure 5A). This could be explained by the fact that the insulin secretion per plasma glucose after oral glucose administration was higher in the GSPE-treated rats (Figure 5B). Instead, the amount of insulin secreted per plasmatic glucose after an intraperitoneal glucose administration was not modified in the GSPE-treated rats when compared to that in control rats. Thus, GSPE-treated rats were more sensitive to orally administered glucose than controls, while the response to intraperitoneal glucose was not changed.

DISCUSSION

Given the fact that procyanidins affect glucose homeostasis[26] and that the enzyme DPP4 plays an important role in glucose homeostasis regulation, we have explored whether procyanidins modulate the enzyme DPP4. Tebib et al.[27] previously showed that very high concentrations of grape seed tannins are able to inhibit DPP4 intestinal activity. In the present study, we used a different approach; we gave moderate grape seed extract concentrations to mimic human daily food intake. Our results show that GSPE inhibits DPP4 activity and down-regulates its gene expression in the intestine.

The in vitro experiments show that GSPE can directly inhibit DPP4 activity. The level of inhibition depends on the source of the enzyme: maximal inhibition was achieved when purified human enzyme was used; when using cell lysates, animal tissue lysates, or fluids (plasma, saliva), the effects were lower. Such differential effects were not species-related, as expected by the high sequence similarity between the human and rat DPP4 sequences. Procyanidins have affinity for salivary and plasma proteins;[28−30] thus, the low inhibition found in in vitro assays with rat plasma and human saliva suggests that procyanidins could have higher affinity for other plasmatic and salivary proteins than for DPP4. This could also take place in vivo, as none of the studies that we have performed showed plasma DPP4 activity inhibition by GSPE. Another explanation would be that the forms in which GSPE reaches the systemic circulation are not the effective forms that directly inhibit DPP4 activity. The grape seed procyanidin extract is a mixture that contains a variety of structures, most of which have still have not been detected in plasma.[31,32] In fact, to our knowledge, the only previous report on the effects of flavonoids in vivo showed a plasma DPP4-inhibitory effect by an extract enriched in some monomeric structures; however, this extract was administered intraperitoneally, not orally.[16] The in vivo experiments performed in this study cover a wide range of conditions including different GSPE doses (acute high-dose to chronic lower-doses treatments) and different periods of time between GSPE administration and blood sample recovery to assay the DPP4 activity (1, 5, 13, or 17 h after GSPE administration), all of which suggest that although a direct inhibition of plasma DPP4 activity by GSPE cannot be fully discarded, it is not a primary mechanism that would explain the procyanidin effects on glucose homeostasis.

Our previous experience has shown that GSPE effects might depend on the experimental model.[26] Therefore, in this study, we have used several different animal models to analyze the effects of GSPE in vivo, including normal-weight and obese (genetically and diet-induced) rats. In the obese animals, we have not found effects of diet or genetic background on DPP4 plasmatic activity. In mice, a high-fat diet also did not modify plasmatic DPP4 activity.[33] Instead, and in contrast to our results, previous studies have shown that plasma activity is increased in rats due to a high fat diet.[34] In Otsuka Long−Evans Tokushima fatty (OLETF) rats, DPP4 plasma activity

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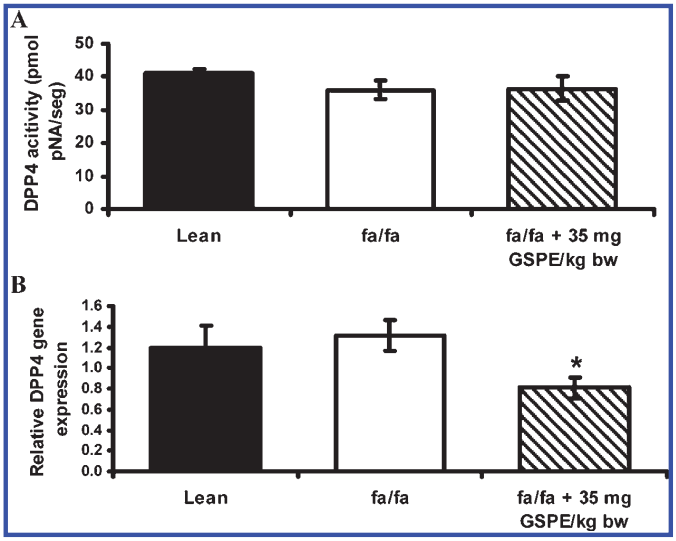

Figure 4. Effect on intestinal DPP4 activity (A) and gene expression (B) on Zucker rats treated for 60 days with 35 mg of GSPE/kg bw. The data are displayed as the mean ± SEM. *, significant differences vs the fa/fa group at $P < 0.05$.

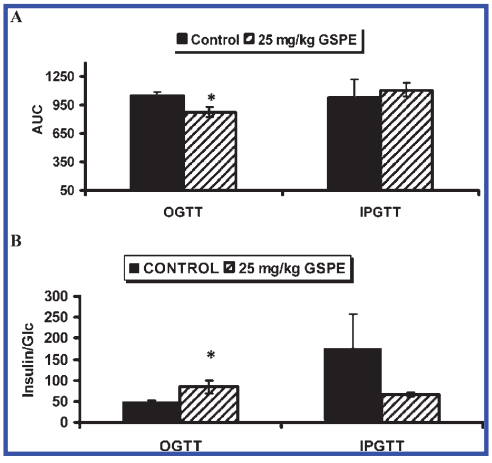

Figure 5. OGTT or IPGTT were performed on healthy animals after 19 days of treatment with 25 mg of GSPE/kg bw. (A) Area under the curve (AUC) of glucose plasma concentrations. (B) Insulin concentration per plasma glucose at $t = 30$ min. The data are displayed as the mean ± SEM. *, significant differences vs the control group at $P < 0.05$.

was only modified during the early phase (insulin-resistant diabetes) but not in hypoinsulinemic diabetes.[35] In humans, there is also controversy as to whether the plasma DPP4 activity is increased in pathologic situations. Some studies showed increased DPP4 plasma activity due to obesity,[36,37] although discrepancies between plasma DPP4 activity and the incretin levels have been found.[36] It therefore has been hypothesized that plasma DPP4 activity is of less relevance for incretin metabolism; instead, DPP4 activity in tissues close to the production site for incretins would be of more relevance.[36,38] Our results showed reduced DPP4 activity in the intestine in most of the experiments. Considering that the experimental procedure included washing the lumen intestine at the moment of sample collection, the mechanisms for such DPP4 inhibition could involve a strong (irreversible) inhibition of the enzyme found at the brush border membrane of the absorptive intestinal cells; it could also be due to direct inhibition of inner intestinal DPP4 by metabolyzed forms of procyanidins. Although absorption of flavonoids is not completely characterized, some flavonoid monomeric forms, like catechin and epicatechin, and also flavonoid metabolites have been reported to be absorbed by the intestinal cells and reach the systemic circulation.[32,39,40,41] Also, dimeric and trimeric procyanidins could be detected in plasma of rats after an acute oral load of procyanidins.[42] In addition, we found that procyanidins down-regulated DPP4 intestinal gene expression, and in fact, gene expression was more sensitive to procyanidins than DPP4 activity. The repression of gene expression could also contribute to the decrease in the intestinal DPP4 activity in chronic treatments, both in healthy and cafeteria-treated rats. Instead, in Zucker rats, GSPE-induced down-regulation of DPP4 gene expression was not enough to modify the enzyme activity. There is not much information concerning the modulation of intestinal DPP4 gene expression. Yang et al.[23] have shown that a high-fat diet increases the intestinal activity and expression of DPP4. We did not find differences in DPP4 activity or expression due to the genetic background or diet. The discrepancies between these and our results could be due to the experimental differences, which include the different genders of animals used (they worked with male rats and we with females), different lengths of the diet treatment (theirs is 12 weeks; ours is 17 weeks), different diets used, and the fact that in our study the samples were obtained after an overnight fasting, whereas in the study of Yang et al., rats seem not to be fasted. However, that study pointed out the importance of DPP4 intestinal gene expression and activity in obesity.

In fact, the exact relationship between DPP4 gene expression regulation and activity is not fully resolved. In Caco-2 cells, high

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glucose concentrations increase DPP4 activity by up-regulating its gene expression.[43] Our experiments in intestinal human Caco-2 cells reinforce the idea that DPP4 gene expression is a target for procyanidins, although the decrease in DPP4 activity does not seem to be dependent on the effects of expression, as low GSPE concentrations decrease DPP4 activity but not gene expression.

In any case, the reduction in intestinal DPP4 activity could be linked to the effects of procyanidins on glucose homeostasis. We have previously shown that 25 mg of GSPE/kg bw in cafeteria-treated rats ameliorate their HOMA-IR index;[11] therefore, modulation of DPP4 activity could also be involved in GSPE effects in addition to the previously suggested effects on glucose absorption and/or action in peripheral tissues. Furthermore, in the present article, we show that chronic GSPE treatment in healthy rats, which decreased intestinal DPP4 activity, increases the plasma insulin/glucose ratio in an oral glucose tolerance test, while it does not do so when glucose is administered intraperitoneally, suggesting an incretin effect. This would support the hypothesis that intestinal DPP4 could be involved in the modulation of insulin production. However, further experiments on the effects of procyanidins in the pancreas, a target of DPP4 inhibitors, as well as more knowledge on the exact relationship between intestinal DPP4 activity and effects on glucose homeostasis, which remain unresolved, will be required to fully elucidate the mechanism of action of procyanidins.

In conclusion, we have found that a grape seed procyanidin extract is able to inhibit DPP4, directly as well as by other mechanisms, such as down-regulation of DPP4 gene expression. GSPE also increases the plasma insulin/glucose ratio in response to orally administered glucose, suggesting an incretin effect that could be responsible for some of the effects of GSPE in glucose homeostasis.

■ ASSOCIATED CONTENT

Supporting Information

Summary of animal experimental procedures; DPP4 protein sequence comparison; and cytotoxicity of Caco-2 cells treated with 100 of GSPE/L during 3 days. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DPP4 dipeptidyl-peptidase 4; GSPE grape seed procyanidin extract; bw body weight; OGTT oral glucose tolerance test; IPGTT intraperitoneal glucose tolerance test

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CHAPTER 3

Grape-seed procyanidins modulate enteroendocrine hormone secretion in STC-1 cells by altering their cellular membrane potential

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Increasing GLP-1 secretion could be another possible mechanism by which procyanidins improve active GLP-1 levels, so the third objective of this thesis was to evaluate the effects of GSPE on enteroendocrine hormone secretion. To do so we used an *in vitro* approach. Several enteroendocrine cell lines are used to evaluate the stimulatory effects of nutrients and the mechanisms involved, such as GLUTag [1,2], NCI-H716 [3] and STC-1 [4-6]. In this study, we used the murine STC-1 cell line.

To assess the effect of procyanidins on GLP-1 secretion, STC-1 cells were treated with several GSPE concentrations in basal or nutrient-stimulated conditions and the amount of GLP-1 secreted to the medium was determined. As STC-1 cells have been reported to secrete other enteroendocrine hormones [7], cholecystokinin (CCK) secretion was also determined to analyze the specificity of the effect of GSPE.

Cellular membrane potential and mitochondrial membrane potential have been reported to be involved in hormone secretion [8,9], therefore they were assayed to assess whether these mechanisms are involved in the modulation of enteroendocrine hormone secretion by GSPE.

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Grape-seed procyanidins modulate enteroendocrine hormone secretion in STC-1 cells by altering their cellular membrane potential

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Abstract

Scope: Grape-seed procyanidins (GSPE) modulate glucose homeostasis, and they have been suggested to act on incretin hormone levels. In the present study we evaluated the effect of GPSE on the secretion of glucagon-like peptide-1 (GLP-1) from the enteroendocrine cell line STC-1, and the underlying mechanisms involved.

Methods and results: We demonstrate that GSPE modulates plasma membrane potential, inducing depolarization at low doses that led to stimulation of cholecystokinin (CCK) secretion, and hyperpolarization at high doses, which led to a reduction of GLP-1 secretion. Furthermore, we explored whether GSPE modifies the effects of nutrients (i.e. glucose, linoleic acid and L-proline) on STC-1, and found that only the high GSPE dose is effective at limiting membrane depolarization and at reducing GLP-1 secretion. Finally, we also examined whether GSPE affects mitochondrial membrane potential, and found that it is altered by GSPE, but such effects do not appear to explain the modulation of plasma membrane potential and GLP-1 secretion.

Conclusion: We have shown that grape-seed procyanidins modulate enteroendocrine hormone secretion in a dose-dependent manner, and that this can be explained by changes in cellular membrane potential.

Keywords: GLP-1/membrane potential/procyanidins/STC-1/CCK

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Introduction

Diabetes mellitus type 2 is a common chronic disease characterized by pancreatic β-cell failure and decrease in insulin release, causing hyperglycaemia. The prevalence of the disease continues to rise significantly [1], and there is a need to develop new therapies which prevent or ameliorate this pathology, including the use of bioactive compounds present in food.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone mainly released from L-cells of the distal intestine (ileum and colon) [2,3]. It has been reported that GLP-1 secretion is impaired in type 2 diabetic patients [4,5] and exogenous GLP-1 infusion improves glycemia [6,7], indicating potential for developing new antidiabetic therapies which enhance GLP-1 secretion.

Several nutrients have been reported to stimulate GLP-1 release by intestinal L-cells *in vitro* and *in vivo*, such as glucose [8,9], fatty acids [10], intact dietary proteins [11] or amino acids [12-14], all of these involving membrane depolarization, calcium entry and calcium-dependent GLP-1 release [15]. However, there are non-nutritive components in food which can also reach these enteroendocrine cells and thus they could modulate GLP-1 secretion. Several reports showed an improvement of GLP-1 levels by some natural compounds. Chlorogenic acid, a major phenol found in coffee, was shown to improve plasma GLP-1 levels [16] and increase GLP-1 secretion and production in STC-1 cells, a murine enteroendocrine cell line [17]. Berberine, a major active constituent of *Rhizoma coptidis*, has been reported to increase portal active GLP-1 levels in healthy and streptozotocin-induced diabetic rats (STZ) and enhance GLP-1 secretion and biosynthesis in NCI-H716 cells, a human enteroendocrine cell line [18,19]. Genistein and daidzen isoflavonoids, derived from soybean fermentation, have been reported to increase GLP-1 secretion from NCI-H716 cells [20]; glyceollins, phytoalexins derived from daidzen in soybean with fungi infection, showed the same effect *in vitro* [21]. Resveratrol, a polyphenolic compound produced by fruits such as red grapes or berries, was found to increase portal active GLP-1 levels and intestinal biosynthesis in high fat diet-fed rats (HFD) [22]. Similarly, cinnamtannin B2, a tretameric procyanidin, was reported to increase GLP-1 secretion *in vivo* [23]. Moreover, a recent paper has reported that curcumin, a phenolic compound isolated from the rhizomes of *Curcuma longa L.*, is able to increase GLP-1 secretion in the murine enteroendocrine cell line, GLUTag [24].

Procyanidins are phenolic compounds present in fruits and vegetables. We have previously reported that a grape seed procyanidin extract can increase the insulin/glucose ratio after oral glucose gavage, suggesting an incretin effect, [25] which could, in part, explain the anti-hyperglycemic effect of GSPE [26,27]. However, the

effects of procyanidins on incretin secretion by enteroendocrine cells have not yet been described. The present study was therefore undertaken to evaluate whether GSPE modulates GLP-1 secretion by L-cells and the mechanisms involved.

Research Design and Methods

Reagents

The grape seed procyanidin extract (GSPE) used in these studies was obtained from *Les Dérivés Résiniques et Terpéniques (Dax, France)* and has underwent detailed characterization by our research group [28].

Linoleic Acid (LA), L-Proline (L-Pro), Methyl α-D-Glucopyranoside and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma-Aldrich (Madrid, Spain). LA was prepared as 100 mM stock in absolute ethanol and CCCP stock was prepared in DMSO to a final concentration of 10 mM. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) was obtained from Biotium (Hayward, CA, USA) and dissolved in DMSO to a final concentration of 10 mM. Rhodamine 123 was purchased from Molecular Probes (Invitrogen, Madrid, Spain) and prepared as 10 mg/mL stock solution in DMSO. All stock solutions were stored at -20 ºC.

Cell culture

The STC-1 clonal cell line was received as a kind gift from Dr. B. Wice (Washington University of St. Louis) with the permission of Dr. D. Hanahan (University of California, San Francisco, CA, USA). This enteroendocrine cell line was originated from a double-transgenic mouse tumor [29]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™ containing 4.5 g/L D-glucose, without sodium pyruvate (Gibco, Madrid, Spain), supplemented with 17.5% fetal bovine serum, 100 U/mL penicillin and 100 mg/L streptomycin (BioWhittaker, Verviers, Belgium) and incubated in a 5% CO_2-humidified atmosphere at 37 ºC. Cells were used between passage numbers 30-50.

Hormone secretion

For secretion experiments, $1.8\text{-}2x10^6$ cells were seeded in 12-well culture plates and allowed to reach 70-90% confluence. On the day of the experiment, cells were incubated for 2h in HEPES buffer (20 mM HEPES, 140 mM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$ (pH 7.4)). After pre-incubation period, buffer was removed and

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cells were incubated with 0.05, 0.5, 5 or 50 mg of GSPE/L for 3h in different conditions: i) Basal conditions: 2.5 mM glucose in HEPES buffer; ii) Glucose stimulation: 10 or 20 mM glucose in HEPES buffer; iii) Fatty acid stimulation: 30 µM Linoleic Acid in HEPES buffer with 10 mM glucose; iv) Amino acid stimulation: 5 mM L-Proline in HEPES buffer with 2.5 mM glucose. At the end of the 3 hour-treatments, medium was collected and centrifuged to remove any cellular debris. Supernatants were stored at -80 ºC until analyses.

Active GLP-1 levels were determined using a glucagon-like peptide-1 (active) ELISA kit (Millipore, Madrid, Spain). Cholecystokinin (CCK) levels were measured using a human/mouse/rat CCK EIA kit from RayBioTech (BioNova cientifica SL, Madrid, Spain).

Cellular membrane potential

Cellular membrane potential ($\Delta\Psi_{cell}$) was measured using the fluorescent probe $DIBAC_4(3)$. 50-60,000 cells were seeded in 96-well culture plates and allowed to reach 70-90% confluence. On the day of the experiment, cells were pre-incubated for 2h with HEPES buffer. Then, cells were labeled with 10 µM $DIBAC_4(3)$ (diluted in HEPES with 2.5 or 10 mM glucose) for 30 minutes at 37 ºC. After labeling, GSPE was added to a final concentration of 0.05, 0.5, 5 or 50 mg/L and the $\Delta\Psi_{cell}$ was monitored with excitation and emission filters set at 493 nm and 516 nm, respectively. After 3 min in basal conditions, cells were stimulated with 10 or 20 mM glucose, 30 µM Linoleic Acid, 5 mM L-Proline or 100 mM Methyl α-D-Glucopyranoside. 5 min later, total cellular membrane depolarization was induced by adding KCl to a final concentration of 75 mM. $\Delta\Psi_{cell}$ was calculated as the difference of membrane potential between the peaks after stimulation (195 sec) and basal (180 sec).

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was determined using the fluorescent probe rhodamine 123. Cells were treated as described for $\Delta\Psi_{cell}$ measurement and labeled with 10 µg/mL of rhodamine 123. $\Delta\Psi_m$ was monitored with excitation and emission filters set at 485 nm and 520 nm, respectively. Total mitochondrial membrane potential depolarization was induced by adding CCCP to a final concentration of 24 µM.

Data analyses

Results are expressed as the mean ± SEM. Effects were assessed by ANOVA and Student's t-test. All calculations were performed with SPSS software (SPSS, Chicago, USA).

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Results

GSPE modulates hormone secretion by changing membrane potential

To assess if GSPE modulated GLP-1 secretion, we determined active GLP-1 levels secreted into the media by STC-1 cells after 3h of treatment with GSPE. As shown in **figure 1a**, 0.05 mg of GSPE/L tended to increase GLP-1 secretion but it was not statistically significant, while the dose of 50 mg of GSPE/L significantly decreased GLP-1 secretion in around a 40%.

To analyze whether GSPE-induced effects on GLP-1 secretion were through its effect on cellular membrane potential, we stimulated STC-1 cells with a range of GSPE concentrations and measured membrane potential changes. Cellular membrane depolarization was induced after stimulation with 0.05 and 0.5 mg of GSPE/L, while 5 mg of GSPE/L did not change membrane potential. Contrarily, 50 mg of GSPE/L caused membrane hyperpolarization (**figures 1b** and **1c)**.

Because STC-1 cells have been reported to secrete other intestinal hormones [30], we also measured CCK levels in media to indicate whether modulation of membrane potential also affects other hormone secretions. As shown in **figure 1d**, 0.05 mg of GSPE/L significantly stimulated CCK secretion.

GSPE alters glucose-induced GLP-1 secretion

To analyze whether GSPE modulates glucose stimulation on enteroendocrine cells, we determined cellular membrane potential and active GLP-1 levels in STC-1 cells stimulated with glucose and different GSPE concentrations. First, we defined the GLP-1 secretion triggered by glucose in STC-1 cells. **Figure 2a** shows that 10 mM glucose caused a higher increase in GLP-1 secretion than 20 mM glucose. This profile was not the same than the effects observed in membrane potential, were 20 mM glucose induced a higher membrane depolarization (**figures 2b** and **2c**).

Then, we assessed the GSPE effects on STC-1 cells stimulated with 20 mM glucose. An inhibition of membrane depolarization was found after treatment with 5 and 50 mg of GSPE/L. In this case, lower doses of GSPE showed no changes on membrane depolarization (**figure 3a** and **3b**). Moreover, secretion of active GLP-1 was determined after simultaneous treatment of 20 mM glucose and the doses of GSPE which modulated membrane potential. A reduction of GLP-1 levels of around a 20 % was found after treatment with 50 mg of GSPE/L. Instead, 5 mg of GSPE/L caused no changes (**figure 3c**).

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To test whether GSPE modulation of glucose-triggered membrane depolarization is due to effects on glucose metabolism pathway, the non-metabolizable glucose analogue methyl α-D-glucopyranoside was used to stimulate STC-1 cells, simultaneously with GSPE, and cellular membrane potential was measured. As shown in **figure 4**, 100 mM methyl α-D-glucopyranoside triggered membrane depolarization and both GSPE treatments (5 and 50 mg of GSPE/L) significantly inhibited this depolarization.

GSPE effects on mitochondrial membrane potential in STC-1 cells

To evaluate whether GSPE affects enteroendocrine cells by modulating mitochondrial membrane potential, we treated STC-1 cells with different concentrations of GSPE in presence or absence of glucose stimulus and measured mitochondrial membrane potential. In basal conditions, 50 mg of GSPE/L significantly hyperpolarized the mitochondrial membrane (**figure 5a** and **5c**). As shown in **figures 5b** and 5**d**, 20 mM glucose seems to trigger mitochondrial membrane depolarization and GSPE showed no effects on $\Delta\Psi_m$.

GSPE alters hormone secretion induced by other nutrients

To test whether GSPE also modulates other nutrients-triggered GLP-1 secretion, we measured cellular membrane potential and GLP-1 secretion by STC-1 cells after stimulation with some nutrients that are well reported to induce membrane depolarization, an amino acid (5 mM L-Proline) and a fatty acid (30 μM of Linoleic Acid). As shown in **figures 6a** and **6b**, L-proline triggered membrane depolarization and this effect was inhibited at 50 mg of GSPE/L. In agreement with membrane depolarization, L-proline tended slightly but not significantly to increase GLP-1 secretion (**figure 6c**) and 50 mg of GSPE/L significantly reduced proline-stimulated GLP-1 levels (**figure 6d**). In the case of linoleic acid, it triggered cellular membrane depolarization (**figures 7a** and **7b**), and it was inhibited by 5 and 50 mg of GSPE/L. Surprisingly, active GLP-1 secretion is decreased after stimulation with linoleic acid (**figure 7c**) and treatment with 50 mg of GSPE/L tended to further decrease it, but was not statistically significant (relative GLP-1 levels: 0.71 *± 0.17* ($P > 0.05$)).

Figure 1. GSPE effects on hormone secretion and cellular membrane potential. Effect of GSPE on GLP-1 secretion (**A**), cellular membrane potential expressed as $\Delta\Psi_{cell}$ (**B**) or % of basal (**C**), and CCK secretion (**D**). The data are displayed as the mean ± *SEM*. **a, b** and **c**, statistically significant differences at $P < 0.05$.

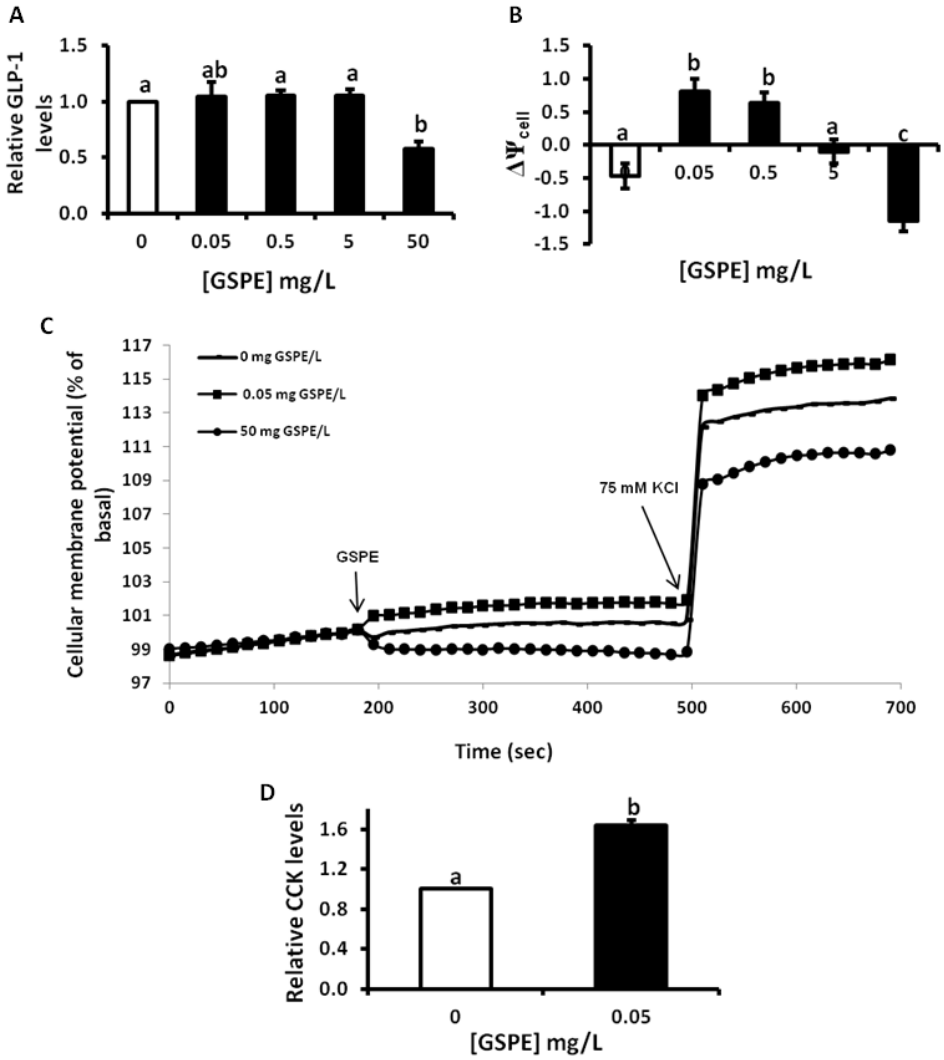

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Figure 2. Glucose modulation of cellular membrane potential and GLP-1 secretion. Effect of 10 and 20 mM glucose on GLP-1 secretion (**A**), and cellular membrane potential expressed as $\Delta\Psi_{cell}$ (**B**) or % of basal (**C**). The data are displayed as the mean ± *SEM*. **a, b** and **c**, statistically significant differences at $P < 0.05$.

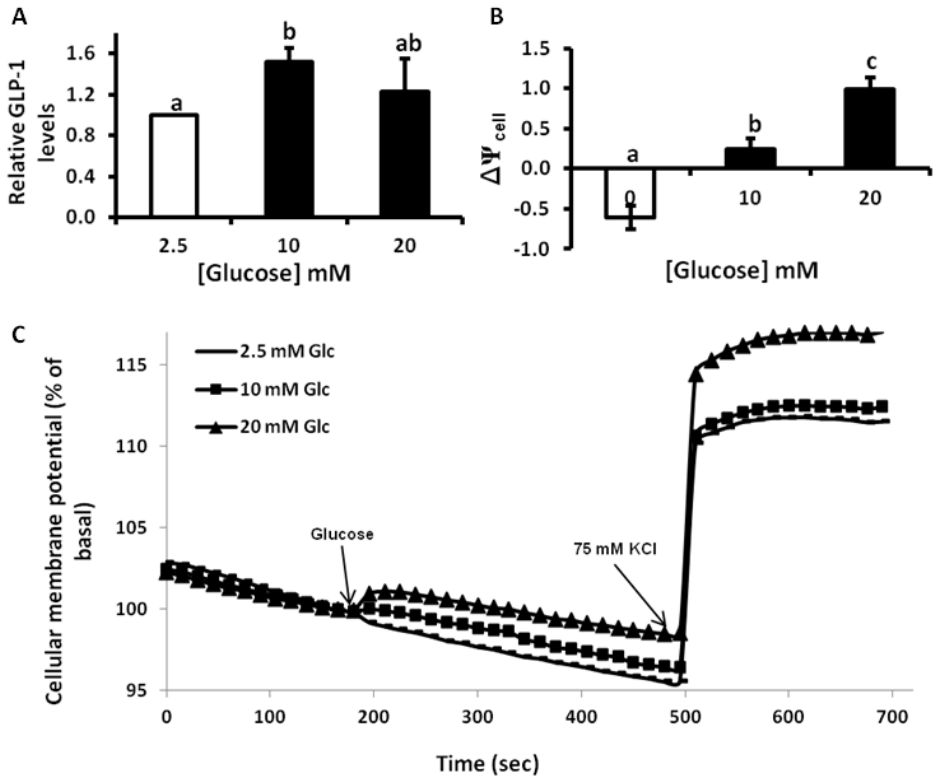

Figure 3. Effect of GSPE on glucose-triggered cellular membrane depolarization and GLP-1 levels. Effect of GSPE after stimulation with 20 mM glucose on cellular membrane potential expressed as % of basal (**A**) or $\Delta\Psi_{cell}$ (**B**), and GLP-1 secretion (**C**). The data are displayed as the mean ± *SEM*. **a, b** and **c**, statistically significant differences at $P < 0.05$.

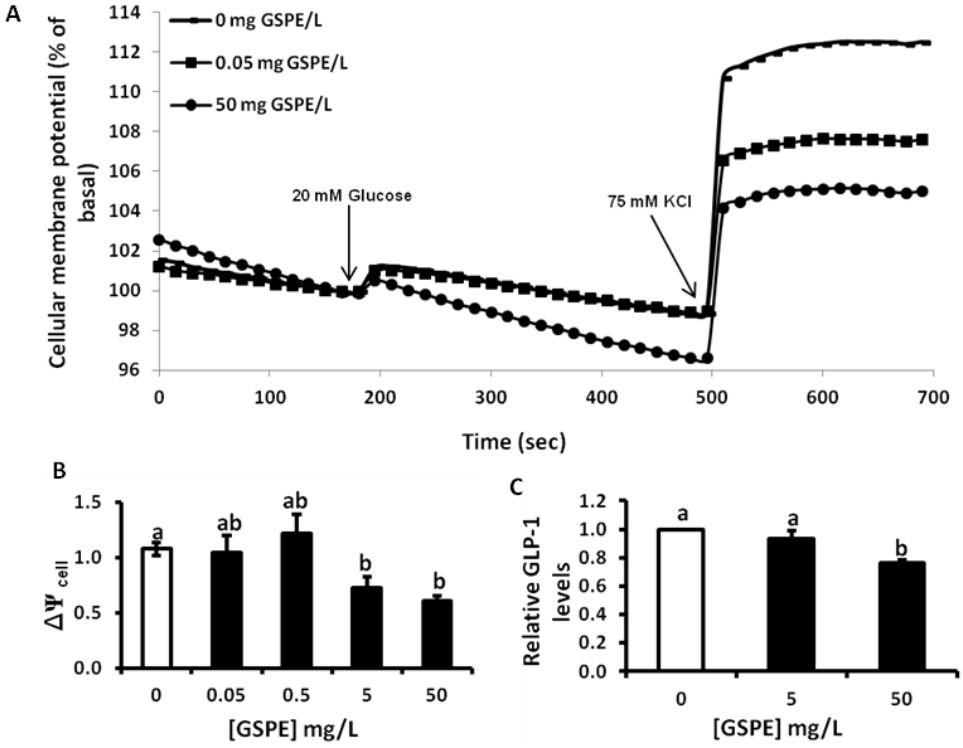

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Figure 4. GSPE modulation of Methyl α-D-Glucopyranoside-triggered cellular membrane depolarization. Effect of GSPE after stimulation with 100 mM methyl α-D-glucopyranoside (MαG) on cellular membrane potential expressed as $\Delta\Psi_{cell}$ (**A**) or % of basal (**B**). The data are displayed as the mean ± *SEM*. **a, b, c** and **d**, statistically significant differences at $P < 0.05$.

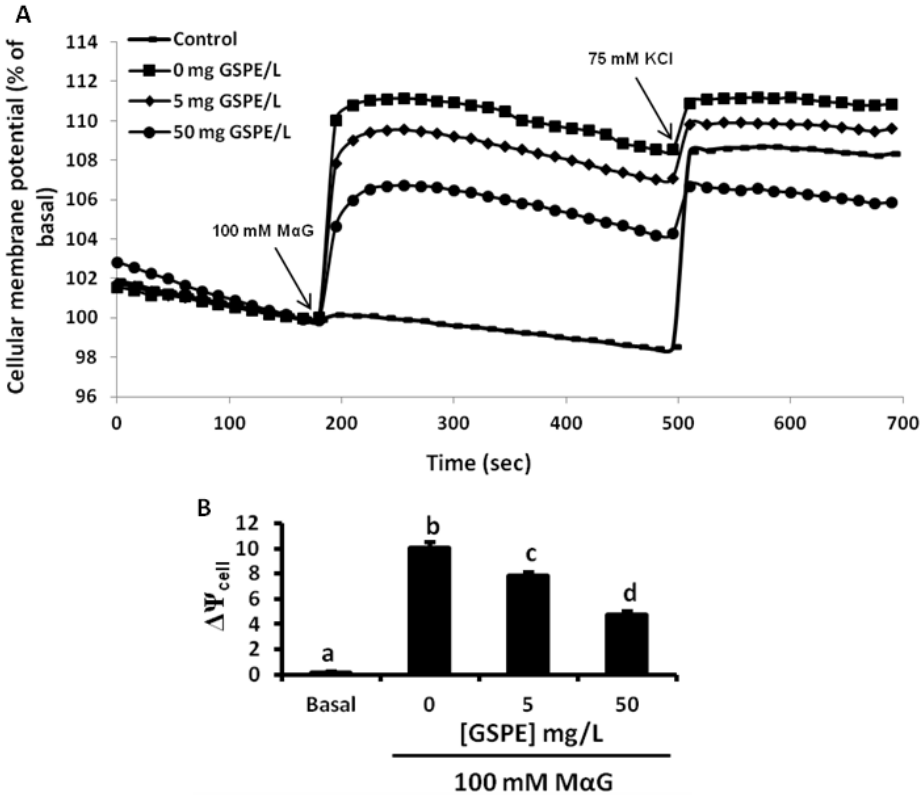

Figure 5. GSPE effects on mitochondrial membrane potential. Effect of GSPE on mitochondrial membrane potential (% of basal) in basal conditions (**A**), and after stimulation with 20 mM glucose (**B**). $\Delta\Psi_m$ in basal conditions (**C**) and after stimulation with 20 mM glucose (**D**). The data are displayed as the mean ± *SEM.* **a** and **b**, statistically significant differences at $P < 0.05$.

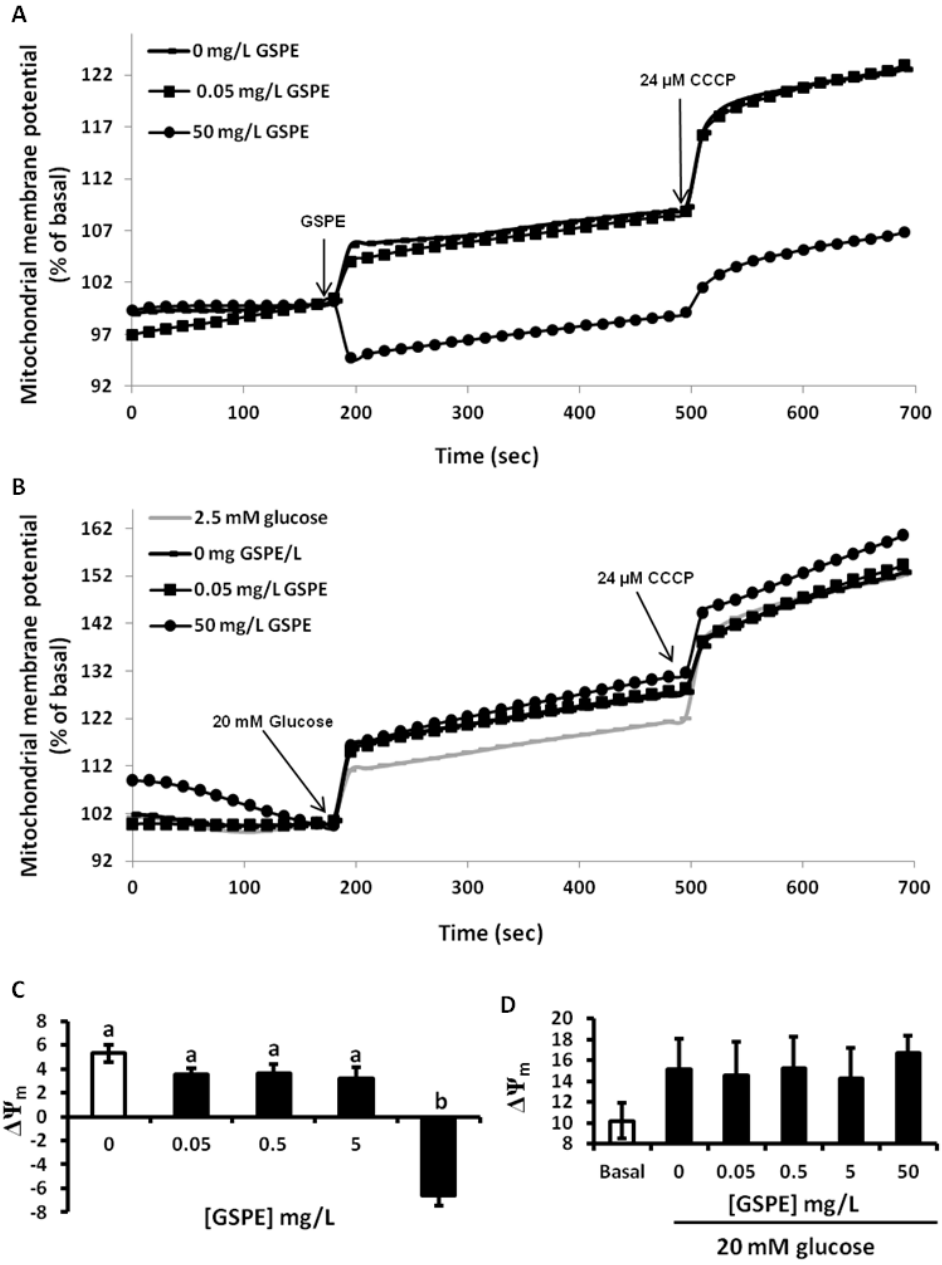

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Figure 6. GSPE modulation on L-proline stimulation. Effect of GSPE after stimulation with 5 mM L-proline (L-Pro) on cellular membrane potential expressed as % of basal (**A**), and $\Delta\Psi_{cell}$ (**B**). GLP-1 secretion after L-pro stimulation (**C**), and after simultaneous treatment with GSPE (**D**)**.** The data are displayed as the mean ± *SEM.* **a, b** and **c**, statistically significant differences at P < 0.05. * P < 0.05 compared to basal.

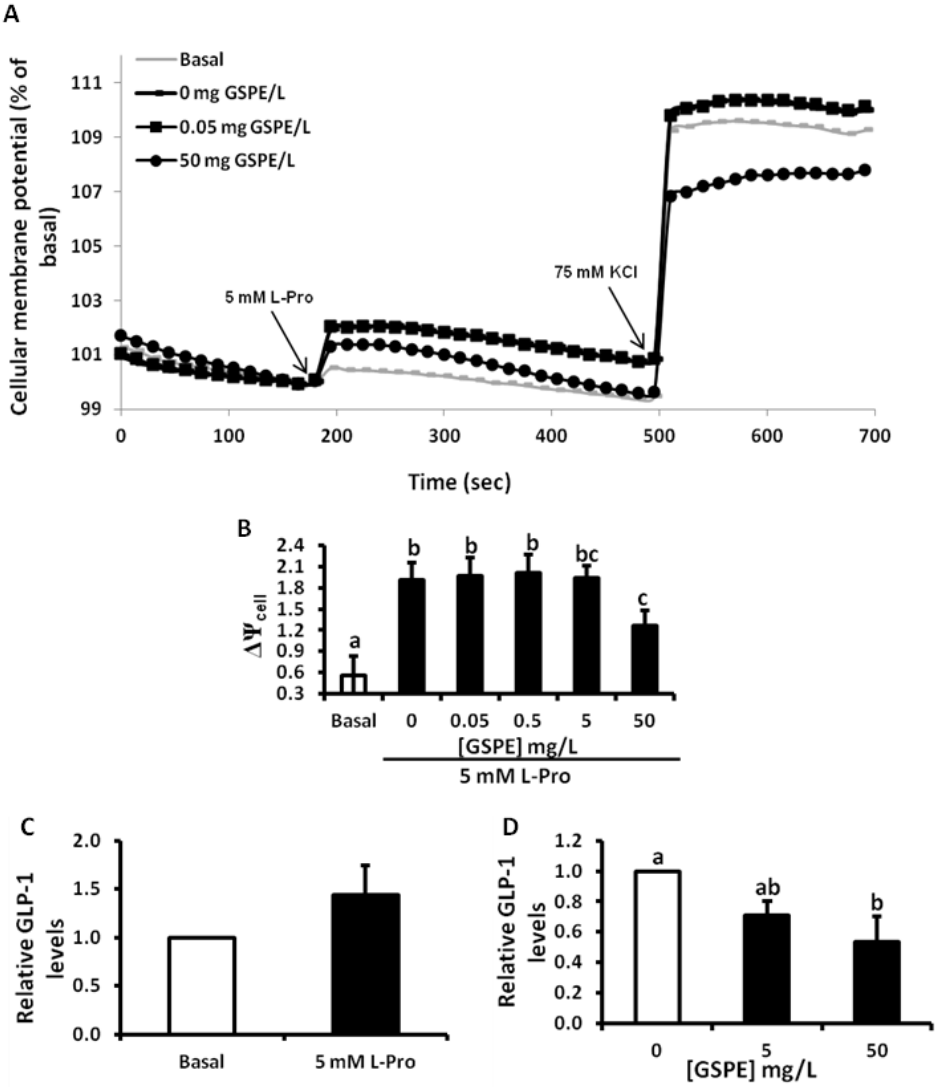
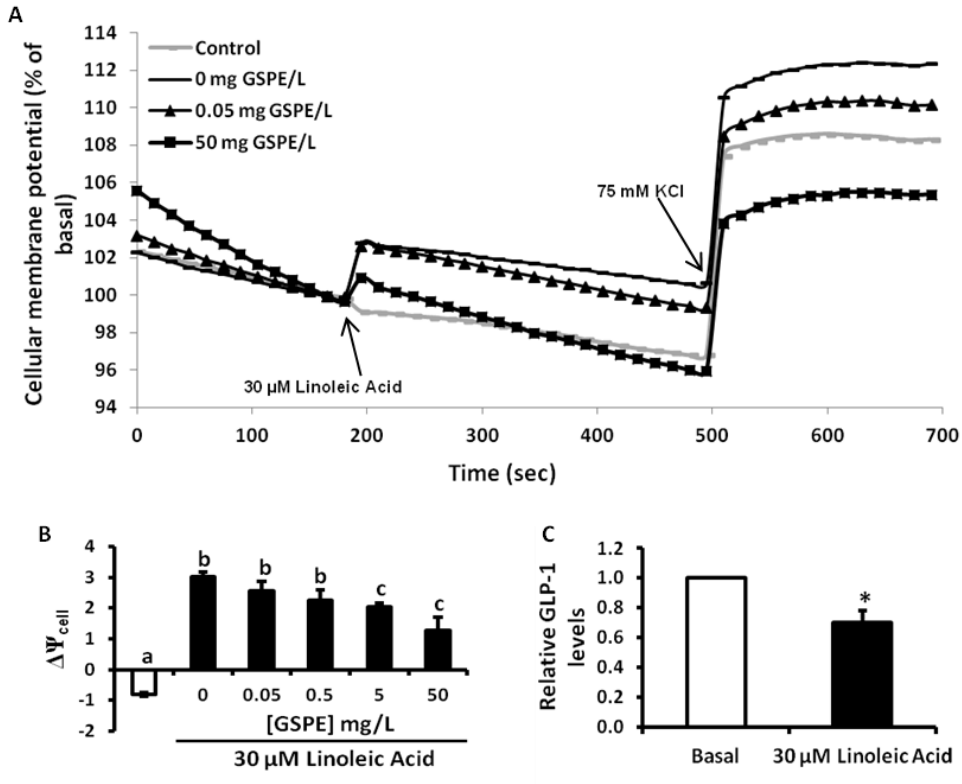


Figure 7. GSPE modulation on linoleic acid stimulation. Effect of GSPE after stimulation with 30 µM linoleic acid (LA) on cellular membrane potential expressed as % of basal (**A**), and $\Delta\Psi_{cell}$ (**B**). GLP-1 secretion after stimulation with LA (**C**).). The data are displayed as the mean ± *SEM.* **a, b** and **c**, statistically significant differences at $P < 0.05$. * $P < 0.05$ compared to basal.



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Discussion

It has previously been shown that a grape-seed procyanidin extract modulates glucose homeostasis [31], and it has been suggested to produce an incretin-like effect [25] through its effects as DPP4 inhibitor, the enzyme responsible for incretin degradation and inactivation. However whether it also affects hormone secretions from intestinal enteroendocrine cells is something that has not previously been investigated. The current study shows that an extract of procyanidins from grape-seed modulates cellular membrane potential of enteroendocrine cells and this influences the secretion of enteroendocrine hormone.

A few studies have shown the effects of phenolic compounds on GLP-1 secretion from enteroendocrine cells, such as genistein and daidzen isoflavonoids, and chlorogenic acid, which have been reported to increase GLP-1 secretion from enteroendocrine NCI-H716 and STC-1 cells, respectively, in the presence of glucose [17,20]. Curcumin has also been reported to increase GLP-1 secretion in the GLUTag cell line [24]. Although in the later an involvement of the Ca^{2+}-CaMKII pathway has been suggested, the mechanisms through which non-nutritive compounds trigger hormone release are still unclear. *In vitro* studies using several enteroendocrine cell line models have found that cellular membrane depolarization is determinant for nutrient-induced hormone secretion [8,9,12,14,32,33]. Our results showed that GSPE extract at low doses (0.05 and 0.5 mg/L) trigger cellular membrane depolarization, leading to a significant increase on CCK secretion and a slight, but not significant, increase on GLP-1.

We also evaluated the interaction of GSPE with different kinds of nutrients to examine whether it could modulate their effects on GLP-1 secretion. Glucose has been reported to be a potent GLP-1 secretagogue (reviewed in [34]). Several studies reported that glucose triggers cellular membrane depolarization, which stimulates Ca^{2+} entry through the voltage-gated calcium channels (VGCCs), inducing GLP-1 secretion [8,9,35]. We reproduced these glucose-induced events in the STC-1 cells, although the degree of depolarization induced by glucose did not correlate with the levels of GLP-1 secretion. Concerning the effects of low doses of GSPE, we also found that GSPE do not enhance glucose-triggered membrane depolarization. Other nutrients, such as fatty acids [10,36-38] and amino acids [12,13], have also been shown to induce hormone secretion from enteroendocrine cells. So, to elucidate whether GSPE effects on cellular membrane potential is dependent on the specific mechanisms activated by the different nutrients, we also evaluated the effects of GSPE with other nutrients that are well reported to trigger cellular membrane depolarization: linoleic acid [33] and L-proline [14]. We found that low GSPE doses did not further enhance membrane depolarization induced by

these agents. Therefore the effects of low GSPE doses on depolarization of the plasma membrane are not additive to any other of the tested stimulus.

In any case we also found that the effects of the nutrients at depolarizing membrane potential are not directly linked to the effects on GLP-1 secretion. Linoleic acid triggered cellular membrane depolarization but, surprisingly, it was accompanied by a decrease on GLP-1 secretion. L-proline also induced cellular membrane depolarization and, in this case, a tendency to increase GLP-1 secretion was found, although it did not reach statistical significance. Few studies on STC-1 cells have shown a further involvement of the G coupled-protein receptor 120 (GPR120) on linoleic acid stimulation [33] and Na^+-dependent neutral aminoacid transporter 2 (SNAT2) in L-proline [14], respectively, but the influence of these on GLP-1 secretion has not previously been studied.

Our results clearly show that the effects of GSPE on membrane potential are dose dependent. Whilst lower doses induced membrane depolarization and hormone release, treatment of STC-1 with higher GSPE doses led to hyperpolarization of the plasma membrane. Such effects on the plasma membrane coincided with reduced levels of GLP-1 in the medium. It could be surmised that alteration of cellular membrane potential is important for GSPE's modulation of hormone secretion. Furthermore, the effects of the higher GSPE doses when treatments were performed simultaneously to stimulation with the different nutrients, showed the same effects, which were clearly significant at the 50 mg GSPE/L dose: reduction in the stimulus-induced membrane depolarization, and decreased or a tendency to decrease GLP-1 in the medium. However, the pathways that use nutrients to stimulate GLP-1 secretion are still understudied. Although there are no studies of the mechanisms involved on glucose-triggered GLP-1 secretion in STC-1 cells, in other cell types and primary L-cell cultures, it has been found that glucose stimulation involves the activation of the sodium/glucose co-transporter 1 (SGLT1) [9,39] and the sweet taste receptors, T1R2 and T1R3 [40]. Our results suggest that GSPE-induced modulation of membrane potential, at higher GSPE doses, is not acting through specific nutrient-receptors but by a more non-specific mechanism.

As previously described, the mechanism for GSPE to act on plasma membrane potential in a pancreatic β-cell line is through induction of mitochondrial membrane depolarization resulting in lower ATP production that limits insulin secretion [41]. Studies in the enteroendocrine GLUTag cell line suggested an analogue mechanism for glucose-induced hormone secretion to that found in pancreatic β-cells [15]. In these cells, glucose-induced insulin release is mediated by glycolysis and mitochondrial oxidation, which generates a rise in ATP/ADP ratio, stimulating ATP-sensitive K^+ (K_{ATP}) channels closure and cellular membrane depolarization (reviewed in [42]). In this way, GSPE effects on STC-1 mitochondrial membrane potential were studied to assess

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whether modulation of cellular membrane potential could be due to these previous reported effects. Our results were quite surprising because in our experimental setup glucose appears to induce mitochondrial membrane depolarization. The effects of glucose on mitochondrial membrane potential have not been reported in enteroendocrine cells, but the observed response is different to that reported in pancreatic β-cells. Furthermore, treatment with 50 mg of GSPE/L in basal conditions hyperpolarized mitochondrial membrane, but no changes were found in glucose-induced conditions. This shows that the mechanisms triggered by high GSPE doses are different from those exerted at the Ins-1 beta-cell line [41]. Besides, it is still debated whether glucose metabolism is a requirement in enteroendocrine cells for stimulation of hormone secretion. Studies with methyl α-D-glucopyranoside, a non-metabolizable glucose analogue, showed that this molecule can also induce membrane depolarization and hormone secretion [9,39]. We have also tested the effects of GSPE concomitant with this molecule, and we found that the high doses of GSPE also limits the high increase in membrane depolarization induced by this agent, discarding the need for glucose metabolism to explain GSPE effects on cellular membrane depolarization.

Other possible general mechanisms for GSPE modulation of plasma membrane need to be explored. One possibility could be that they affect membrane fluidity, as has been shown for polyphenols from tea (epigallocatechin gallate), grape seeds (quercetin), and *Curcuma longa* (curcumin) which decrease membrane fluidity in a dose-dependent manner, thus decreasing membrane depolarization [43].

In summary, this study indicates that grape seed procyanidins modulate hormone (GLP-1 and CCK) secretion from intestinal endocrine cells. Furthermore, we show that GLP-1 secretion by STC-1 cells is modulated by grape-seed procyanidins in a dose-dependent manner, which could be explained by changes in cellular membrane potential. Finally, we also have described the influences of a number of nutrients on the enteroendocrine cell line STC-1. Further work is required to understand all the physiological effects of dietary consumption of grape seed procyanidins.

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contributed to research data, M. B. contributed to discussion, B D. G., M. P. and A. A. contributed to the experimental design and discussion and reviewed the manuscript.

The authors declare that they have no conflict of interest.

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CHAPTER 4

Grape-seed procyanidins prevent the cafeteria diet-induced decrease of glucagon-like peptide-1 production

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Once the effects on GLP-1 levels, including its secretion and degradation, had been determined, we focused on our last objective that was to evaluate whether GSPE affects GLP-1 secretion and production in a preventive manner in a model of insulin resistance. Hence, rats were treated with GSPE simultaneously with insulin resistance induction by a cafeteria diet.

In this study, the effect of the extract on plasma active GLP-1 levels was assessed. To understand the mechanisms by which GSPE exerts its effect, GLP-1 production was determined in colon, where L-cells are mainly localized [1], as well as changes on the enteroendocrine cell amount and DPP4 activity on plasma and intestine were also evaluated. To further evaluate the effects of GSPE on GLP-1 production and sensitivity, GLP-1 and its receptor gene expression was determined in another tissue that is well known to express them, the hypothalamus [2].

GSPE has been previously reported to improve insulin resistance and insulinemia in cafeteria-fed rats after a corrective treatment [3,4], so we also assessed whether these effects are reproduced after a preventive treatment.

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Abstract

Grape-seed procyanidins (GSPE) have been reported to improve insulin resistance in cafeteria rats. Glucagon-like peptide-1 (GLP-1) is involved in glucose homeostasis and is impaired in type 2 diabetes and insulin resistant states. Thus, we evaluated the preventive effects of GSPE on GLP-1 production, secretion, and elimination. The cafeteria diet decreased active GLP-1 plasma levels, which is attributed to a decreased intestinal GLP-1 production, linked to reduced colonic enteroendocrine cell populations. Such effects were prevented by GSPE. In the same context, GSPE induces dipeptidyl-peptidase 4 (DPP4) activity. In the hypothalamus, we did not observe an apparent effect of the cafeteria diet but GSPE treatment increased GLP-1 production and down-regulated GLP-1 receptor gene expression. In conclusion, the preventive treatment with GSPE abrogates the effects of the cafeteria diet on intestinal GLP-1 production and DPP4 activity. Besides, the hypothalamic GLP-1 system is more sensitive to GSPE treatment than to the cafeteria diet.

Keywords: grape-seed procyanidin extract/glucagon-like peptide-1/dipeptidyl-peptidase 4/insulinemia

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Introduction

Procyanidins, a class of phenolic compounds, have been shown to improve glucose homeostasis in several models of glucose-homeostasis disruption [1]. Chronic corrective treatment using grape-seed procyanidins ameliorates insulin resistance in a cafeteria diet model [2]. These effects might be partially explained by their insulin-like effect on insulin-sensitive cell lines [3,4] as well as through their effects on pancreatic β-cell function, and by decreasing insulin secretion and production and lipid accumulation in the pancreas that is induced by the cafeteria diet [5]. Moreover, we have previously reported that the chronic treatment of healthy rats with GSPE increases the insulin/glucose ratio after oral glucose administration compared to intraperitoneal glucose infusion, suggesting an incretin-like effect [6]. However, the effect of procyanidins on incretins in an insulin resistance context has not been described yet.

The main incretin hormones are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), which are secreted by the intestinal L- (ileum and colon) and K-cells (duodenum and jejunum), respectively [7]. Plasma levels of GLP-1 have been reported to be reduced in type 2 diabetic patients [8,9] and in mice with high-fat diet (HFD)-induced insulin resistance [10], and exogenous infusions of GLP-1 were shown to improve glycemia [11,12] and insulin resistance [13] in these contexts. Along this line, therapies, including the use of bioactive food components, which would enhance the action of GLP-1, are of great interest to diminish the development of these pathologies. An *Ilex paraguariensis* leaf extract, which is rich in the phenolic compound 3,5-*O*-dicaffeoyl-*D*-quinic acid, was previously shown to improve glycemia and insulinemia in HF-fed mice, a result that was correlated with increased plasma levels of active GLP-1 [14]. Moreover, Dao et al. reported that chronic treatment with resveratrol, a polyphenolic compound found in red grapes, improves portal and intestinal levels of active GLP-1 and pro-glucagon gene expression in HF-fed mice [10].

The aim of this study was to assess whether preventive treatment with GSPE can counteract the effects of a cafeteria diet on glucose homeostasis, focusing on the GLP-1 system.

Research Design and Methods

Reagents

The grape seed procyanidin extract (GSPE) was obtained from *Les Dérivés Résiniques et Terpéniques* (Dax, France) and was previously fully characterized by our research group [15].

Animal experimental procedures

Female Wistar rats were purchased from Charles River Laboratories (Barcelona, Spain), housed in animal quarters at 22ºC with a 12-h light/ 12-h dark cycle and maintained for 1 week in quarantine. The animals were divided into the following three groups (8 animals/group): a control group fed with standard chow; a group fed with a cafeteria diet (bacon, biscuits with pâté, muffins, carrots and milk with sugar) and water plus the standard diet and were vehicle-treated; and a group fed with the cafeteria diet and treated with 25 mg GSPE/kg of body weight (bw) per day. The cafeteria diet-fed animals also had access standard chow (Panlab, Barcelona, Spain) and water, and the treatments were administered by voluntary feeding of the GSPE dose using sweetened condensed milk, diluted 1:10 with water, as a vehicle. Every day, the food was withdrawn at 9 a.m. and was replaced at 4 p.m.

At the 8th week of treatment, the rats were fasted overnight and an oral glucose tolerance test (OGTT) was performed. Briefly, 2 g glucose/kg bw was dissolved in water, administered by oral gavage, and tail blood samples were taken at 0, 15, 30 and 120 minutes after the administration of the glucose load. The plasma glucose and insulin levels were measured.

At the 9th week of treatment, the rats were fasted overnight, tail blood samples were taken and insulin levels were measured.

After 12 weeks of GSPE treatment, the overnight fasted animals, were anesthetized with 50 mg of pentobarbital/kg body weight and sacrificed by bleeding. The blood was collected and treated with a commercial DPP4 inhibitor (Millipore, Madrid, Spain) and a serine protease inhibitor, Pefabloc SC, (Roche, Barcelona, Spain) to prevent the inactivation of active GLP-1 and ghrelin, respectively. The animal tissue specimens were immediately frozen in liquid nitrogen and stored at -80 ºC until further analysis.

All of the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili.

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Plasma parameter measurements

The glucose plasma concentrations were assayed using an enzymatic colorimetric kit (GOD-PAP method from QCA, Tarragona, Spain). Insulin and plasma concentrations were determined using Rat Insulin ELISA/Ultrasensitive and Rat Insulin ELISA (Mercodia, Uppsala, Sweden). Total GIP and active GLP-1 plasma levels were measured using Rat/Mouse GIP (total) ELISA and GLP-1 (active) ELISA kits, respectively (Millipore, Madrid, Spain). All of the procedures were performed following the manufacturers' protocols.

AUC and HOMA-IR were calculated as previously described by Ou et al. and Matthews et al., respectively [16,17].

Intestinal GLP-1 extraction

Active GLP-1 was extracted from colon as previously described [18]. Briefly, colon samples were homogenized using an ethanol-acid solution (100% ethanol-sterile water-12 M HCl, 74:25:1), placed for 24 hours at 4ºC and centrifuged at 2,000 x g for 20 minutes at 4ºC. Supernatants were collected, diluted in 1X PBS and stored at -80ºC until further analysis. Active GLP-1 was measured as described above for the plasma samples.

Measurement of DPP4 activity

DPP4 was extracted from rat intestine as previously described [6]. Briefly, intestine samples were homogenized using lysis buffer (PBS containing 100 KIU/mL aprotinin and 1% Triton X-100), centrifuged at 1,000 x g at 4ºC for 10 minutes to eliminate the cellular debris, and centrifuged twice at 20,000 x g at 4ºC for 10 minutes. The supernatants were stored at -80º C until further analysis.

To determine the activity of DPP4 in the intestinal lysates and the rat plasma samples, the specimens were incubated with 0.2 mM H-glycylprolyl-pnitroanilide p-tosyalte (Bachem, Bubendorf, Switzerland) in Tris-HCl buffer at 37ºC, and the release of p-nitroanilide absorbance was measured every minute at 405 nm for 30 minutes.

Western Blot

Protein was extracted from the intestine using RIPA lysis buffer (15 mM Tris-HCl, 1% Triton X-100, 0,1% SDS, 167 mM NaCl and 0.5% Na-deoxycholate) with a protease inhibitor cocktail (diluted 1:1000, Sigma-Aldrich) and 1 mM PMSF. The total protein levels of the lysates were determined using the Bradford method [19]. The proteins were loaded and run on 10% SDS-polyacrylamide gels. The samples were transferred

onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA), blocked at room temperature using 5% (w/v) non-fat milk in TBST buffer (Tris-buffered saline with 0.2% (v/v) Tween-20) and incubated overnight at 4ºC with rabbit anti-β-actin antibody (Sigma-Aldrich) or anti-CD26 (Abcam, Cambridge, UK). After washing with TBST, the blots were incubated at room temperature with peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK). The blots were washed thoroughly in TBST, followed by TBS after immunoblotting, and the immunoreactive proteins were visualized using the ECL Plus Western blotting detection system (GE Healthcare). Densitometric analysis of the immunoblots was performed using ImageJ 1.44p software; all proteins were quantified relative to the loading control.

Quantitative RT-PCR

The total RNA from the hypothalamus was extracted using an RNAeasy Kit (Qiagen, Hilden, Germany), and the total RNA from the duodenum and colon was extracted using the TRIzol reagent following the manufacturers' protocols cDNA was generated using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). Quantitative PCR amplification and detection were performed using the following TaqMan assay-on-demand probes (Applied Biosystems): Rn00562910_m1 for DPP4, Rn00571500_m1 for GIP, Rn00562293_m1 for proglucagon, Rn01460420_g1 for PYY, Rn00572200_m1 for Chromogranin A (CgA), and Rn00562406_m1 for GLP-1 receptor (GLP-1R). β-actin was used as the reference gene (Rn00667869_m1). The relative mRNA expression levels were calculated using the ΔΔCt method.

Data analyses

The results are expressed as the mean values ± SEM. The effects were assessed by ANOVA and Student's t-test. All calculations were performed using SPSS software (SPSS, Chicago, USA).

Results

GSPE limits the cafeteria diet-induced decrease in intestinal GLP-1 biosynthesis

To analyze whether a preventive treatment with procyanidins modulates the incretin levels in cafeteria diet-fed rats, basal GIP and GLP-1 levels in plasma were assessed in rats fed a cafeteria diet for 12 weeks simultaneously with 25 mg of GSPE/kg of bw. As shown in **figure 1a**, cafeteria-fed rats exhibited reduced plasma levels of active GLP-1, although GSPE did not alter the active GLP-1 levels compared to the vehicle-treated

cafeteria-fed rats. Total GIP levels were neither modified by the cafeteria diet nor by the GSPE treatment (**figure 1b**).

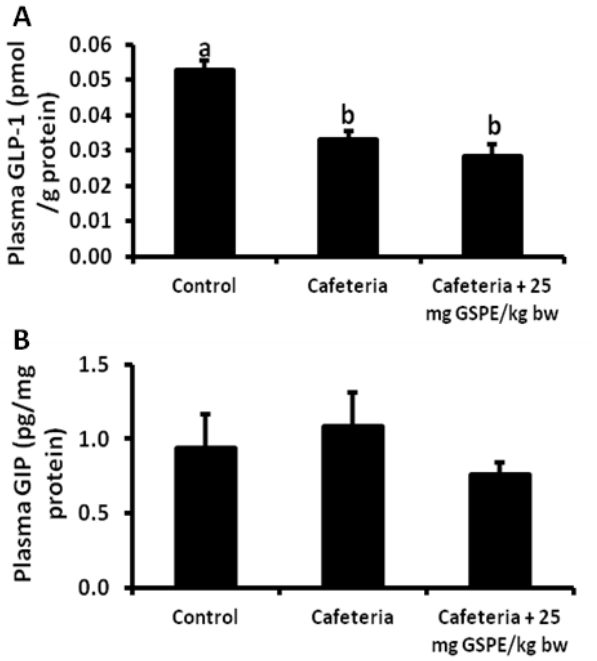

Figure 1. Effect of cafeteria diet and GSPE on incretin plasma levels. Effects on plasma active GLP-1 levels (**A**), and total GIP levels (**B**) were assayed after 12 weeks of cafeteria diet and preventive treatment with 25 mg of GSPE/kg of bw. The data are displayed as the mean ± *SEM*. **a** and **b**, statistically significant differences at $P < 0.05$.

To assess the effect of the cafeteria diet and GSPE treatment on incretin biosynthesis, we measured the GLP-1 levels and pro-glucagon gene expression in colon, as well as GIP gene expression in duodenum. As shown in **figure 2**, the colon levels of GLP-1 were decreased by the cafeteria diet by approximately 30% compared to the control diet (**figure 2a**), a reduction that was accompanied by a significant down-regulation of the pro-glucagon gene expression (**figure 2b**). The GSPE treatment prevented the decrease in colon levels of GLP-1 produced by cafeteria-diet, although it did not reach control levels (**figure 2a**). This effect was also observed for the expression of the pro-glucagon gene as shown in **figure 2b**. GIP gene expression in the duodenum was unaffected either by the cafeteria diet or the GSPE treatment (0.93 *± 0.17* and 0.89 *± 0.10*, respectively, versus control (1.03 *± 0.09*) ($P > 0.05$)).

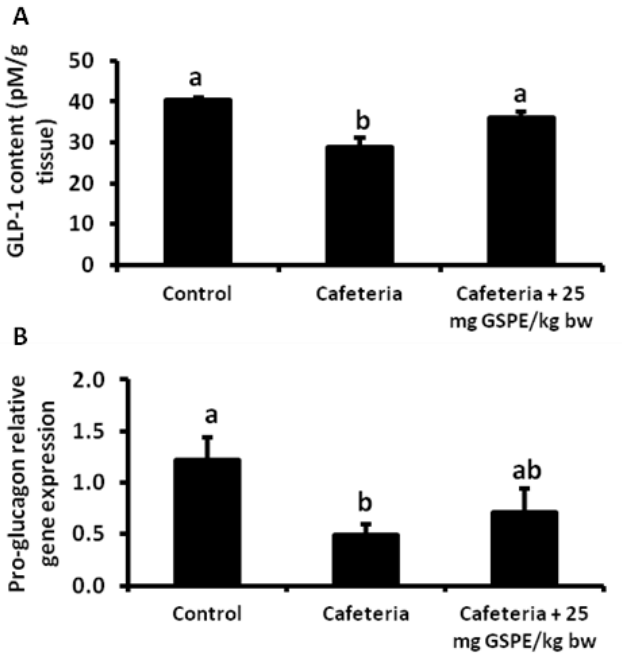

Figure 2. Effect of cafeteria diet and GSPE on GLP-1 production in colon. Effects on colon active GLP-1 content (**A**), and proglucagon gene expression (**B**) were determined after 12 weeks of cafeteria diet and simultaneous treatment with 25 mg of GSPE/kg of bw. The data are displayed as the mean ± *SEM*. **a**, **b** and **c**, statistically significant differences at $P < 0.05$.

GSPE prevents the cafeteria diet-induced decrease of enteroendocrine cells in the colon

To determine whether the reduction in GLP-1 is due to a modulation of GLP-1 gene expression or to a general effect on GLP-1 producing cells, we tested the effects of the cafeteria diet and GSPE on PYY, another hormone that is also secreted by intestinal L-cells. We evaluated PYY gene expression, and as shown in **figure 3a,** it was significantly down-regulated due to the cafeteria diet, whereas GSPE treatment partially blocked this effect.

Next, we analyzed the gene expression of CgA, a marker of endocrine differentiation. **Figure 3b** shows that the expression of CgA gene in colon was significantly decreased in the vehicle-treated cafeteria diet-fed rats, whereas GSPE treatment prevented the effect of the cafeteria diet, similar to the effect observed for GLP-1 and PYY. The expression of CgA gene in the duodenum was also assessed, but it was unaffected by the cafeteria diet or by the GSPE treatment (0.88 *± 0.11* and 1.13 *± 0.27*, respectively, versus control (1.16 *± 0.22*) ($P > 0.05$)).

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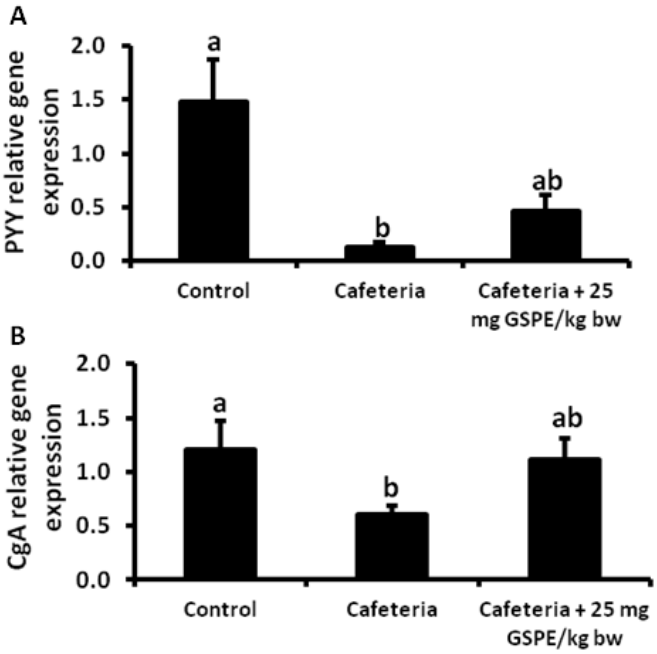

Figure 3. Effect of cafeteria diet and GSPE on intestinal PYY and CgA gene expression. Gene expression of colon PYY (**A**) and CgA (**B**) were determined after 12 weeks of cafeteria diet and simultaneous treatment with 25 mg of GSPE/kg of bw. The data are displayed as the mean ± *SEM*. **a** and **b**, statistically significant differences at $P < 0.05$.

GSPE modulates intestinal DPP4

To assess whether the cafeteria diet and the GSPE treatment could also modify the enzyme responsible for incretin degradation, DPP4, the plasma and intestinal levels of DPP4 activity were measured at the end of the treatment. As shown in **figure 4a**, plasma DPP4 activity was slightly but significantly decreased by the cafeteria diet, whereas it was unaffected by the GSPE treatment. Intestinal DPP4 activity was also reduced by the cafeteria diet, whereas in contrast to the effects in plasma, the simultaneous GSPE treatment prevented this decrease (**figure 4b**). To analyze whether the preventive effect elicited by GSPE was due to a modulation of DPP4 production, its gene and protein expression were also evaluated in the intestine. As shown in **figures 4c** and **4d**, DPP4 gene expression and protein levels were reduced by the cafeteria diet, which is consistent with what was observed in terms of its activity. GSPE prevented the cafeteria-induced decrease in DPP4 protein expression, but elicited no effect at the gene expression level.

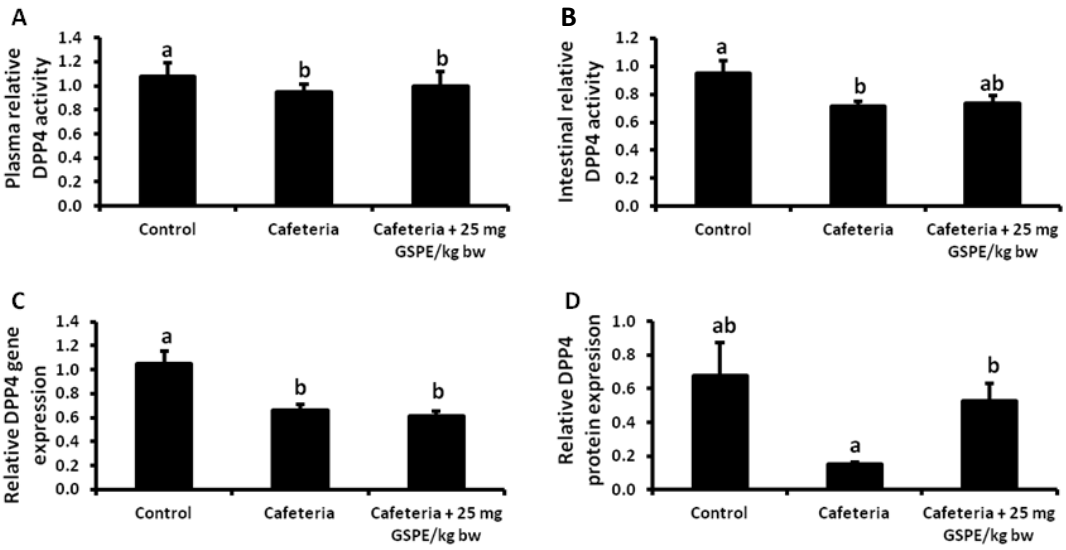

Figure 4. Effect of cafeteria diet and GSPE on DPP4. After 12 weeks of cafeteria diet and simultaneous treatment with 25 mg of GSPE/kg of bw, DPP4 activity was determined in plasma (**A**), and intestine (**B**). Intestinal DPP4 gene expression (**C**) and protein expression (**D**) were also assessed by RT-PCR and Western Blot, respectively. The data are displayed as the mean ± *SEM*. **a**, **b** and **c**, statistically significant differences at $P < 0.05$.

GSPE treatment modulates the expression of GLP-1 and GLP-1 receptor genes in the hypothalamus

To evaluate the systemic preventive effects of GSPE on GLP-1 production, pro-glucagon gene expression was also assessed in the hypothalamus. As shown in **figure 5a**, the cafeteria diet tended to up-regulate pro-glucagon expression and GSPE treatment further enhanced this effect. We also evaluated the sensitivity to GLP-1 by assaying GLP-1 receptor (GLP-1R) gene expression, and we found that its expression was unaffected by the cafeteria diet, whereas it was down-regulated by the GSPE treatment compared to cafeteria-fed rats treated with the vehicle (**figure 5b**).

GSPE modulates insulinemia after 9 weeks of treatment

To assess whether a preventive GSPE treatment can modulate insulinemia, an OGTT of 2 hours was performed at the 8th week of treatment. As shown in **figure 6a**, insulin AUC (area under the curve) was increased in cafeteria diet-fed rats, whereas a preventive GSPE treatment prevented this effect.

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Moreover, fasting plasma insulin and glucose levels were measured after 9 and 12 weeks to test whether preventive GSPE treatment affects insulin resistance induced by the cafeteria diet. As shown in **figure 6b**, HOMA-IR was significantly increased after 9 weeks of cafeteria diet, whereas a simultaneous treatment with 25 mg of GSPE/kg of bw prevented this effect, resulting in a healthier HOMA-IR. However, after 12 weeks, GSPE treatment was unable to prevent the increase in HOMA-IR induced by the cafeteria diet (**figure 6c**).

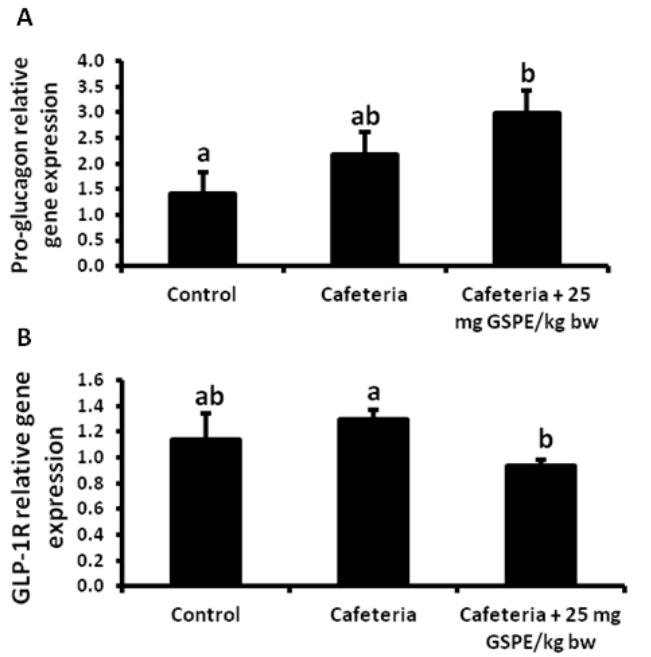

Figure 5. Effect of cafeteria diet and GSPE on hypothalamic GLP-1 and GLP-1R.. Effects on hypothalamic gene expression of pro-glucagon (**A**), and GLP-1R (**B**) were assessed after 12 weeks of cafeteria diet and preventive treatment with 25 mg of GSPE/kg of bw. The data are displayed as the mean ± *SEM*. **a** and **b**, statistically significant differences at $P < 0.05$.

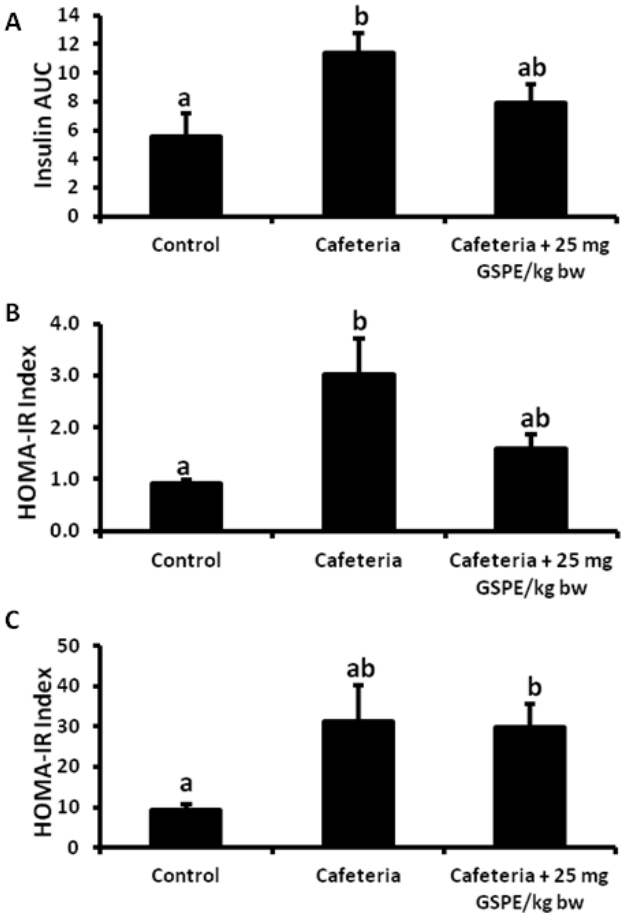

Figure 6. Effect of cafeteria diet and GSPE on insulinemia. After 8 weeks of cafeteria diet and a preventive treatment with 25 mg of GSPE/kg of bw, a 2 hour-OGTT was performed, insulin was analyzed at time 0, 15, 30 and 120 minutes and the AUC was calculated (**A**). Plasma glucose and insulin levels were also determined and HOMA-IR was calculated after 9 (**B**), and 12 weeks (**C**). The data are displayed as the mean ± *SEM*. **a** and **b**, statistically significant differences at $P < 0.05$.

Discussion

Procyanidins from grape seed have been shown to improve insulin resistance, eliciting a corrective effect when the glucose homeostasis of the animal has been slightly disturbed by a cafeteria diet [1,2,5,20]. However, whether GSPE can act in a preventive way has not yet been described. Moreover, GSPE has been shown to elicit an incretin-like effect in healthy rats [6]. However, our understanding of the effects of GSPE on the incretin system in animals with disturbed glucose homeostasis remains limited. In this study, we developed a 12-week cafeteria diet-fed model that caused a decrease in GLP-1 plasma levels and production in the intestine, with no effects on GLP-1 production and detection in the hypothalamus. The cafeteria diet also caused a decrease in the amount of DPP4 enzyme at the plasma level and in the intestine, and the HOMA-IR clearly revealed the induction of insulin-resistance. We found that procyanidins were able to counteract several of these effects, which were predominantly found in the intestine.

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Regarding GLP-1 production, the GSPE-treated animals exhibited an increased amount of GLP-1 in colonic cells, with a simultaneous increase in GLP-1 mRNA. Similarly, Dao et al. reported that resveratrol increased the colon levels of active GLP-1, together with an increase in pro-glucagon gene expression, in mice fed with a HFD [10]. Dao et al. also suggested that the increase in GLP-1 production by resveratrol might explain its preventive effects on plasma levels of GLP-1, which are reduced in the vehicle-treated HFD-fed mice after the administration of an oral glucose load but are increased in the resveratrol-treated HFD-fed animals [10]. GSPE has also been found to effectively improve glucose-stimulated GLP-1 secretion. In healthy rats, we previously showed that an acute dose of GSPE increased the glucose-stimulated plasma levels of GLP-1 (submitted results). Some other proanthocyanidins (i.e., berry purees [21] and the tetrameric procyanidin cinnamtannin A2 [22]) have also been shown to elicit this effect.

Our results showed that treatment with GSPE also up-regulated the colon expression of the PYY gene, a hormone that has been reported to be co-expressed with GLP-1 in enteroendocrine L-cells [23]. Enteroendocrine cells actively self-renew and differentiate throughout the life of an animal [24], and procyanidins are known to modify cell proliferation [20,25]. Therefore, we assessed whether GSPE modulated CgA levels, a marker of endocrine cells [26]. We found that GSPE again counteracted the effects of the cafeteria diet. Our results suggest that the modulation on GLP-1 production is not only due to a direct modulation of its gene expression but also to changes in the amount of enteroendocrine cells, which were reduced by the cafeteria diet. There are no previous studies that have analyzed the effects of a cafeteria diet on the number of enteroendocrine cells. A high-fat diet was suggested to increase the proliferation of L-cells, causing a dysfunction and reducing GLP-1 secretion [27]. The different results might be attributed to differences in experimental conditions (type of diet, animal gender, length of the study) between studies. Moreover, that study analyzed the effects within the duodenum, whereas we found effects that were limited to the colon, the predominant location for GLP-1 production [28]. In contrast, we found that the expression of CgA and GIP genes in the duodenum, where GIP-producing cells are predominantly found [29], was unchanged in our experiment, which is consistent with the unaltered plasma levels of GIP.

The effects of GSPE on DPP4 are consistent with our previous results, which demonstrated the sensitivity of intestinal DPP4 to GSPE and a lack of GSPE effects on plasma DPP4 [6]. In this study, we found that GSPE prevents the decrease in intestinal DPP4 activity and protein caused by a cafeteria diet. These results differ from previous observations because we had shown an inhibitory effect of 25 mg of GSPE/kg of bw after 45 days of treatment in healthy animals or after 30 days in animals with cafeteria diet-induced insulin resistance [6]. The effect that a cafeteria diet elicited on DPP4 is

unclear. Several authors have reported an increase in circulating DPP4 activity in humans with obesity or type 2 diabetes [30-32] and in rats with type 1 diabetes or impaired glucose tolerance [33,34]. However, limited studies in humans have shown decreased plasma DPP4 activity in diabetic subjects [35] and unchanged activity in obese, diabetic and impaired-glucose tolerance subjects [31,36]. Taking together, these data indicate that a severe degree of hyperglycemia is required to induce an increase on circulating DPP4 activity, whereas mild hyperglycemia is insufficient to induce its increase [31,36]. Our model supports this conclusion because the cafeteria diet did not affect glycemia, which is consistent with the lack of increased DPP4. In contrast, we found a decrease that is partially prevented by GSPE. In a completely different context, the flavonoid apigenin has been reported to counteract the decrease in DPP4 activity caused by cancer in a colorectal carcinoma cell line, an observation that is in line with the effects on DPP4 observed in our study [37]. However, there is limited information regarding DPP4 activity in the intestine. Thus, it is difficult to make further conclusions. Considering that this is the first preventive study on GSPE effects, further studies are warranted to fully assess the implications of the DPP4 inhibition in the cafeteria diet-fed model and of the opposing effects elicited by GSPE.

To evaluate the systemic preventive effects of GSPE on GLP-1, we analyzed GLP-1 and its receptor in the hypothalamus, where they are also expressed [38]. Previous studies have shown that brain pro-glucagon gene expression is up-regulated by HFD [39]. We observed a tendency to increase hypothalamic pro-glucagon gene expression. However, we found that GLP-1 expression was stimulated in GSPE-cafeteria-fed rats. This stimulation is consistent with the reduced expression of the GLP-1R gene. Knauf et al. found that blockade of brain GLP-1Rs in HF-fed mice improves insulin resistance and enhances glucose utilization by muscle [39]. The available evidence suggests that enteric glucose absorption activates GLP-1R-sensitive CNS networks that promote enhanced glucose disposal. Furthermore, brain GLP-1R signaling controls peripheral blood flow and insulin sensitivity predominantly under hyperinsulinemic, hyperglycemic conditions. The relative importance of central versus peripheral GLP-1 action for the control of glucose homeostasis remains unclear [38].

All of these results demonstrate that GSPE interacts with the GLP-1 system, predominantly preventing the negative effects induced by a cafeteria diet. Therefore, the GSPE-treated animals were expected to be less insulin-resistant than those of the cafeteria diet group. At the 8^{th} week, we performed an OGTT and found that the GSPE treatment corrected the glucose-induced increase in insulin AUC as expected. Indeed, these results are consistent with the action of a GSPE corrective treatment of 4 weeks, which was previously shown to improve peripheral insulin resistance at the same dose [2]. This effect appeared to be maintained at 9 weeks, when we observed an improved

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HOMA-IR due to GSPE pretreatment. However, surprisingly, at 12 weeks, the HOMA-IR was no longer different between the vehicle and GSPE cafeteria-treated rats. We had found a high variability of the GSPE effect on the insulin levels depending on the GSPE dose received [40], and our current results reinforce this earlier finding. In addition, we previously explained that GSPE elicits its effects via its action on insulin synthesis and on the insulin degradation enzyme. Together with the results of this study, we now include new GSPE targets that might be helpful in the further elucidation of how procyanidins act in organisms.

In conclusion, we have found that a cafeteria diet induces a decrease in active GLP-1 levels by decreasing GLP-1 production in the colon, which might be attributed to a loss of enteroendocrine cells, an effect that is prevented by simultaneous treatment with GPSE. GSPE treatment also increased hypothalamic GLP-1 production, and down-regulated GLP-1Rs, opposing the effects of the cafeteria diet. This preventive action also impacts intestinal DPP4, predominantly by preventing the decrease in its activity and protein levels. Our results show that preventive GSPE treatment is effective for correcting HOMA-IR in cafeteria fed rats only for a limited period of time.

Acknowledgments

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The authors declare that they have no conflict of interest.

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IV. SUMMING-UP

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Previous studies done in our research group reported that a procyanidin extract from grape-seed (GSPE) can improve a situation of insulin resistance induced by a cafeteria diet. A decrease on the fasting insulinemia and the HOMA-IR index have been found in animals treated with GSPE [1], which was linked to the procyanidins insulin-like effect in adipose tissue [2]. These results also suggested an action on pancreas functionality, which was subsequently confirmed, evidencing that procyanidins modulate insulin production and secretion [3,4], as well as pancreatic β-cell proliferation [5,6]. Because the mechanisms for hormone secretion are very similar between the different endocrine cells [7] and the fact that GLP-1 is a key hormone for glucose homeostasis regulation, enteroendocrine cells were suggested as a possible target for procyanidins. Besides, it is known that procyanidins have a low bioavailability *in vivo* [8-10], pointing out that they might be directly interacting with the intestine, the first tissue involved on sensing food components. Moreover, procyanidins have been found to have affinity for salivary and plasma enzymes [11-13] and an epicatechin derivative found in *Vitis vinifera* has been predicted *in silico* to inhibit DPP4, the GLP-1 degrading enzyme [14], suggesting that procyanidins might inhibit the DPP4 activity, leading to a lower GLP-1 inactivation. However, the effects of GSPE on GLP-1 secretion, production and/or cleavage have not been reported yet. Hence, this doctoral thesis was developed to verify whether modulation of active GLP-1 levels is involved on the antihyperglycemic effect of GSPE. To achieve our objectives, we used *in vivo* models of healthy and insulin resistant rats induced by a cafeteria diet or by genetic factors (*Zucker fatty*); and *in vitro* models, for which the intestinal absorptive CaCo-2, endothelial HUVEC and enteroendocrine STC-1 cell lines were used.

Initially, our first objective was to demonstrate whether GSPE modulates active GLP-1 levels. Since GLP-1 secretion is induced by nutrient consumption, mainly glucose [15], the *in vivo* effect of an acute GSPE treatment was evaluated on healthy rats after a simultaneous oral glucose administration. We found a significant increase on plasma active GLP-1 levels, in concordance with other polyphenols that have been reported to enhance glucose-triggered GLP-1 levels [16-18], reinforcing the idea that GLP-1 is a potential target for procyanidins. GSPE also produced an enhancement of the insulin/glucose plasma profile after the oral glucose load, which might be produced by the higher levels of active GLP-1, highlighting the relevance of the GLP-1 modulation on the GSPE antihyperglycemic effect. The mechanisms by which GSPE enhances active GLP-1 levels could be due to its action on GLP-1 production, secretion, and/or degradation.

Regarding to its degradation, limiting the GLP-1 cleavage by inhibiting its degrading enzyme (DPP4) is one of the current strategies used for the pharmacological treatment of type 2 diabetes [19,20]. Therefore, the effects of GSPE on DPP4 activity were assessed

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in several *in vivo* experimental models and, in all cases, plasma DPP4 activity was unaffected. As shown in *in vitro* assays, high GSPE concentrations are needed to direct inhibit DPP4 activity, but their low bioavailability *in vivo* [8-10] impedes to achieve such concentrations in plasma, which could explain this lack of effect. Although commercial DPP4 inhibitors have been commonly reported to exert their effect by inhibiting plasma DPP4 activity (reviewed in [21]), Dai et al. showed a non-proportional relationship between plasma DPP4 inhibition and the increase on active GLP-1 levels [22]. Otherwise, Hansen et al. reported that a half of the newly secreted GLP-1 is degraded before reaching the systemic circulation and suggested an involvement of the DPP4 present in the endothelium of the capillaries next to the GLP-1-secreting cells [23]. Thus we determined intestinal DPP4 activity that was decreased after a chronic treatment with GSPE in healthy animals and in a model of insulin resistance induced by diet. As we determined intestinal DPP4 activity using lysates from the whole intestine, an *in vitro* approach was performed to mimic the intestinal environment and assess whether the inhibition found is produced in inner tissues, such as the endothelium of the capillaries. We indirectly co-cultured CaCo-2 (enterocytes) and HUVEC (endothelial) cells and found that the GSPE compounds absorbed and/or metabolized by the intestinal barrier were able to inhibit the endothelial DPP4, suggesting that GSPE might act on the DPP4 present on the endothelium of the capillaries, leading to an increase on active GLP-1 levels.

Conversely, intestinal DPP4 activity was unaffected by GSPE in *Zucker fatty* rats and increased after a preventive treatment in rats with a diet-induced-insulin resistance, thus counteracting the effect provoked by the cafeteria diet. These data suggest that the effect of procyanidins on intestinal DPP4 activity depends on the treatment and the degree of obesity achieved, reinforcing our group previous experience that showed different effects of procyanidins depending on the dose and the animal model [24].

Because GSPE is a mixture of different phenolic compounds [25], we further examined the molecules responsible for the DPP4 inhibition. To do that, CaCo-2 cells were treated with GSPE on its apical side and the basolateral media containing the metabolites and absorbed compounds was analyzed by chromatographic analyses. The major molecules found were catechin, epicatechin, procyanidin dimer and gallic acid, which were used for *in vitro* direct inhibition assays. The obtained results suggest that the inhibition found on the endothelium of the capillaries is due to an additive effect of the absorbed molecules.

As explained above, another target for GSPE could be the GLP-1-producing cells in the intestine. Previous studies in our group have reported that GSPE modulates insulin secretion and production from pancreatic β-cells by limiting mitochondrial membrane

hyperpolarization, which was linked to changes on cellular membrane potential [4]. Therefore, considering that the mechanisms acting on endocrine cells are very similar between the different types of hormone-producing cells [7] and that glucose triggered-GLP-1 levels are higher after GSPE treatment, we analyzed the effect of procyanidins on GLP-1 secretion and the mechanisms involved. Because enteroendocrine cells are scattered as individual cells along the whole intestine [26], we could not perform *in vivo* secretion studies, hence we used an *in vitro* model, the enteroendocrine STC-1 cell line. Since these cells are in direct contact with the intestinal lumen [27], they might be able to sense food components before they were absorbed, so STC-1 cells were treated with the whole extract of procyanidins, in absence and presence of nutrients. Low doses of GSPE caused a tendency to increase GLP-1 secretion in absence of nutrients, whereas high doses significantly inhibited it. In the presence of different types of nutrients (glucose, fatty acids or amino acids), high doses of GSPE also decreased GLP-1 secretion, while low doses did not provoke any change.

Several authors have found that cellular membrane depolarization is determinant for nutrient-induced hormone secretion [28-33], so a modulation of the cellular membrane potential might be involved in the GSPE effect on GLP-1 secretion. To test that, we assessed plasma membrane potential and found that GSPE modulates it in parallel to changes on GLP-1 secretion in a dose-dependent manner. Accordingly, we concluded that the modulation of GLP-1 secretion by GSPE might be due to changes on the cellular membrane potential. The mechanisms by which procyanidins modulate membrane potential has not been elucidated, but Margina et al. reported that several polyphenols from tea, grape seeds and *Curcuma longa* decrease membrane depolarization in a dose-dependent manner by affecting its fluidity [34], which could be an explanation for the effects of GSPE.

Finally, we also wanted to test whether GSPE modulates GLP-1 production in a situation in which GSPE effects had not been tested before: a **preventive** treatment with GSPE in a model of insulin resistance induced by diet. We found that, after 12-weeks, the cafeteria diet-fed animals had lower active GLP-1 levels in plasma, which was linked to a decrease on its production in colon, effect that was partially avoided by the treatment with GSPE. Our results are similar to those shown by Dao et al., who found that resveratrol, polyphenolic compound present in red grapes, is able to increase portal active GLP-1 levels due to an increase on its colonic production [17]. Moreover, we assessed whether these effects were linked to a direct modulation of GLP-1 gene expression or to a general effect on GLP-1-producing cells, finding that the cafeteria diet induces a decrease on the amount of enteroendocrine cells in colon and GSPE can prevent it. This could be due to a modulation of proliferation and/or apoptosis, effect

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that has been previously described by our group on pancreatic β-cells [5,6,35], although further studies should be performed to confirm it.

In this preventive experiment, a sensitivity to GSPE was also found in hypothalamus, a tissue that also expresses GLP-1 and its receptor [36,37]. We observed that the cafeteria diet tended to increase pro-glucagon expression, similarly to previous studies performed in HFD-fed animals [38,39], and preventive GSPE treatment enhanced it. Regarding to GLP-1R, its expression was down-regulated by GSPE compared to cafeteria-fed rats. These results show that the hypothalamus is a potential target for procyanidins too, although whether it is a direct or indirect effect is still unclear. The decrease provoked by GSPE on GLP-1R gene expression in hypothalamus suggests an attempt to counteract the increased GLP-1 production, which has been reported to increase hepatic glycogen production and insulin resistance in muscle [38,39]. Moreover, it has been reported that brain GLP-1R blockage improves insulin resistance in HFD-fed mice [38], suggesting a new mechanism by which GSPE might counteract the cafeteria diet-induced insulin resistance.

Moreover, in the same experiment, oral glucose-induced insulin secretion and insulin resistance were improved after 8 and 9 weeks of preventive treatment with GSPE, respectively. Since peripheral GLP-1 infusion has been reported to improve insulin sensitivity [40], our results suggest that this improvement on insulinemia could be, in part, due to the increased GLP-1 production. However, after 12 weeks, the preventive effect of GSPE on insulin resistance was lost, suggesting that a preventive treatment with procyanidins only corrects the HOMA-IR for a limited period of time. Nevertheless, the improvement on the HOMA-IR index in insulin resistant animals has only been reported after a corrective treatment with 25 mg of GSPE/kg of bw (reviewed in [24]), so this is the first study of the preventive effects of 25 mg of GSPE/kg of bw and more studies are needed to make further conclusions.

In conclusion, GSPE modulates active GLP-1 levels that could, in part, explain its antihyperglycemic effect. This increase on GLP-1 levels might be due to the modulation of colonic GLP-1 production and secretion, as well as by its modulation of intestinal DPP4 activity. Otherwise, considering that procyanidins are absorbed and metabolized in several steps since their ingestion, it could be suggested that the molecules that reach each tissue are different, so the GSPE effects at different levels of the GLP-1 system, such as the intestine and the hypothalamus, could be due to different molecules and/or metabolites.

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V. CONCLUSIONS

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1. **An oral administration of GSPE enhances the increase on active GLP-1 levels induced by an oral glucose challenge. This effect can, in part, explain the higher plasma insulin levels caused by GPSE.**

2. **DPP4 is a target for GSPE action.**

   - In healthy animals, intestinal DPP4 activity is inhibited by GSPE.

   - The inhibition found on the DPP4 present on the endothelium of the capillaries in the intestine are explained by the inhibitory action of the pure molecules present in the original extract (catechin, epicatechin, procyanidin B2 and gallic acid).

   - In obesity models, induced by diet as well as by genetic factors, the GSPE effect depends on the treatment and the degree of obesity that is reached.

3. **Procyanidins modulate *in vitro* GLP-1 secretion from the enteroendocrine cell line STC-1.**

   - GSPE modulates GLP-1 secretion from enteroendocrine cells in a dose-dependent manner, increasing it at low doses and inhibiting it at high doses.

   - Procyanidins act modulating cellular membrane potential, conditioning GLP-1 secretion.

   - High doses of GSPE modulate the effect of nutrients on GLP-1 secretion. This effect is due to a blocking on the nutrient-triggered membrane depolarization.

4. **A preventive treatment with GSPE modulates the cafeteria diet effects on GLP-1 production.**

   - GSPE limits the decrease on intestinal GLP-1 production induced by cafeteria diet through acting on the number of enteroendocrine cells.

   - In hypothalamus, GSPE treatment enhances the increase on GLP-1 production induced by cafeteria diet and decrease gene expression of its receptor.

V. Conclusions

1. **Una càrrega oral de GSPE potencia l'increment dels nivells de GLP-1 activa induïts per una càrrega oral de glucosa. Aquest efecte pot explicar, en part, l'increment produït per GSPE en la insulina plasmàtica.**

2. **DPP4 és una diana de l'acció del GSPE.**

   - En animals sans, l'activitat DPP4 intestinal és inhibida per GSPE.

   - La inhibició de la DPP4 present a l'endoteli del capil·lars a l'intestí s'explica per l'acció inhibitòria de les molècules pures originàries de l'extracte (catequina, epicatequina, procianidina B2 i àcid gàlic).

   - En models d'obesitat, ja sigui induïda per la dieta o per causes genètiques, l'acció del GSPE depèn del tractament i el grau d'obesitat assolit.

3. **Les procianidines modulen la secreció de GLP-1 *in vitro* en la línia de cèl·lules enteroendocrines STC-1.**

   - GSPE modula la secreció de GLP-1 per part de les cèl·lules enteroendocrines d'una manera dosi-depenent, incrementant-la a dosis baixes i inhibint-la a dosis altes.

   - Les procianidines actuen modulant el potencial de membrana cel·lular, condicionant la secreció de GLP-1.

   - Dosis altes de GSPE modulen l'efecte dels nutrients sobre la secreció de GLP-1. Aquesta acció passa per impedir la despolarització que aquests causen a la membrana plasmàtica.

4. **El tractament preventiu amb GSPE modula els efectes de la dieta de cafeteria sobre la producció de GLP-1.**

   - GSPE limita la disminució en la producció intestinal de GLP-1 induïda per una dieta de cafeteria mitjançant la acció sobre la quantitat de cèl·lules enteroendocrines.

   - A hipotàlem, el tractament amb GSPE accentua l'increment produït per la dieta de cafeteria en la producció de GLP-1 i disminueix la expressió gènica del seu receptor.

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VI. ANNEXES

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Identification of novel human dipeptidyl peptidase-IV inhibitors of natural origin (Part I): Virtual screening and activity assays

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Identification of Novel Human Dipeptidyl Peptidase-IV Inhibitors of Natural Origin (Part I): Virtual Screening and Activity Assays

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Abstract

Background: There has been great interest in determining whether natural products show biological activity toward protein targets of pharmacological relevance. One target of particular interest is DPP-IV whose most important substrates are incretins that, among other beneficial effects, stimulates insulin biosynthesis and secretion. Incretins have very short half-lives because of their rapid degradation by DPP-IV and, therefore, inhibiting this enzyme improves glucose homeostasis. As a result, DPP-IV inhibitors are of considerable interest to the pharmaceutical industry. The main goals of this study were **(a)** to develop a virtual screening process to identify potential DPP-IV inhibitors of natural origin; **(b)** to evaluate the reliability of our virtual-screening protocol by experimentally testing the *in vitro* activity of selected natural-product hits; and **(c)** to use the most active hit for predicting derivatives with higher binding affinities for the DPP-IV binding site.

Methodology/Principal Findings: We predicted that 446 out of the 89,165 molecules present in the natural products subset of the ZINC database would inhibit DPP-IV with good ADMET properties. Notably, when these 446 molecules were merged with 2,342 known DPP-IV inhibitors and the resulting set was classified into 50 clusters according to chemical similarity, there were 12 clusters that contained only natural products for which no DPP-IV inhibitory activity has been previously reported. Nine molecules from 7 of these 12 clusters were then selected for *in vitro* activity testing and 7 out of the 9 molecules were shown to inhibit DPP-IV (where the remaining two molecules could not be solubilized, preventing the evaluation of their DPP-IV inhibitory activity). Then, the hit with the highest activity was used as a lead compound in the prediction of more potent derivatives.

Conclusions/Significance: We have demonstrated that our virtual-screening protocol was successful in identifying novel lead compounds for developing more potent DPP-IV inhibitors.

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Introduction

Type 2 diabetes mellitus (T2DM) is considered to be the "epidemic of the 21st century" and, consequently, the development of new therapies is one of the main challenges in drug discovery today [1]. While current T2DM therapies that increase insulin secretion have proven to have beneficial therapeutic effects, these treatments often suffer from undesirable side effects such as hypoglycemia and weight gain [2]. Therefore, there is a significant unmet medical need for better drugs to treat T2DM.

Recently, the inhibition of human dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) has emerged as a new treatment option for T2DM [3]. This enzyme belongs to the serine protease family and selectively removes N-terminal dipeptides from substrates containing proline or alanine as the second residue. The most important substrates of DPP-IV are incretins, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [4]. GLP-1 is released from intestinal L-cells in response to meals and performs the following actions: GLP-1 stimulates insulin biosynthesis and secretion, reduces glucagon release, slows gastric emptying, reduces appetite, and stimulates the regeneration and differentiation of islet B-cells [5]. Alternatively, GIP is produced by the duodenal K-cells and is extensively involved in glucose metabolism by enhancing insulin secretion [6]. Both peptides have very short half-lives (4 min for GIP and only 1–2 min for GLP-1) because of their rapid degradation by DPP-IV. Inhibiting DPP-IV prolongs the action of GLP-1 and GIP, which, in turn, improves glucose homeostasis with a lower risk of hypoglycemia.

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Consequently, DPP-IV inhibitors are of considerable interest to the pharmaceutical industry [7], and intense research activities in this area have resulted in the launch of sitagliptin, saxagliptin, alogliptin, linagliptin and vildagliptin to the market [8].

The DPP-IV binding site is highly druggable in the sense that tight and specific binding to the enzyme can be achieved with small molecules with drug-like physicochemical properties [9,10]. The different interaction motifs used by these DPP-IV ligands include Ser630 (that together with Asp708 and His740 form the enzyme catalytic triad), the hydrophobic S1 pocket (formed by Tyr631, Val656, Trp659, Tyr662, Tyr666 and Val711), the hydrophobic S2 pocket (formed by Arg125, Phe357, Arg358, Tyr547, Pro550 and Asn710) and the N-terminal recognition region (formed by Glu205, Glu206 and Tyr662) [9,11]. Based on the analysis of the DPP-IV crystal structures [12–18] and interpretation of the structure-activity relationship (SAR) data, both the lipophilic S1 pocket and the Glu205/Glu206 dyad can be considered as crucial molecular anchors for DPP-IV inhibition [9].

The large scaffold diversity and properties of natural products (NPs), such as structural complexity and drug similarity, makes these molecules ideal starting points for drug design. The main goal of this paper is to apply a virtual screening (VS) protocol to identify NPs with DPP-IV inhibitory activity as well as different scaffolds relative to known DPP-IV inhibitors that could be used as lead compounds in drug-design. In order to achieve this goal, we first identified complexes between DPP-IV and potent reversible inhibitors of non-peptide nature in the PDB. After validating the fit of the coordinates of binding site residues and inhibitors onto the corresponding electron density map, the validated DPP-IV complexes were overlapped to get the experimental poses of the inhibitor in the same orientation. Subsequently, the relative contribution of the different intermolecular interactions to the protein-ligand binding affinity was quantified to derive structure-based pharmacophores. The resulting energetically optimized pharmacophores were used to derive a structure-based common pharmacophore that contained key intermolecular interactions between DPP-IV and the inhibitors. The exclusion volumes were also determined and added to the pharmacophore. Then, the previous structure-based pharmacophore and a VS protocol were used to look for DPP-IV inhibitors in a NPs database [19], and the reliability of the prediction was demonstrated using *in vitro* testing to determine the DPP-IV inhibitory effects of representative VS hits. Lastly, the hit with the highest activity was used as a lead compound in a combinatorial screen for the prediction of more potent DPP-IV inhibitors.

Results and Discussion

Common Structure-based Pharmacophore Building and Description

There are currently 54 entries for DPP-IV in the Protein Data Bank (PDB; http://www.pdb.org; see Table 1) [20] but only 10 of those entries correspond to validated complexes of the native enzyme with potent reversible inhibitors of a non-peptide nature (see Figure 1). As a result, only these 10 entries are suitable for deriving reliable structure-based pharmacophores that capture the key intermolecular interactions needed for drugs to inhibit DPP-IV. In order to define a common background for DPP-IV inhibition, we identified features of inhibitors that make the most important contributions to the bioactivity of the ligand by first superposing all 10 PDB files. Then, the energetic pharmacophores were derived from the resulting coordinates, and energetically relevant pharmacophore sites were visually inspected for finding common or frequent ones. Figure 2 shows that all 10 pharmacophores have two sites in common (one positive/donor and one hydrophobic/aromatic ring) that often make the most important contribution to the protein-ligand binding affinity (see data for sites **P/D** and **H/R1** in Table 2). From these data, we inferred that these two sites are essential for the inhibition of DPP-IV and considered them to be required in the common structure-based pharmacophore (see Figure 3). Interestingly, previous studies have identified the lipophilic S1 pocket (formed by Tyr631, Val656, Trp659, Tyr662, Tyr666 and Val711) and the Glu205/Glu206 dyad as crucial molecular anchors for inhibition [9,21,22] and, in coherence with this, the mandatory hydrophobic/aromatic ring and positive/donor sites interact with the S1 pocket and Glu205/Glu206, respectively. Table 2 also shows that there are two other hydrogen-bond acceptors (**A1** and **A2**) and three hydrophobic/aromatic ring sites (**H/R2**, **H/R3** and **H/R4**) that, although not common to all experimental poses, could increase either protein-ligand binding affinity or drug-specificity. Moreover, it is remarkable that these sites correspond to interactions with other relevant areas from the DPP-IV binding site. For example, the **H/R2** site interacts with Phe357, Arg358 and Tyr547 in the S2 pocket (known to preferentially recognize large hydrophobic and aromatic side chains [11]). Therefore, these sites were also included as optional sites in the common structure-based pharmacophore (see Figure 3).

VS Workflow Description and Application to the NP Subset of the ZINC Database

The VS workflow (see Figure 4) consisted of several sequential steps where the output molecules of one step were the input molecules for the next step and so on. The NP subset of the ZINC database was used as the source of molecules to which our VS schema was applied to search for new DPP-IV inhibitors. Initially, these 89,165 molecules were submitted to an ADME/Tox filter with the FAF-Drugs2 tool [23] aimed at discarding molecules that were either potentially toxic or exhibited poor ADME properties.

Conformers for molecules with appropriate ADME/Tox properties were then filtered with Phase through the structure-

Table 1. Codes for DPP-IV structures currently available at PDB.

Valid PDB Structures			Discarted PDB Structures				
1N1M	**2OPH**	**2RIP**	**(a)**	**(b)**	**(c)**	**(d)**	**(e)**
2FJP	2OQI	3C43	1J2E	1TKR*	1R9N	1RWQ	1X70
2HHA	2OQV	**3C45**	1NU6	2AJL	1WCY	2BUB	2OAG
2I78	2P8S	3CCC	1NU8	2G5T	2BGN	2JID	3CCB
2IIT	2QJR	3D4L	1PFQ	2G5P	2BGR		3EIO
2IIV	2QOE	3F8S	1R9M	2G63			
2OGZ	**2QT9**	**3H0C**	1TK3	2I03			
2OLE	**2QTB**	**3HAB**	1U8E	2QKY			
2ONC	**2RGU**	**3HAC**	1W1I	3BJM*			

Some PDB structures were discarded for the following reasons: (a) the structures were of apo forms without inhibitor, (b) inhibitors were covalently linked with Ser630, (c) inhibitors were of oligopeptide nature, (d) there were no structural factors available in the PDB or (e) the scripts in the EDS failed to produce the map from the structural factors. PDB structures marked with an asterisk (*) have mutations in the enzyme to modify the activity. Only the PDB files from the "**Valid PDB Structures**" section with IC_{50} values $\leq$10 nM (in bold) were used to derive the corresponding structure-based common pharmacophore for DPP-IV inhibition (see Figure 1).
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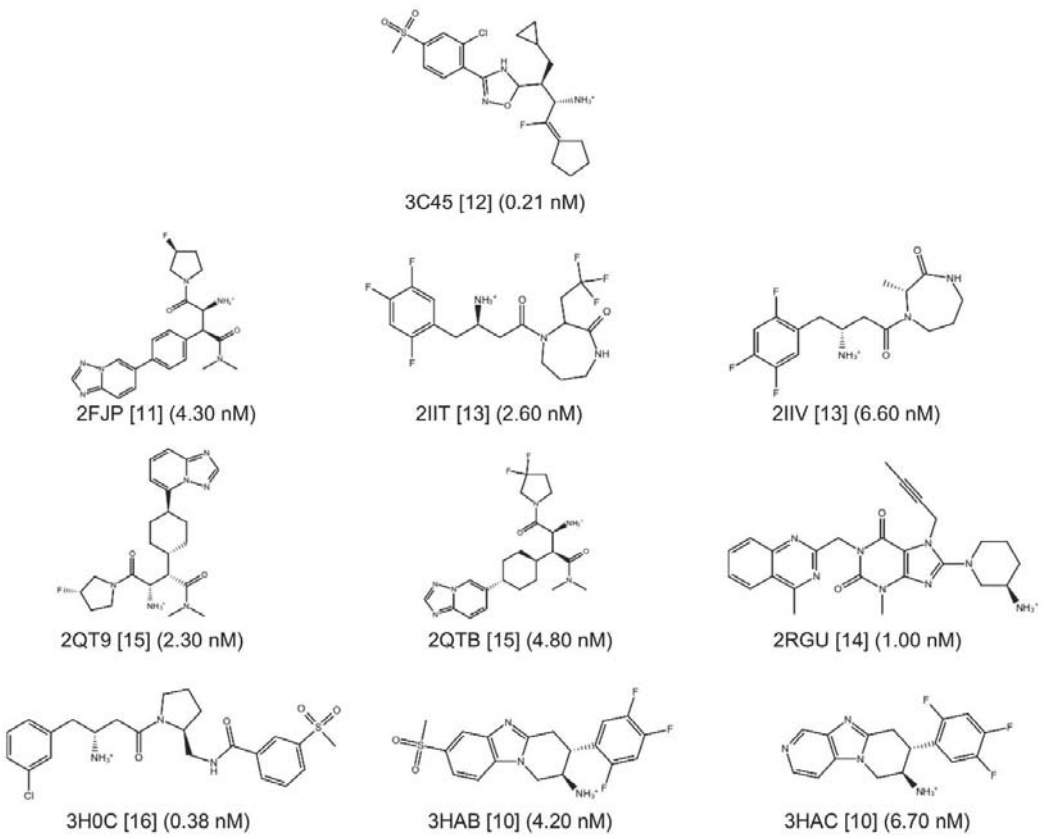

Figure 1. Drug-like reversible DPP-IV inhibitors used for the generation of the common structure-based pharmacophore with their corresponding IC_{50} values. The codes of the PDB complexes from which the ligand poses were used are also shown.
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based common pharmacophore. Ligands with at least one hit in the Phase search were then used in a protein-ligand rigid-docking study and docked onto the ligand binding site of the DPP-IV conformation present in the 3C45 PDB file [14]. In order to find docking poses that were compatible with the pharmacophore, the resulting ligand poses were filtered again with Phase through the structure-based common pharmacophore using the same filtering conditions as in the first Phase run but without reorienting the poses (*i.e.*, the score in place option was used). From these two pharmacophore screens, we obtained 4,952 compounds (see Figure 4) with at least one pose that was both compatible with the DPP-IV active site and had functional groups that match the 3D location of the two compulsory sites and at least one of the optional sites of the structure-based common pharmacophore.

Finally, the poses for the 4,952 compounds from the second pharmacophore screen were submitted to a shape and electrostatic-potential comparison with the experimental pose of the DPP-IV inhibitor at the PDB file 3C45 (that has the smallest IC_{50} for all the non-peptide reversible inhibitors found in DPP-IV-inhibitor complexes at the PDB [14]; see Figure 1). The shape and electrostatic-potential comparison identified 446 hit molecules with potential DPP-IV inhibitory activity (see Figure 4).

Finding New Scaffolds of Natural Origin for DPP-IV Inhibitors

One of the most important challenges of any VS workflow is the ability to find molecules with the required activity but without trivial similarity (in terms of chemical structure) to known active compounds. To determine which of the 446 potential DPP-IV inhibitors predicted by our VS workflow could be considered as new lead molecules, we merged the 446 potential DPP-IV inhibitors with 2,342 known DPP-IV inhibitors that were obtained from the BindingDB database [24]. After calculating the 2D fingerprints of these inhibitors, the resulting set was classified into 50 clusters by means of a hierarchical cluster analysis (data not shown). Notably, 12 out of the 50 clusters obtained consisted exclusively of NPs that were previously unidentified as DPP-IV inhibitors. The 219 molecules that belong to these 12 clusters are scaffold-hopping candidates for DPP-IV inhibition (see Table S1). To prove the reliability of our predictions, we selected 9 molecules (**C1** and **C2** from cluster 30, **C3** from cluster 36, **C4** from cluster

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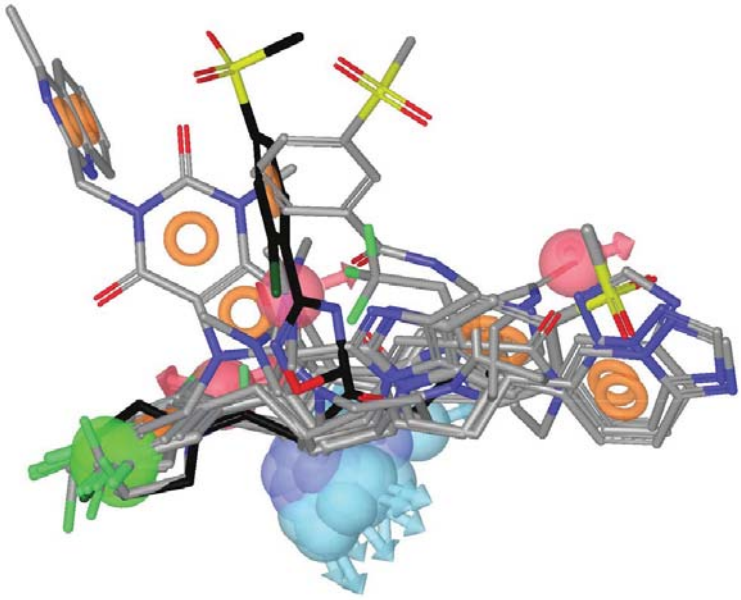

Figure 2. The relative location of the experimental poses of the ligands in Figure 1 after DPP-IV superposition. The experimental pose for the most potent inhibitor (*i.e.,* the one at 3C45) is shown in black for reference. For each ligand, the energetically relevant pharmacophore sites are shown. Light red and light blue spheres represent the acceptor and donor features, respectively. The green spheres and orange torus display the hydrophobic regions and aromatic rings, respectively. Blue spheres represent positively charged regions.
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37, **C5** and **C6** from cluster 41, **C7** from cluster 45, **C8** from cluster 49 and **C9** from cluster 50) from 7 of these 12 clusters (see Figure 5) and tested their effects on the DPP-IV activity using an *in vitro* assay. The results of this experiment demonstrated that 7 out of the 9 molecules (**C1**, **C2**, **C3**, **C5**, **C7**, **C8** and **C9**) inhibit DPP-IV (see Figure 6). The remaining molecules, **C4** and **C6**, could not be solubilized, preventing the evaluation of their DPP-IV inhibitory activity. The lack of DPP-IV inhibitory activity for **C5**, **C7** and **C9** at 1mM was also due to insolubility (see Figure 6). Furthermore, Figure 6 shows that from all the tested molecules, **C5** is the most potent inhibitor with an IC_{50} of 61.55 μM (see Figure 7). With the exception of **C1**, which significantly inhibited DPP-IV only at 1 mM, the rest of the molecules significantly inhibit DPP-IV at 0.25 mM (see Figure 6) showing a dose-response effect. Moreover, a SciFinder search (Chemical Abstracts Service, Columbus, Ohio, USA; http://www.cas.org/products/sfacad) of the literature revealed that none of these 7 molecules

Table 2. Site contribution to the energy-optimized pharmacophores obtained from PDB complexes in bold from Table 1.

PDB	2FJP	2IIT	2IIV	2QT9	2QTB	2RGU	3C45	3H0C	3HAB	3HAC
P/D	−4,6	−4,13	−4,45	−4,09	−4,54	−1,66	−4,54	−4,81	−4,54	−4,29
H/R1	0,77	−1,29	−1,36	−1,25	−1,68	−0,075	−0,64	−1,18	−1,1	−1,25
H/R2	−0,69			−0,66	−0,68				−0,9	−0,69
H/R3						−1,94				
H/R4							−0,85			
H/R5						−0,56				
A1	−0,64			−0,4	−0,59					
A2			−0,62							
A3	−0,35				−0,35					
A4						−0,44				

Required and optional sites at the structure-based common pharmacophore are shown in bold and italics, respectively. The other sites are not part of the structure-based common pharmacophore. Data at the same raw for different PDB complexes indicate that the pharmacophore site is shared by these complexes.
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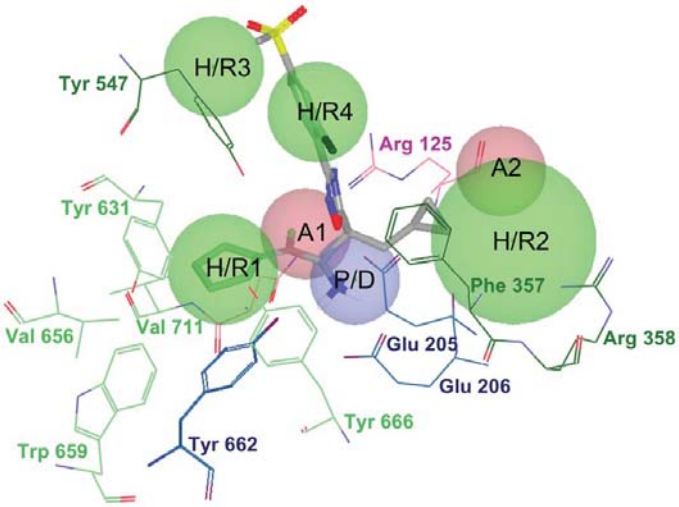

Figure 3. The structure-based common pharmacophore derived from the alignment of the poses in Figure 2 and shown in the context of the 3C45 active site. The pharmacophore is formed by two hydrogen-bond acceptors (*i.e.,* **A1** and **A2**), one positive/hydrogen-bond donor feature (*i.e.,* **P/D**) and 4 hydrophobic/aromatic ring sites (*i.e.,* **H/R1**, **H/R2**, **H/R3** and **H/R4**). The associated tolerances (*i.e.,* radii) of the pharmacophore are 1.8Å for **P/D**, **A1** and **A2**, 2.0Å for **H/R1**, **H/R3** and **H/R4** and 3.3Å for **H/R2**. Two out of these seven sites (*i.e.,* **P/D** and **H/R1**) are required during pharmacophore-based searches whereas the remaining five are optional. The **P/D** site interacts with the Glu205/Glu206 dyad whereas the **H/R1** site potentially fills the S1 pocket. The residues are colored according to the type of intermolecular interactions involved. For example, blue residues interact with donor sites, pink residues interact with acceptor sites and green residues are involved in hydrophobic contacts. Light green residues are a part of the S1 pocket.
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have been reported as antidiabetic drugs. In fact, no bioactivity has been described for these 7 molecules.

Structural Analysis of the Inhibition of DPP-IV by C1, C2, C3, C5, C7, C8 and C9

The docking of **C1**, **C2**, **C3**, **C5**, **C7**, **C8** and **C9** in the DPP-IV binding site of the 3C45 structure demonstrated that these molecules match the structure-based common pharmacophore in the same orientation, sharing the same intermolecular interactions with DPP-IV (see Figures 8 and 9A). With the exception of **C7** in which the positive charge of the tertiary amine forms a salt bridge with Glu205/Glu206 (see Figure 8D), all compounds use primary or secondary amines to form hydrogen bond interactions with either Glu206 or with the Glu205/Glu206 dyad side chains (see Figures 8 and 9A). Additionally, all molecules filled the S1 pocket (partially in the case of **C1** and **C8,** which could explain why these two molecules have lower activities as DPP-IV inhibitors; see Figure 6) establishing one intermolecular interaction that corresponds to the compulsory **H/R1** site of our common structure-based pharmacophore (see Figure 3). Moreover, it is worthwhile to mention that some molecules could potentially form additional hydrogen bonds with DPP-IV. For example, the hydroxyl and the methoxy groups of **C1** could hydrogen bond with the side chains of Glu206 and Ser630, respectively (see Figure 8A). **C8** forms two additional hydrogen bonds with the side chains of Arg358 and Tyr666 (see Figure 8E). Finally, **C9** could form three additional hydrogen bonds with the side chains of Tyr547, Ser630 and Tyr662 (see Figure 8F).

Figure 9A shows the best docking pose of **C5** in the DPP-IV binding pocket where its tertiary amine hydrogen bonds with Glu206. The carbonyl oxygen of the 7-hydroxy-2H-chromen-2-one moiety could also hydrogen bond with the Tyr666 side chain. The S1 pocket is occupied by the **C5** butyl chain that could form hydrophobic interactions with Tyr662, Tyr666 and Val711. Finally, the chromene ring of the 7-hydroxy-2H-chromen-2-one moiety forms π-π interactions with Phe357. Interestingly, this interaction with Phe357 has been shown to be directly related to the increased potency of synthetic DPP-IV inhibitors relative to those that lack this interaction [13,15,25–27]. Therefore, the fact that this interaction is only present at **C5** (see Figures 8 and 9A) would explain why this molecule shows higher bioactivity than the other compounds assayed (see Figure 6). Moreover, an electrostatic and shape comparison of the 7 poses in Figures 8 and 9A revealed that the molecule with the highest similarity to the 3C45 ligand (with the lowest IC_{50}; see Figure 1) is **C5** (results not shown). The ET_combo score for this comparison is 1.050, which corresponds to a shape and electrostatic contribution of 0.628 and 0.422, respectively. Remarkably, the same analysis with **C2** (which shows a significant bioactivity as DPP-IV inhibitor; see Figure 6), also has a significant ET_combo score of 1.038.

Predicting ZINC02132035 Derivatives with Higher Binding Affinity on DPP-IV

Although none of the seven *in vitro* assayed VS hits showed activity in the nanomolar range, these hits incorporate scaffolds with no previously described effects on DPP-IV activity and, consequently, are of interest from a medicinal chemistry point of view as lead compounds for more potent DPP-IV inhibitors. With this goal in mind, we have predicted derivatives for the most potent DPP-IV inhibitor found in our dose-response studies (*i.e.*,

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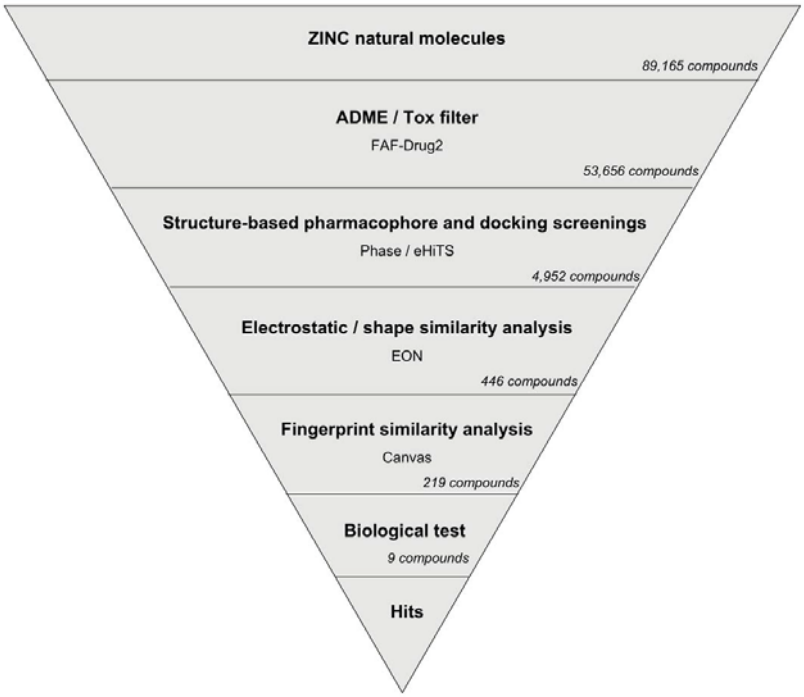

Figure 4. Schematic overview of the VS workflow and the procedure used for selecting the VS hits that were tested for DPP-IV inhibitory activity. For the VS, the number of compounds that passed each step and the programs used are showed. For the selection of VS hits for bioactivity testing, the numbers show either how many VS hits are scaffold-hopping candidates for DPP-IV inhibition (*Fingerprint similarity analysis* step) or how many molecules were experimentally tested for bioactivity (*Biological test* step).
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C5; see Figure 6) by **(1)** using a fragment-based docking approach to identify which intermolecular interactions with the DPP-IV binding site could improve the binding affinity of **C5** derivatives relative to **C5**; **(2)** using this information to identify where changes in **C5** should be made; and **(3)** accordingly building **C5** derivatives and predicting their relative binding affinities.

The comparison of the XP descriptors from **C5** and from the docked poses of the fragments showed that while some of the terms of the scoring function are 0.00 Kcal/mol for **C5**, their corresponding value for 13 out of 50 fragments is in the [-2.48, -0.83] Kcal/mol range (see Table S2). Interestingly, 12 out of 13 of these fragments bind at the locations of three of the sites of our structure-based common pharmacophore (*i.e.*, **H/R1**, **H/R2** and **H/R4**), whereas the remaining fragment is close to the **H/R1** site (see Table S2). These findings demonstrate that our pharmacophore is able to *capture* all of the essential features for an inhibitor interaction with the DPP-IV binding-site, which would explain why all of the assayed molecules show activity as DPP-IV inhibitors (see Figure 6). Moreover, the analysis in Table S2 shows that **C5** activity can be improved if **(1)** its butyl group matching the **H/R1** site is replaced by a group that is able to interact with the lipophilic atoms of the S1 pocket either by producing the so-called *hydrophobic enclosure reward* (that would consist of enclosing the two sides of the substituent –at a 180° angle– on the hydrophobic environment of the S1 pocket) or by making π-cation interactions with the aromatic side chains in this pocket and **(2)** groups that match the **H/R2** site (optional in our pharmacophore but present in half of the ligands used to derive the pharmacophore; see Table 2) and that are able to make hydrophobically packed correlated H-bonds are added to **C5**.

The substituents that were attached to the **C5** core to obtain the top five derivatives with the highest predicted binding affinity for the DPP-IV binding site are shown in Table S3. None of the five molecules are currently registered in ChemSpider (http://www.chemspider.com), whereas their analysis with FAF-Drugs2 shows that all of these derivatives exhibit the proper ADMET properties. Therefore, these derivatives are undescribed drug-like molecules that, according to their XP GScores (see Table S3), would show a significant increase in their binding affinity relative to **C5** (*i.e.*, -4.2 Kcal/mol).

Figure 9 shows the docked poses for **C5** derivatives compared with **C5** and can be used to explain the structural basis of the expected increase in binding affinity. Remarkably, the XP GScores for these poses are in the -9.5 to -11.8 Kcal/mol range (see Table S3), whereas the GScores for the experimental poses of the DPP-IV inhibitors shown in Figure 1 are in the -5.8 to -11.0 Kcal/mol range (results not shown). Therefore, the **C5** derivatives reported in Table S3 are likely to exhibit nanomolar activity as DPP-IV inhibitors.

As shown in Figure 9, the **C5** derivatives usually maintained the most important protein-ligand interactions found for the **C5** core. Moreover, Table S3 also shows that all of the substituents that

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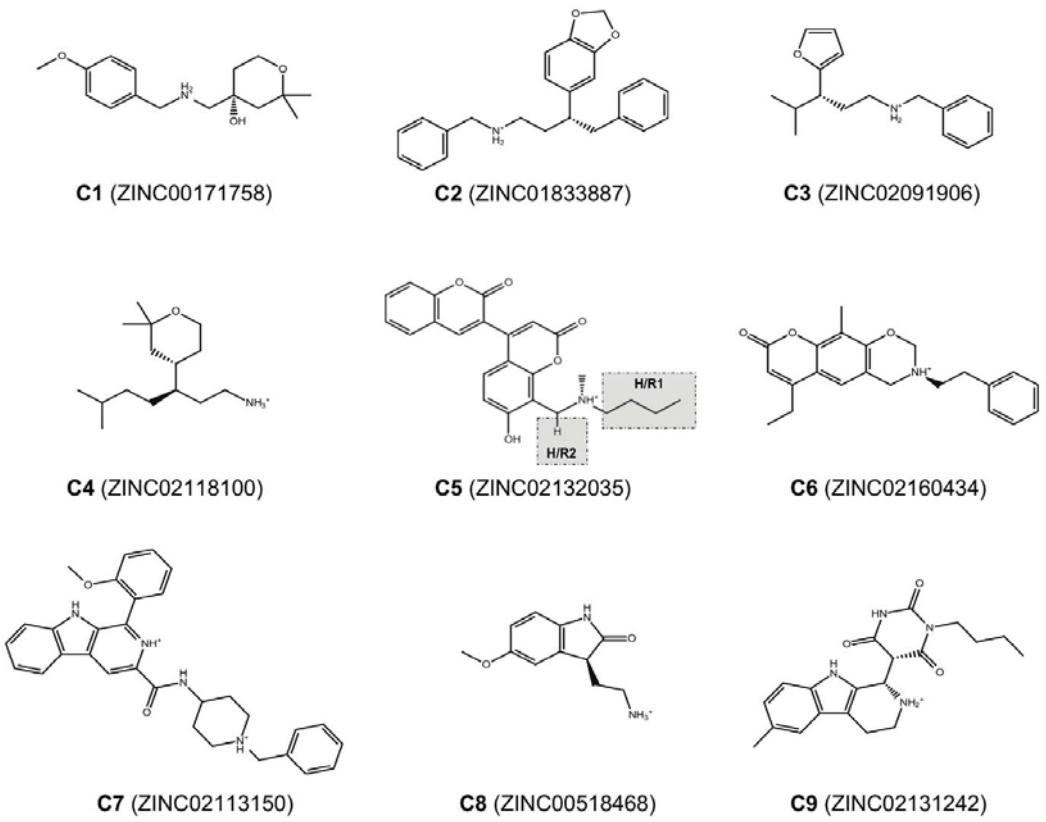

Figure 5. Chemical structures and ZINC codes for the 9 molecules selected for experimentally testing whether these compounds exhibited DPP-IV inhibitory activity. The insolubility of **C4** and **C6** prevented these compounds from being assayed for DPP-IV inhibitory activity. Positions in the **C5** structure that will be replaced by substituents to identify derivatives with higher binding affinity on the DPP-IV binding site are **(a)** indicated with a grey background and **(b)** annotated with the label of the corresponding site in the common structure-based pharmacophore (see Figure 3).
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have replaced the original **C5** butyl group (*i.e.*, at the **H/R1** site) have a common positive formal charge that, according to results shown in Figure 9, allows them to form π-cation interactions with two of the aromatic residues in the S1 pocket (*i.e.*, Tyr662 and Tyr666). Additionally, some of the substituents at this location (*i.e.*, **97** in **C5-97-786**, **100** in **C5-100-563** and **274** in **C5-274-536**; see Table S3) also aid in increasing the protein-ligand binding affinity by enclosing the two sides of the corresponding ring in the lipophilic protein environment in the S1 pocket (results not shown). Furthermore, all substituents at the **H/R2** site (except the one in **C5-309-787**) are able to make hydrogen bonds either with the S2 pocket residue Arg358 (*i.e.*, **786** in **C5-97-786**, **784** in **C5-137-784** and **563** in **C5-100-563**; see Figures 9B, 9C and 9D) or with Arg669 (*i.e.*, **536** in **C5-274-536**; see Figures 9F). The **786** substituent in **C5-97-786** is also able to make a hydrogen bond with the Ser209 side chain (see Figure 9B). Remarkably, there are SAR studies with a structurally distinct series of DPP-IV inhibitors that show (**1**) a 4-fold loss of potency when substituents that interact with the side chains of Ser209 and Arg358 are removed [28], (**2**) a 2-fold increase in inhibition when a carboxylic acid that interacts with Arg358 is introduced [27], and (**3**) a 6-fold increase in inhibition when a 3-pyridyl group that interacts with Ser209 is introduced [29]. Therefore, the substituents selected for the **H/R2** site by the combinatorial screen are able to form the intermolecular interactions with the S2 pocket that previous SAR studies with anti-diabetic drugs have shown to increase the affinity for the DPP-IV binding site.

Conclusions

The challenge of any VS protocol consists of using *in silico* tools to predict which molecules in a database have the required activity against a specific target. The results of the present study demonstrate that our VS protocol is highly successful in the non-trivial identification of DPP-IV inhibitors with no chemical-structure similarities to known activities. Therefore, scaffold hopping on this target can be achieved. Moreover, this is the first time that anti-diabetic activity has been described for **C1** (*i.e.*, ZINC00171758), **C2** (*i.e.*, ZINC01833887), **C3** (*i.e.*,

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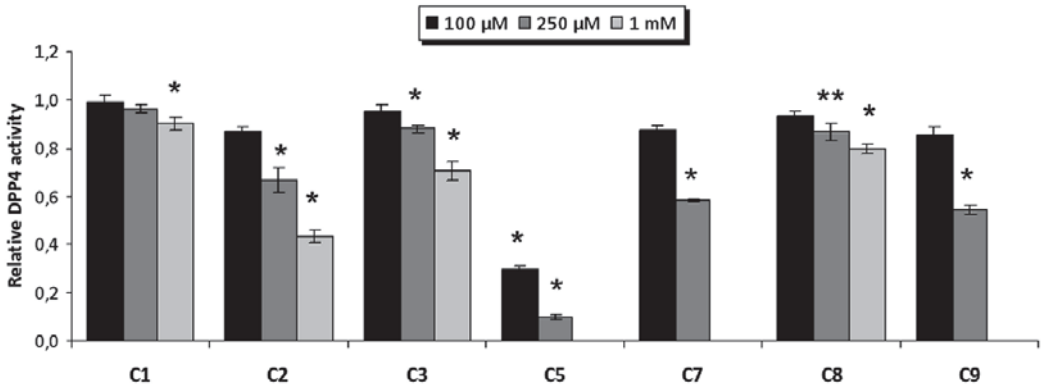

Figure 6. Dose-response results for the *in vitro* inhibition of DPP-IV by C1, C2, C3, C5, C7, C8 and C9. The relative DPP-IV inhibitory activity with or without the selected NPs (vehicle, 1% DMSO) is shown where each column represents the average ± SEM (n = 3 or 4). The insolubility of **C5**, **C7** and **C9** in DMSO at 1 mM prevented the measurement of DPP-IV inhibitory activity. *p<0.05 **p<0.1 vs vehicle, T-student.
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ZINC02091906), **C5** (*i.e.*, ZINC02132035), **C7** (*i.e.*, ZINC02113150), **C8** (*i.e.*, ZINC00518468) and **C9** (*i.e.*, ZINC02131242).

Although the IC_{50} of the 7 hit molecules indicates their *in vitro* activity is significantly lower than that of most known DPP-IV inhibitors used to derive the structure-based common pharmacophore (see Figure 1), it is important to remark that these molecules can be used as lead compounds for developing more potent inhibitors by means of SAR studies. Furthermore, these 7 molecules were selected based on their commercial availability, cost and purity with the primary goal of testing the performance of our VS protocol. Therefore, it is possible that there are other molecules among the remaining 210 molecules in clusters 10, 29, 30, 36, 37, 38, 40, 41, 44, 45, 49 and 50 (see Table S1) that could be better starting points than **C5** for the rational drug design of potent and selective DPP-IV inhibitors with new chemical scaffolds. Remarkably, our work makes a significant contribution to the discovery of DPP-IV inhibitors of natural origin (described, at present, for only few NPs [21,30–32]) from a quantitative point of view. Moreover, this work is also applicable to screen synthetic molecules databases when looking for antidiabetic activity.

Finally, we would like to note the high degree of agreement between our predictions (without making any prior knowledge-based assumptions that could bias our decisions) about the derivatization of **C5** to increase the binding affinity (*e.g.*, introducing side chains that could interact with Ser209 and Arg358) and what SAR studies have reported in the literature for achieving this increase. Therefore, this strongly supports the reliability of our combinatorial screening results.

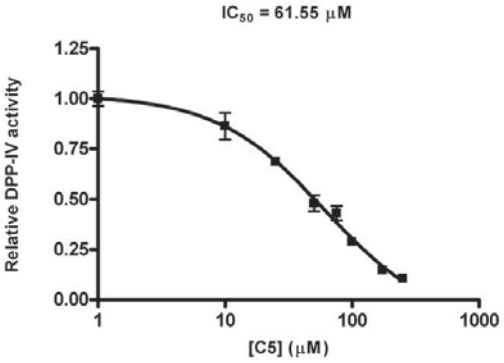

Figure 7. DPP-IV inhibitory dose-response curve obtained for C5 via a competitive binding assay.
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Methods

Criteria for Selecting the 3D Structures for DPP-IV Complexes used to Derive the Common Structure-based Pharmacophore

Coordinates for complexes between DPP-IV and potent reversible inhibitors were obtained from the PDB with the help of the following information: (a) LigPlot [33] schemes downloaded from the PDBsum website (http://www.ebi.ac.uk/pdbsum/) that were used to confirm the non-peptide and reversible character of the DPP-IV inhibitor present in each complex and; (b) IC_{50} values directly extracted from the literature describing the complexes (only complexes with inhibitors with $IC_{50} \leq 10$ nM were considered). Furthermore, the complexes with at least one mutation in their amino acid sequences were discarded. The reliability of the binding-site residues and inhibitor coordinates was assessed for the remaining complexes by visually inspecting their degree of fitness to the corresponding electron density map available from the Uppsala Electron Density Server (EDS; http://eds.bmc.uu.se/eds/) [34].

Superposition of the Selected DPP-IV Structures

The coordinates from the PDB complexes that met all the mentioned requirements were superposed with the DeepView v3.7 program (http://spdbv.vital-it.ch/) [35] to have the complexes in the same relative orientation. Only the resulting re-oriented coordinates for these PDB files were used during the subsequent structure-based pharmacophore generation and in the steps of the VS workflow (*i.e.*, pharmacophore-based searches, protein-ligand

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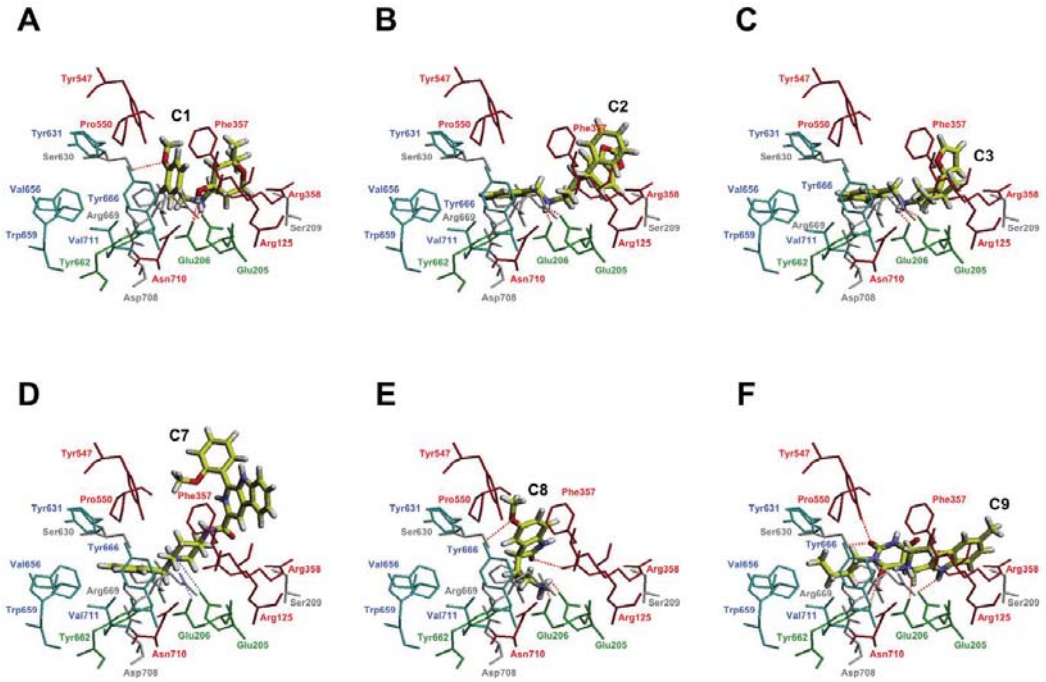

Figure 8. Docking poses for C1, C2, C3, C7, C8 and C9 at the 3C45 binding site. All of the panels in this figure and in Figure 9 are in the same relative orientation to allow for easier comparisons between the predicted poses. Residues at the DPP-IV binding site are colored according to the subsite where they belong (*i.e.*, residues from the S1 pocket are colored in cyan, those from the S2 pocket are red and those from the N-terminal recognition region are green). Other important residues that have not been classified in any pocket are colored in white. Dashed lines are used to show intermolecular hydrogen bonds (in red) or charge-charge interactions (in blue).
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docking studies and shape and electrostatic-potential comparisons) where spatial orientation is crucial.

Common Structure-based Pharmacophore for DPP-IV Inhibition

Energetic structure-based pharmacophores were built from the superposed coordinates of the previously selected complexes by means of the Glide-based procedure developed by Schrödinger (Schrödinger LLC., Portland, USA; http://www.schrodinger.com) [36]. According to this procedure, pharmacophore sites are ranked based on the Glide XP energies with the advantage that each contribution to the protein-ligand interactions is quantified. Therefore, energetically favorable features can be incorporated into the pharmacophore with preference over energetically weaker features. The resulting individual energetic pharmacophores were used for the construction of a common structure-based pharmacophore for DPP-IV reversible inhibition. This pharmacophore consists on two compulsory sites (one positive/donor and one hydrophobic/aromatic ring) whereas the remaining acceptor and hydrophobic/aromatic ring sites are optional. The associated tolerances for the different sites are 1.8Å for **P/D**, **A1** and **A2**, 2.0Å for **H/R1**, **H/R3** and **H/R4** and 3.3Å for **H/R2**. The pharmacophore was completed with receptor-based excluded volumes that schematically represent the location of the DPP-IV residues that form the binding pocket by applying the ***Receptor-Based Excluded Volumes*** graphic front-end from Phase v3.1 (Schrödinger LLC., Portland, USA; http://www.schrodinger.com) [37] to the PDB file 3C45. The ***Sphere filters*** parameter values were set to the following criteria: (a) ignoring receptor atoms whose surfaces were within 0.25 Å of ligand surface; and (b) limit excluded volume shell thickness to 10 Å. Otherwise, the remaining parameter values used were the default values.

Ligand Selection for VS Purposes

Ligands for VS purposes were downloaded from the Natural Products subset of the ZINC database (http://wiki.bkslab.org/index.php/Natural_products_database) [19]. This dataset contains 89,165 commercially available natural products and natural-product derivatives, making the dataset suitable for experimentally testing the success of a VS workflow.

ADME/Tox Filter

The ADME/Tox filter was carried out with the FAF-Drugs2 tool [23]. The drug-like properties of a compound were evaluated using the Lipinski rule [38]. The Lipinski rule is based on a set of property values, such as the number of hydrogen-bond donors and acceptors, the molecular weight and the logP, that were derived from drugs with good ADME characteristics. Molecules that

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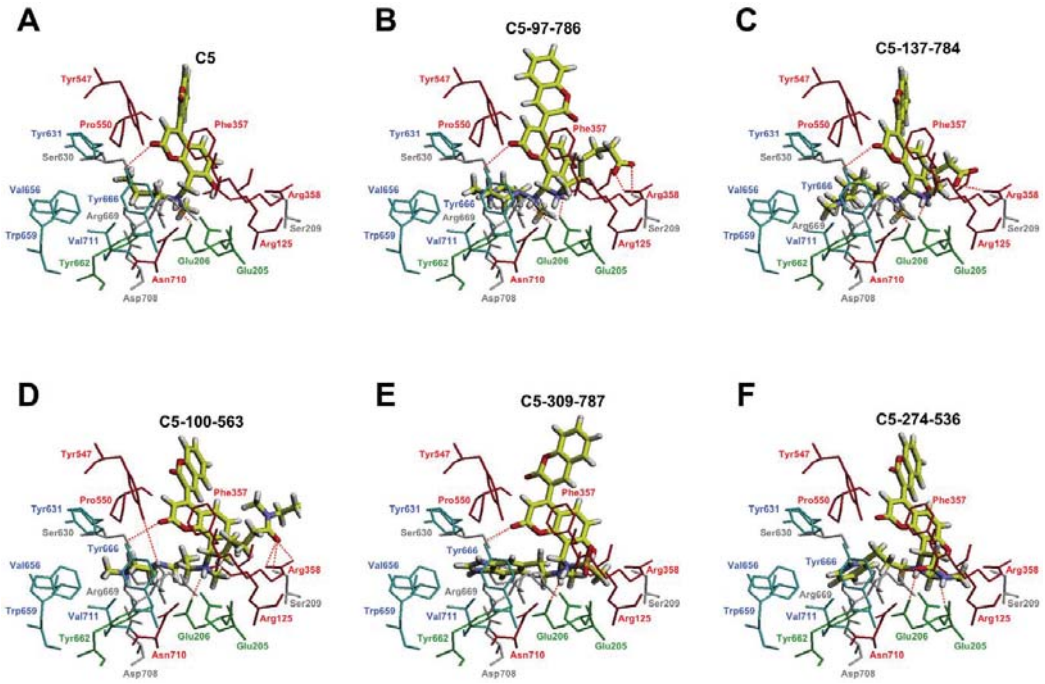

Figure 9. Docked poses for C5 (panel A) and the five C5 derivatives with the highest predicted affinities (panels from B to F) at the 3C45 binding site. All of the panels in this figure and in Figure 8 are in the same relative orientation to allow for easier comparisons between the predicted poses. Residues at the DPP-IV binding site are colored by the same criteria described in Figure 8. Dashed lines are used to show intermolecular hydrogen bonds.
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adhere to the Lipinski rule are expected to be active in humans after oral admission. Only one violation of this rule was allowed. Molecules containing toxic groups were filtered using the 204 substructures for "warhead" chelators, frequent hitters, promiscuous inhibitors and other undesirable functional groups available in the FAF-Drugs2 tool [23].

Ligand Setup

The 3D structures of the ligands for VS purposes were incorporated into LigPrep v2.3 (Schrödinger LLC., Portland, USA; http://www.schrodinger.com) and improved by cleaning. The cleaning process was carried out using the following parameters: (a) the force field used was OPLS 2005; (b) all possible ionization states at pH 7.0±2.0 were generated with Ionizer; (c) the desalt option was activated; (d) tautomers were generated for all ionization states at pH 7.0±2.0; (e) chiralities were determined from the 3D structure; and (f) one low-energy ring conformation per ligand was generated. Conformations and sites for the resulting ligand structures were determined during the generation of the corresponding Phase [37] databases with the ***Generate Phase Database*** graphic front-end. Default parameter values were used during this conformer generation with the exception of the maximum number of conformers per structure, which increased from 100 (the default value) to 200. The conformer sites were generated with definitions made by adding the ability to consider aromatic rings as hydrophobic groups to the default built-in Phase definitions.

Structure-based Pharmacophore Screening

The initial filtering through the structure-based common pharmacophore was performed with Phase v3.1 using the following steps: (a) search in the conformers database, (b) do not score in place the conformers into the structure-based common pharmacophore (*i.e.*, allow reorientation of the conformers to determine if they match the pharmacophore or not), (c) match the two compulsory sites of the structure-based common pharmacophore and at least one of the optional sites, (d) do not have a preference for partial matches involving more sites and (e) use the excluded volumes from the structure-based common pharmacophore. Default values were used for the rest of the options and parameter values used during this search. For the second pharmacophore screening, the same filtering options of the first pharmacophore matching were applied with the exception that now no re-orientation of the poses was allowed during the search (*i.e.*, the *score in place* option was used) because it was performed by using docked poses.

Protein-ligand Docking during the VS

During the VS, the protein-ligand docking was performed with eHiTS v2009 (SimBioSys Inc., Toronto, Canada; http://www.simbiosys.ca/ehits) [39], and ligands were docked into the ligand

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binding site of the DPP-IV conformation present in the 3C45 PDB file [14]. The receptor was considered to be a rigid body and the ligands as flexible such that free rotation was allowed around the single bonds of the ligand. Default docking conditions were selected with the exception of the size of the sides of the cubic box encompassing the DPP-IV binding site, which was increased from 10 Å to 15 Å.

Electrostatic and Shape Similarity Screening

The software EON v2.0.1 (OpenEye Scientific Software, Inc., Santa Fe, New Mexico, USA; http://www.eyesopen.com) determines the electrostatic potentials of two compounds and consequently calculates the Electrostatic Tanimoto combo score (ET_combo). The ET_combo is the sum of the Shape Tanimoto (ST) and the Poisson-Boltzman Electrostatic Tanimoto scores. The Shape Tanimoto (ST) score is a quantitative measure of three-dimensional overlap where 1 corresponds to a perfect overlap (*i.e.*, the same shape) [40]. The Poisson-Boltzman Electrostatic Tanimoto score (ET_pb) compares the electrostatic potential of two small molecules where 1 corresponds to identical potentials and negative values correspond to the overlap of positive and negative charges [41]. Only those molecules that have both ET_pb and ST score values higher than 0.623 and 0.244, respectively, were selected and visualized with VIDA v4.0.3 (OpenEye Scientific Software, Inc., Santa Fe, New Mexico, USA; http://www.eyesopen.com). These threshold values were chosen after analyzing which ET_pb and ST score values are obtained when the DPP-IV inhibitor in PDB file 3C45 is compared with the experimental poses of the rest of the inhibitors from which the common pharmacophore was derived (see Figure 1).

Hit Selection for Further Experimental Assays on DPP-IV Activity

The molecules that survived the electrostatics/shape similarity filter were merged with 2,342 known inhibitors obtained from the BindingDB database [24], and then clustered using Canvas v1.2 (Schrödinger LLC., Portland, USA; http://www.schrodinger.com). MOLPRINT2D fingerprints [42], using a fingerprint precision of 32 bits, were calculated for each molecule and then hierarchical clustering, based on Tanimoto similarities, was performed resulting in 50 clusters. Nine compounds from 7 of the 12 clusters exclusively formed by NPs that were previously unidentified as DPP-IV inhibitors were selected based on their commercial availability, cost and purity (≥92%) for *in vitro* assays of DPP-IV inhibitory activity. These compounds were ZINC00171758 (*i.e.*, **C1**), ZINC01833887 (*i.e.*, **C2**), ZINC02091906 (*i.e.*, **C3**), ZINC02118100 (*i.e.*, **C4**), ZINC02132035 (*i.e.*, **C5**) and ZINC02160434 (*i.e.*, **C6**), ZINC02113150 (*i.e.*, **C7**), ZINC00518468 (*i.e.*, **C8**) and ZINC02131242 (*i.e.*, **C9**), which were all purchased from InterBioScreen, Ltd (http://www.ibscreen.com).

In vitro Assay of the Effect of Selected Compounds on the DPP-IV Activity

The *DPP-IV Drug Discovery Kit-AK499* (Enzo Life Sciences International, Inc.) was used to conduct DPP-IV inhibition assays. Briefly, 10 µL of each compound were added to commercial recombinant human DPP-IV. Stock solutions of the assayed compound were made in DMSO and diluted in buffer (50 mM Tris-HCl) to final concentrations ranging from 10–1000 µM in the assay. The final concentration of DMSO in the assay was 1%. After 10 minutes of incubation at 37°C, the reaction was initiated by the addition of the fluorimetric substrate H-Gly-Pro-AMC. Fluorescence was measured continuously for 30 minutes at Ex: 380 nm/Em: 460 nm in a Biotek FLx800 Fluorescence Microplate Reader. At least three independent assays were performed, each with two technical replicates. A standard DPP-IV inhibitor (P32/98 from Biomol, Germany) served as positive control.

IC_{50} Calculation

IC_{50} was determined using GraphPad Prism v4.0 for Windows (GraphPad Software, San Diego CA, USA; http://www.graphpad.com) by fitting the experimental data from the *in vitro* assay to a nonlinear regression function using a four-parameter logistic equation.

Docking of Novel DPP-IV Ligands

Docking studies of DPP-IV inhibitors **C1**, **C2**, **C3**, **C5**, **C7**, **C8** and **C9** were performed with the software Glide v5.6 (Schrödinger LLC., Portland, USA; http://www.schrodinger.com) using the DPP-IV coordinates that can be found using the 3C45 PDB code. The binding site was defined using the default options of the *Receptor Grid Generation* panel. Standard-precision (SP) docking was initially used to screen the ligands. The flexible docking mode was selected such that Glide internally generated conformations during the docking process. No constraints were selected for docking. Each docking run recorded at most ten poses per ligand that survived the post-docking minimization. The best docking poses for the novel DPP-IV ligands were selected by not only considering the docking scores but also by taking into account the results of the visual inspection of all docking poses. This visualization was performed with Maestro v9.2 (Schrödinger LLC., Portland, USA; http://www.schrodinger.com). Further, the location of the selected poses within the binding site was refined with extra-precision (XP) to maximize the intermolecular interactions between **C1**, **C2**, **C3**, **C5**, **C7**, **C8** and **C9** and the DPP-IV binding site. The resulting **C5** docked pose was subsequently used for lead-optimization.

Lead-optimization from the Most Active Compound

Improvement of the binding affinity of **C5** was performed in two steps. Initially, a library formed by 50 fragments (and available with the last version of the Schrödinger suite) was docked at the 3C45 binding site using the Glide XP mode. Then, the XP visualizer tool (Schrödinger LLC., Portland, USA; http://www.schrodinger.com) was used to compare the values for the different XP descriptors between the **C5** docked pose and the highest score pose for each fragment. We focused the comparisons on XP descriptors that have no contributions to the XP GScore of **C5** but instead show significant values for some fragments (*i.e.*, the **PhobEn**, **PhobEnHB**, **PhobEnPairHB** and **πCat** descriptors; see Table S2). This comparison resulted in potential attachment positions of **C5** for testing substituents that could improve the DPP-IV inhibitory activity by increasing the corresponding affinity for the target.

The substituents available in the CombiGlide Diverse Side-chain Collection v1.2 (which contains all reasonable ionization and tautomeric states for a collection of 817 representative functional groups commonly found in pharmaceuticals, with linkers of variable lengths) were used to replace the original substituents of **C5** at each attachment point (see Figure 5). This replacement was carried out using the Virtual Combinatorial Screening workflow available in CombiGlide v2.7 (Schrödinger LLC., Portland, USA; http://www.schrodinger.com). During the docking step of this workflow, docked poses were restricted to be within a maximum RMSD of 1.0 Å relative to the **C5** core in the **C5** predicted pose (see Figure 9A). Moreover, those **C5** derivatives

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resulting from a single substitution at any position on the core structure were docked, and those reagents at each position that did not seem promising were screened out. This elimination significantly reduced the number of fully substituted structures to be docked. The remaining options during the combinatorial screening were set by default. Finally, the top 100 scored poses for the **C5** derivatives were selected for refinement with Glide XP using the default options, and the resulting top-five ranked poses were chosen for further analyses (see Table S3).

Supporting Information

Table S1 Predicted scaffold-hopping candidates for DPP-IV inhibition. This table shows ZINC codes for the 219 hit molecules predicted to inhibit DPP-IV that belong exclusively to clusters containing NPs that were previously unidentified as DPP-IV inhibitors. The best results of the shape and electrostatic-potential comparisons for each hit molecule with the ligand of 3C45 crystallized structure are shown. The Tanimoto values for the comparison between the electrostatic potentials of the molecules (using an outer dielectric of 80) are shown in the ET_PB columns. Furthermore, the values for the comparison between shapes are shown in the ET_Shape columns. The sum of the ET_PB and ET_Shape values is reported in the Combo columns. Hits from each cluster are sorted according to their decreasing combo value. ZINC00171758 and ZINC01833887 (cluster 30), ZINC02091906 (cluster 36), ZINC02118100 (cluster 37), ZINC02132035 and ZINC02160434 (cluster 41), ZINC02113150 (from cluster 45), ZINC00518468 (cluster 49) and ZINC02131242 (cluster 50) were tested in an *in vitro* assay to validate the success rate of our predictions (in bold in Table S1). Due to the insolubility, ZINC02118100 (cluster 37) and ZINC02160434 (cluster 41) could not be tested.
(PDF)

Table S2 Docked fragments that have significant contributions to the GScore for XP descriptors that are 0.00 Kcal/mol for C5. The most potent DPP-IV inhibitor found by our dose-response studies (*i.e.*, **C5**) has no contribution to the GScore by the following XP descriptors: **(a)** PhobEn (*i.e.*, hydrophobic enclosure reward); **(b)** PhobEnHB (*i.e.*, reward for hydrophobically packed H-bond); **(c)** PhobEnPairHB (*i.e.*, reward for hydrophobically packed correlated H-bond); and **(d)** πCat (*i.e.*, reward for π-cation interactions). This table displays the docked fragments showing the highest values for these XP descriptors and the common pharmacophore sites of Figure 3 that are matched to the corresponding fragment, if any.
(DOC)

Table S3 Top five C5 derivatives according to their XP GScores. The top five C5 derivatives according to their XP GScore values. The structures of the substituents that were attached to the **C5** core at the two replacement sites (see Figure 5) are shown. The code for each molecule is obtained by adding the CombiGlide Diverse Side-chain Collection code for the substituents at the H/R1 and at the H/R2 sites to **C5**.
(DOC)

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Author Contributions

Conceived and designed the experiments: GP SGV MP AA. Performed the experiments: LG MJO NGA. Analyzed the data: GP SGV MP AA LG ES AC. Contributed reagents/materials/analysis tools: MM CV. Wrote the paper: GP SGV LG.

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4. Martínez-Micaelo N., **González-Abuín N**., Pinent M., Ardévol A., Blay M. Omega-3 docosahexaenoic acid and procyanidins inhibit cyclooxygenase activity and attenuate NF-κB activation through a p105/p50 regulatory mechanism in macrophage inflammation. Biochemical Journal 441, 653-663 (PuMed ID: 21954853) 2012.

Contribution to congresses

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2. **González-Abuín, N.**; Martínez-Michaelo, N.; Blay, M.; Ardévol, A; Pinent, M. Incretin-like effect of grape-seed derived procyanidins. *1ª Jornada Tècnica sobre la Recerca en Enologia i Viticultura a Catalunya*. Tarragona, Spain, 2013.
3. **González-Abuín, N.**; Martínez-Michaelo, N.; Blay, M.; Ardévol, A; Pinent, M. Incretin-like effect of grape-seed derived procyanidins. *World Forum for Nutrition Research Conference.* Reus, Tarragona, Spain, 2013.
4. **González-Abuín, N.**; Martínez-Michaelo, N.; Serrano-López, J.; Blay, M.; Green, B.D.; Ardévol, A; Pinent, M. Grape-seed derived procyanidins inhibit GLP-1 secretion from enteroendocrine cells. *5th International Congress on Prediabetes and the Metabolic Syndrome.* Viena, Austria, 2013.
5. **González-Abuín, N.**; Martínez-Michaelo, N.; Blay, M.; Pinent, M.; Ardévol, A. Grape-seed derived procyanidins decrease intestinal dipeptidyl-peptidase 4 (DPP4) activity showing an incretin effect. *Phenotypic Flexibility Symposium.* Madrid, Spain, 2013.
6. **González-Abuín, N.**; Martínez-Michaelo, N.; Blay, M.; Ardévol, A; Pinent, M. Grape-seed derived procyanidins decrease intestinal dipeptidyl-peptidase 4 (DPP4) activity and improve glycemia. *22nd IUBMB and 37th FEBS Congress.* Sevilla, Spain, 2012.

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7. **González-Abuín, N.**; Martínez-Michaelo, N.; Blay, M.; Ardévol, A; Pinent, M. Grape-seed derived procyanidins decrease intestinal dipeptidyl-peptidase 4 (DPP4) activity and improve glycemia. *CEICS Nobel Campus: Chemistry for life.* Salou, Tarragona, Spain, 2012.
8. **González-Abuín, N.**; Martínez-Michaelo, N.; Blay, M.; Pinent, M.; Ardévol, A. Grape-seed derived procyanidins decrease intestinal dipeptidyl-peptidase 4 (DPP4) activity and expression. *36thFEBS Congress.* Torino, Italy, 2011.
9. **González-Abuín, N.**; Martínez-Michaelo, N.; Blay, M.; Ardévol, A; Pinent, M. Grape-seed derived procyanidins inhibit dipeptidyl-peptidase 4 (DPP4) activity. *35thFEBS Congress.* Gothenburg, Sweeden, 2010.

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PROCEDIMENTS NORMALITZATS DE TREBALL DE METODOLOGIES (PNTMs)

The techniques used in this doctoral thesis are already described in each result chapter. The following documents are the Normalized Working Procedures that I have written following the Normative ISO 9001 (2000) from the Nutrigenomics group Quality Management System.

UNIVERSITAT ROVIRA I VIRGILI
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Nº Pàgina

Redactat per: Noemi González	Revisat per: Anna Ardévol	Aprovat per: Anna Ardévol
Data: 3 Setembre 2013	Data: 4 Setembre 2013	Data: 4 Setembre 2013

UNIVERSITAT ROVIRA I VIRGILI
MODULATION OF ACTIVE GLUCAGON-LIKE PEPTIDE-1 (GLP-1) LEVELS BY GRAPE-SEED PROCYANIDINS
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1. Objectius

Protocol per treballar adequadament amb la línia cel·lular HUVEC. Cultiu, subcultiu, manteniment, congelació i descongelació de la linia cel·lular HUVEC.

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

Linia cel·lular HUVEC, corresponent a cèl·lules endotelials humanes. HUVEC (Human Umbilical Vein Endothelial Cells). Catalog Number: C-015-10C (Cascade Biologics™, Carlsbad, CA, USA) (>10^6 viable cells/vial)

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	
Microscopi invertit amb contrast de fase KYOWA	Cultius	007408	058	
Congelador -20ºC Liebherr	Cultius	005536	015	
Incubador CO_2	Cultius	005584	030	

SANYO-MCO				
Nevera Liebherr	Cultius	005576	069	
Bany termostàtic selecta precisterm	Cultius	005480	039	
Bomba buit	Cultius	007411		
Pipetus AKKU	Cultius		075	
Pipetus Pipetboy	Cultius	500058	075	
Vórtex	Cultius			
Centrífuga Eppendorf 5804 R	Lab 115	062679	043	

6.2 Solucions i materials

Nom: EGM-2 BulletKit (Lonza, Ref. CC-3162)				
Volum final: 500 ml				
Manipulació prèvia: Atemperar en un bany a 37ºC abans d'addicionar al cultiu cel·lular				
Conservació: 4ºC				
Reactiu	**Nº CAS**	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
EBM-2(1)		CC-3156 Lonza		500 ml
EGM-2 SingleQuots(2)		CC-4176 Lonza	-2% v/v Fetal Bovine Serum	10 ml
			-hydrocortisone	0,2 ml
			-human epidermal growth factor	0,5 ml
			-Ascorbic acid	0,5 ml
			-Heparin	0,5 ml
			-Antibiotics (gentamicin sulfate/amphotericin B)	0,5 ml
			-vascular endothelial growth factor	0,5 ml
			-human fibroblast growth factor-B	2 ml
			-R3-IGF-1	0,5 ml

(1) *Emmagatzemat a la nevera de cultius a 4ºC*
(2) *Emmagatzemat al congelador de cultius a -20ºC.*
El medi es suplementa amb tots els reactius que conté el kit una vegada s'han deixat descongelar a temperatura ambient.

Solució stock: Trypsin Neutralizing Solution, CC-5002 (Lonza)	
Volum final: 100 ml	
Manipulació: aliquotar y congelar a -20ºC	
Conservació: -20ºC, Congelador LIEBHERR	

Solució stock: EmbryoMax 0.1% Gelatin Solution, ES-006-B (Millipore)	
Volum final: 500 ml	
Manipulació:	
Conservació: temperatura ambient	

Solució stock: PBS 1X	
Volum final:	**pH :** 7,4
Manipulació: dilució de la solució mare	
Conservació: temperatura ambient	

Referència protocol sala cultius

Per preparar PBS 1X: Diluir 10 vegades el PBS 10X en una probeta amb aigua MQ. Ajustar a pH 7,4 amb HCl 0,5M. Autoclavar en ampolles de 0,5L. Després aliquotar en Falcons de 50mL i conservar a temperatura ambient. Una vegada oberts guardar a la nevera.

Solució stock: PBS 10X			
Volum final: 2L		**pH :** 7,4	
Manipulació:			
Conservació: temperatura ambient			
Solució mare	**Referència/marca**	**Concentració final**	**Quantitat (unitats)**
NaCl	Panreac 131659	1,4M	160g
KCl	Panreac 131494	27mM	4g
KH2PO4	Merck 4873	15mM	4g
Na2HPO4.2H2O	Panreac 131678	80mM	28,5g

Enrasar a 2L amb aigua MQ

Material	Capacitat	Tractament Previ	Observacions
Criotubs	2ml		Estèrils
Flascons Greiner	25, 75 y 150 cm^2		Estèrils
Plaques Greiner	6, 12 y 24 pozos		Estèrils
Eppendorf	1,5mL	Autoclavades	
Tubs Falcon	15mL y 50mL		Estèrils
Puntes amb filtre			Estèrils
Pipetes	2, 5, 10 i 25 ml		Estèrils

6.3 Precaucions

-No utilitzar les cèl·lules quan tinguin un passi superior a 20.
-Posar les cèl·lules en tripsina durant un temps entre 1 i 3 minuts quan es faci subcultiu.
-Donar uns copets al flascó de cultiu i/o pipetejar amb pipeta de 2 mL per acabar de desenganxar les cèl·lules de la superfície del flascó.
-S'aconsella fer subcultiu o congelació de les cèl·lules a una densitat cel·lular del 80% de confluència, una densitat superior pot provocar un retard en el seu creixement posterior.

6.4 Protocol

Els flascons i/o plaques s'han de pretractar amb gelatina (EmbryoMax 0.1% Gelatin Solution) abans de sembrar les cèl·lules. Afegir el volum necessari per a cobrir tota la superfície del flascó o placa i incubar-ho durant 30 minuts, com a mínim, a temperatura ambient. Aspirar la gelatina just abans de sembrar les cèl·lules per evitar que s'assequi la superfície.

Protocol de Congelació:

El cultiu ha d'estar a una confluència del 80% o inferior.

Per un flascó de 25 cm^2 :

1) Aspirar el medi de cultiu i rentar dues vegades amb 2 mL de PBS 1x, atemperat a 37ºC en un bany, per eliminar les restes de serum que contenen inhibidor de tripsina.

2) S'aspira el PBS 1x de rentat i s'afegeix 1 mL de Tripsina- EDTA. S'observen sota el microscopi. El cap d'uns 30 segons les cèl·lules passen d'una morfologia allargada tipus fibroblast a una morfologia arrodonida. Es comencen a desenganxar de la superfície del flascó. La tripsina tarda en actuar entre 1 i 3 min (es preferible el menor temps possible). Per ajudar a que les cèl·lules es desenganxin completament es donen uns copets per la part de baix del flascó. Tot a Tªamb.

3) En aquest moment s'ha de neutralitzar la tripsina perquè no faci malbé les cèl·lules. S'afegeixen 4 ml de Trypsin Neutralizing Solution (CC-5002, Lonza), posant aquesta suspensió cel·lular en un tub de rosca estèril de 15 ml.

4) S'afegeixen 4 ml més de Trypsin Neutralizer per acabar de recollir les cèl·lules que hagin pogut quedar al flascó. Aquest volum també es posa al tub de 15 ml.

5) A continuació es centrifuga durant 7 minuts a 180 g (Centrifuge 5804 R, Eppendorf). Aquest pellet s'ha de ressuspendre en medi de cultiu al 5% de DMSO, de manera que s'obtingui una concentració de cèl·lules entre $5*10^5$ i $3*10^6$.

6) Distribuir en criotubs (1 mL) y posar-los en gel durant 15-30 minuts.

7) Aquests criotubs s' introduiran al congelador a -20ºC fins que s' observi que s'han congelat (entre 2 i 4 hores). Transcorregut aquest temps, es posaran al congelador a -80ºC durant 24 hores, i finalment en fase vapor de nitrogen líquid, on romandran fins al

moment de la seva utilització. *Segons el protocol de Cascade Biologics:* els criotubs s'han de congelar fent baixar la temperatura 1ºC per min fins aconseguir els -40ºC. A partir d'aquest punt, s'ha de fer baixar 2ºC per min fins arribar als -90ºC. Finalment en fase vapor de nitrogen líquid, on romandran fins al moment de la seva utilització.

8) Els vials s'han de rotular amb el nº de pase, data i nom de la linia cel·lular mentre estan en gel.

Protocol de Descongelació:

1) Les cèl·lules estan emmagatzemades en un critotub en fase vapor de nitrogen líquid.

2) Per descongelar les cèl·lules, s'ha d' introduir la meitat del criotub dins d'un bany a 37ºC i agitar-lo suaument; ha de ser una descongelació ràpida, inferior a 2 minuts.

3) Treure el vial fora de l'aigua una vegada que el contingut s'hagi descongelat. Ruixar el vial amb etanol 70% abans d'introduir-lo a la cabina de flux laminar. A partir d'ara es treballarà sota condicions asèptiques.

4) S'obre el vial i es pipeteja amb una micropiepeta d'1 ml la suspensió cel·lular per dispersar les cèl·lules. Després fiquem la suspensió cel·lular en un flascó de 25 cm^2 i afegim 4 ml de medi de cultiu.

5) Es recomana no tocar el cultiu fins que no hagin passat 24 h després d'haver sembrat. Quan s'ha establert el cultiu, s'ha de canviar el medi cada 48 h. Una vegada les cèl·lules arriben al 80% de confluència, s'ha de canviar el medi de cultiu cada 24 h, en aquest punt però es recomana fer subcultiu. Incubar a 37ºC amb 5% CO_2 .

Protocol per subcultiu:

Al flascó s'escriurà: DATA, LINIA CEL·LULAR, PASE I NOM DE L'INVESTIGADOR. Es recomana fer subcultiu una vegada les cèl·lules han arribat al 80% de confluència. Una confluència superior pot provocar dificultats en el procés de tripsinització, seguit d'un retard en el cicle de creixment cel·lular, és a dir, les cèl·lules tardarien més temps a arribar una altra vegada a un 80% de confluència.

Per flascons de 25 cm^2:

1) Aspirar el medi de cultiu i rentar dues vegades amb 2 mL de PBS 1x, atemperat a 37ºC en un bany, per eliminar les restes de sèrum que contenen inhibidor de tripsina.

2) S'aspira el PBS 1x de rentat i s'afegeix 1 mL de Tripsina- EDTA. S'observen sota el microscopi. El cap d'uns 30 segons les cèl·lules passen d'una morfologia allargada tipus fibroblast a una morfologia arrodonida. Es comencen a desenganxar de la superfície del flascó. La tripsina tarda en actuar entre 1 i 3 min (es preferible el menor temps possible). Per ajudar a que les cèl·lules es desenganxin completament es donen uns copets per la part de baix del flascó. Tot a Tªamb.

3) En aquest moment s'ha de neutralitzar la tripsina perquè no faci malbé les cèl·lules. S'afegeixen 4 ml de Trypsin Neutralizing Solution (CC-5002, Lonza), posant aquesta suspensió cel·lular en un tub de rosca estèril de 15 ml.

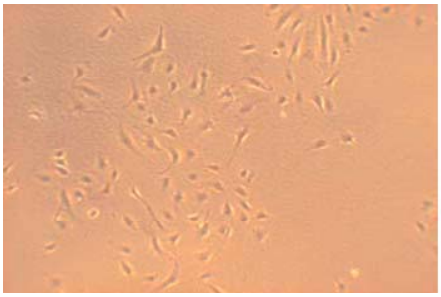
4) S'afegeixen 4 ml més de Trypsin Neutralizer per acabar de recollir les cèl·lules que hagin pogut quedar al flascó. Aquest volum també es posa al tub de 15 ml.

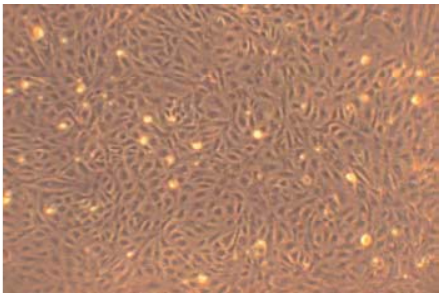
5) A continuació es centrifuga 7 min a 180 g (Centrifuge 5804R, Eppendorf). S'aspira el sobrenedant i el pellet obtingut es ressuspen en 4 ml de medi suplementat, fins a obtenir una suspensió cel·lular homogènia.

6) Es fa un comptatge cel·lular a la cambra de Neubauer per determinar-ne la concentració. Es recomana sembrar com a mínim $2,5*10^3$ cèl·lules/cm^2, encara que es pot sembrar una quantitat superior per arribar al 80% de confluència en un temps inferior. Es mantindran en un incubador a 37 ºC amb una atmosfera al 5% de CO_2.

6.5 Verificació de la tècnica i interpretació de resultats

Sempre s'ha de comprovar la morfologia de les cèl·lules sota el microscopi:





A més de la morfologia cel·lular, es necessari observar sota el microscopi les condicions del medi de cultiu. La observació a temps de microorganismes es fonamental per evitar la propagació d'aquests als altres cultius cel·lulars.

6.6. Guia de problemes ("troubleshooting")

Problema	Possible causa	Solució
Mort cel·lular	Passi antic	Descongelar passi nou
	Contaminació del medi de cultiu	Llençar les cèl·lules contaminades i fer neteja d'incubadors amb p-formaldehid

7. Referències

S'han seguit els protocols de la casa comercial Cascade Biologics
www.cascadebiologics.com

8. Control de la documentació

El PNTM una vegada aprovat es guardarà en format electrònic (.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.

UNIVERSITAT ROVIRA I VIRGILI
MODULATION OF ACTIVE GLUCAGON-LIKE PEPTIDE-1 (GLP-1) LEVELS BY GRAPE-SEED PROCYANIDINS
Noemi González Abuin
Dipòsit Legal: T. 80-2014

Nº Pàgina

Redactat per: Noemi González	Revisat per: Montserrat Pinent	Aprovat per: Montserrat Pinent
Data: 27 Juliol 2012	Data: 27 Juliol 2012	Data: 27 Juliol 2012

UNIVERSITAT ROVIRA I VIRGILI
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Noemi González Abuin
Dipòsit Legal: T. 80-2014

1. Objectius

Protocol per treballar adequadament amb les línies cel·lulars STC-1 i pGIP/Neo. Cultiu, subcultiu, manteniment, congelació i descongelació de les línies cel·lulars STC-1 i pGIP/Neo.

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 STC-1, corresponent a cèl·lules enteroendocrines originades a partir d'un tumor de ratolí doble transgènic [Rindi et al, 1990]. La línia cel·lular pGIP/Neo es correspon a la línia cel·lular STC-1 transfectada amb el plasmidi GIP/Neo.

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	
Microscopi invertit amb contrast de fase KYOWA	Cultius	007408	058	
Congelador -20ºC Liebherr	Cultius	005536	015	
Incubador CO_2	Cultius	005584	030	

SANYO-MCO				
Nevera Liebherr	Cultius	005576	069	
Bany termostàtic selecta precisterm	Cultius	005480	039	
Bomba buit	Cultius	007411		
Pipetus AKKU	Cultius		075	
Pipetus Pipetboy	Cultius	500058	075	
Vórtex	Cultius			
Centrífuga Eppendorf 5804 R	Lab 115	062679	043	

6.2 Solucions i materials

Nom: DMEM + GlutaMAX^{Tr2} 1X (Gibco, Ref. 61965)
Volum final: 500 ml
Manipulació prèvia: Atemperar en un bany a 37ºC abans d'addicionar al cultiu cel·lular
Conservació: 4ºC

Reactiu	Nº CAS	Referència/marca	Concentració final	Quantitat (unitats)
DMEM + GlutaMAX^{Tr2} 1X[(1)]		61965, Gibco		500 ml
FBS [(2)]		DE-14-802F, Lonza	10 %	105 ml
P/S [(3)]		DE-17-602E, Lonza	100U/ml / 100µg/ml	5,8 mL
Bicarbonat sódic [(4)]		S6014, Sigma	0,075 g/mL (en aigua miliQ)	10 mL
G418 [(4)]		A1720, Sigma	0,2 mg/ml	2 mL

(1) *Emmagatzemat a la nevera de cultius a 4ºC*
(2) *Inactivar seguint el protocol, alicuotar i emmagatzemar al congelador de cultius a -20ºC.*
(3) *Aliquotar i emmagatzemar al congelador de cultius a -20ºC.*
(4) *Preparar la solució, filtrar i addicionar al medi de cultiu. S'utilitzaran només en el cas de la línia cel·lular pGIP/Neo.*

Solució stock: DMSO, D2650
Volum final: 100ml
Manipulació:
Conservació: Temperatura ambient

Solució stock: Tripsina-EDTA, B-17-161F
Volum final: 500ml
Manipulació: descongelar tota la nit a 4ºC (una vegada descongelat, aliquotar i guardar a -20ºC)
Conservació: -20ºC, Congelador LIEBHERR

Material	Capacitat	Tractament Previ	Observacions
Criotubs	2 ml		Estèrils
Flascons Greiner	25, 75 y 150 cm^2		Estèrils
Plaques Greiner	6, 12 y 24 pozos		Estèrils
Eppendorf	1,5mL	Autoclavades	
Tubs Falcon	15 mL y 50 mL		Estèrils
Puntes amb filtre			Estèrils
Pipetes	2, 5, 10 i 25 ml		Estèrils

6.3 Precaucions

-No utilitzar les cèl·lules quan tinguin un passi superior a 50.
-Posar les cèl·lules en tripsina durant un temps entre 1 i 3 minuts quan es faci subcultiu.
-Donar uns copets al flascó de cultiu i/o pipetejar amb pipeta de 2 mL per acabar de desenganxar les cèl·lules de la superfície del flascó.
-S'aconsella fer subcultiu o congelació de les cèl·lules a una densitat cel·lular del 80% de confluència, una densitat superior pot provocar un retard en el seu creixement posterior.

6.4 Protocol

Protocol de Congelació:

El cultiu ha d'estar a una confluència del 80% o inferior.

Per un flascó de 75 cm^2:

1) Aspirar el medi i rentar amb 5 mL de PBS 1X dues vegades.

1) Afegir 2 mL de Tripsina- EDTA i posar 3 minuts a l'incubador a 37ºC. Per ajudar a que les cèl·lules es desenganxin completament es donen uns copets per la part de baix del flascó.

2) En aquest moment s'ha de neutralitzar la tripsina perquè no faci malbé les cèl·lules. S'afegeixen 8 ml medi de cultiu i les cèl·lules es pipetegen up-and-down unes 15-20 vegades. Després fiquem la suspensió cel·lular en un tub de rosca estèril de 15 ml.

3) Fer recompte de cèl·lules amb la cambra de Neubauer.

4) Per descartar les restes de tripsina centrifuguem els 10 ml durant 5 min a 1000 rpm. Després de centrifugar visualitzem un pellet i un sobrenedant. El sobrenedant el descartem.

5) Es resuspen el pellet en el volum de FBS, que conté un 10% de DMSO (9 ml de medi + 1 ml de DMSO), necessari per tenir 10 milions de cèl·lules per ml. Pipetejar up-and-down 15-20 vegades per disgregar les cèl·lules.

6) Afegir el mateix volum de FBS. La concentració final de cèl·lules es de 5 milions per mL i la de DMSO es del 5%.

7) Es distribueix 1 ml ($5*10^6$ cèl·lules/mL) en cada criotub. Els criotubs s'han de rotular amb el nº de pase, data i nom de la linia cel·lular i ficar-los en gel durant 15-30 min.

8) Després es deixen a -20ºC durant 2 hores.

9) Després es fiquen a -80ºC durant 24 hores.

10) Finalment es guarden en nitrogen líquid.

Protocol de Descongelació:

1) Les cèl·lules estan emmagatzemades en un critotub en fase vapor de nitrogen líquid.

2) Per descongelar les cèl·lules, s'ha d' introduir la meitat del criotub dins d'un bany a 37ºC i agitar-lo suaument; ha de ser una descongelació ràpida, inferior a 2 minuts.

3) Treure el vial fora de l'aigua una vegada que el contingut s'hagi descongelat. Ruixar el vial amb etanol 70% abans d'introduir-lo a la cabina de flux laminar. A partir d'ara es treballarà sota condicions asèptiques.

4) S'obre el vial i es pipeteja amb una micropiepeta d'1 ml la suspensió cel·lular per dispersar les cèl·lules. Després fiquem la suspensió cel·lular en un tub de rosca estèril de 15 ml i afegim 9 ml de medi de cultiu.

5) Per descartar el DMSO, centrifuguem els 10 ml durant 5 min a 1000 rpm. Després de centrifugar visualitzem un pellet i un sobrenedant.

6) El sobrenedant el descartem i resuspenem el pellet en 5 ml de medi de cultiu suplementat, pipetejant up-and-down 15-20 vegades per disgregar les cèl·lules.

7) Finalment, fiquem la suspensió cel·lular en un flascó de 25 cm^2.

8) Es recomana no tocar el cultiu fins que no hagin passat 24 h després d'haver sembrat. Quan s'ha establert el cultiu, s'ha de canviar el medi cada 48 h. Una vegada les cèl·lules arriben al 80% de confluència, s'ha de fer subcultiu. Incubar a 37ºC amb 5% CO_2 .

Protocol per subcultiu:

Al flascó s'escriurà: DATA, LINIA CEL·LULAR, PASE I NOM DE L'INVESTIGADOR. Es recomana fer subcultiu una vegada les cèl·lules han arribat al 80% de confluència. Una confluència superior pot provocar dificultats en el procés de tripsinització, seguit d'un retard en el cicle de creixment cel·lular, és a dir, les cèl·lules tardarien més temps a arribar una altra vegada a un 80% de confluència.

Per flascons de 75 cm^2:

1) Aspirar el medi i rentar amb 5 mL de PBS 1X dues vegades.

2) Afegir 2 mL de Tripsina- EDTA i posar 3 minuts a l'incubador a 37ºC. Per ajudar a que les cèl·lules es desenganxin completament es donen uns copets per la part de baix del flascó.

3) En aquest moment s'ha de neutralitzar la tripsina perquè no faci malbé les cèl·lules. S'afegeixen 8 ml medi de cultiu i fiquem la suspensió cel·lular en un tub de rosca estèril de 15 ml.

4) Per descartar les restes de tripsina centrifuguem els 10 ml durant 5 min a 1000 rpm. Després de centrifugar visualitzem un pellet i un sobrenedant. El sobrenedant el descartem.

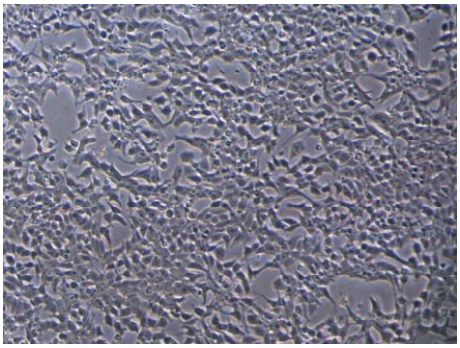
5) Es resuspen el pellet en 10 ml de medi de cultiu i es pipeteja up-and-down unes 15-20 vegades per a que no es formin grumolls.

6) Fer recompte de cèl·lules amb la cambra de Neubauer per determinar-ne la concentració.

7) Es recomana sembrar com a mínim $8*10^6$ cèl·lules/flascó, encara que es pot sembrar una quantitat superior per arribar al 80% de confluència en un temps inferior. Es mantindran en un incubador a 37 ºC amb una atmosfera al 5% de CO_2.

6.5 Verificació de la tècnica i interpretació de resultats

Sempre s'ha de comprovar la morfologia de les cèl·lules sota el microscopi:



A més de la morfologia cel·lular, es necessari observar sota el microscopi les condicions del medi de cultiu. La observació a temps de microorganismes es fonamental per evitar la propagació d'aquests als altres cultius cel·lulars.

UNIVERSITAT ROVIRA I VIRGILI
MODULATION OF ACTIVE GLUCAGON-LIKE PEPTIDE-1 (GLP-1) LEVELS BY GRAPE-SEED PROCYANIDINS
Noemi González Abuin
Dipòsit Legal: T. 80-2014

6.6. Guia de problemes ("troubleshooting")

Problema	Possible causa	Solució
Mort cel·lular	Passi antic	Descongelar passi nou
	Contaminació del medi de cultiu	Llençar les cèl·lules contaminades i fer neteja d'incubadors amb p-formaldehíd

7. Referències

- Acute and chronic effects of dietary fatty acids on cholecystokinin expression, storage and secretion in enteroendocrine STC-1 cells. *Katharine V. Hand et al.* Mol. Nutr. Food Res. 2010, 54:S93-S103

- Hormone profiling in a novel enteroendocrine cell line pGIP/neo: STC-1. *Katharine V. Hand et al.* Metabolism. 2012 Dec;61(12):1683-6

8. Control de la documentació

El PNTM una vegada aprovat es guardarà en format electrònic (.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.