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Gemma Montagut Pino

Procyanidin effects on an impaired glucose metabolism: A further insight into procyanidin signalling in adipose cells

PhD DOCTORAL THESIS

codirected by Dr. Anna Ardévol Grau
and Dr. Montserrat Pinent Armengol

Bioquímica i Biotecnologia Department

UNIVERSITAT ROVIRA I VIRGILI

Tarragona
2009

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Anna Ardévol Grau, professora titular d'universitat i Montserrat Pinent Armengol, professora lector del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili,

CERTIFIQUEM:

Que aquest treball, titulat **"Procyanidin effects on an impaired glucose metabolism: A further insight into procyanidin signalling in adipose cells",** que presenta Gemma Montagut Pino per a l'obtenció del títol de Doctor, 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, 28 d'abril de 2009

Anna Ardévol Grau Montserrat Pinent Armengol

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"Satisfaction lies in the effort, not in the attainment.

Full effort is full victory."

Mahatma Gandhi (1869-1948)

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THANKS, GRÀCIES, GRACIAS, MERCI…

És dimarts matí i jo a casa barallant-me amb la introducció de la tesi. L'he apartat un moment i intento desconnectar i penso com m'agradaria dir gràcies a la gent que m'ha ajudat, acompanyat i acollit durant tot aquest temps. No començaré dient les típiques paraules de que m'ha costat molt escriure aquesta part perquè, en definitiva, totes les parts de la tesi, inclosos els agraïments, costen (... i molt!) d'escriure. Plasmar sobre paper els resultats d'assajos i estudis no ha estat feina fàcil. Però què t'he d'explicar a tu, Albert, que acabes de llegir la tesi fa pocs mesos o a tu, Ximena, que has compartit amb mi aquesta tortura que ha estat alguns cops el fet d'escriure la tesi? Bueno, com podeu comprovar, començo tan positiva com sempre…

No sé si podré incloure en aquests agraïments a totes les persones que d'alguna manera han contribuït a que arribem a llegir impreses aquestes línies. Primer de tot, he d'agrair a les meves directores de tesi el seu suport durant tot aquest temps. Gràcies Montse, pels teus consells, pels teus ànims, per la teva col·laboració i direcció en aquesta tesi. Gràcies Anna, per la confiança que has dipositat en mi, tot i que he de reconèixer que no sempre t'ho he posat fàcil (vull dir amb lo de veure el got mig buit!). Te'n recordes com va començar tot? A la facultat vella, vas ser la primera que em va animar a fer el doctorat després del treball experimental, i ja veus, poc a poc amb el teu suport i la teva ajuda, amb la teva dedicació i amb el teu recolzament has fet possible que aquest projecte hagi arribat fins aquí. Gràcies Anna.

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La tesi engloba un procés d'aprenentatge tan a nivell conceptual com a nivell personal, ja que aquests 4 anys, a banda de centrar-me en "fer ciència" també m'han ajudat a madurar i a intentar veure les coses des d'una altra perspectiva. Però quan la ciència no entèn el significat de paraules com treball,

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esforç o sacrifici els que t'envolten, el teu grup de companys i d'amics amb els qui convius cada dia es converteixen amb el teu gran suport. Una conversa d'un et distreu, et fa riure i t'anima, un altre imita un jugador o directiu del Barça, algú t'invita a passar el cap de setmana a casa seva o bé dinant et parlen de coses tan variades com menjars, gossos, cavalls, música, roba o fins i tot de "resines epoxi"! Per tots aquests, la meva idea inicial era escriure un GRÀCIES general, amb lo qual però qui més qui menys se sentiria decebut. No ens enganyem, sempre fa gràcia veure què han posat de tu als agraïments!! Així doncs, comencem "la dura tasca" de recordar a tots els que heu compartit amb mi aquests anys de tesi.

El primer agraïment personal, com no, és pel Josep, més conegut per mi com Xusepill o Xusepet, amb el qui m'ho he passat molt bé durant tot aquest temps a banda d'aprendre moltíssimes coses. Sempre recordaré una cançó que xiulaves al laboratori (no escric el nom del grup perquè sé que et farà vergonya reconèixer que xiulaves una cançó dels "backstritboys"...ups, se m'ha escapat!) i "l'O de Xusepill" que tan sovint elaboraves!! No t'ho he dit mai, però t'admiro. Gràcies Josep, de "gran" vull ser com tu! No canviïs.

Gràcies Albert i Lídia per compartir amb mi el nostre dia a dia, esmorzars, dinars, llargues xerrades amb cafetons (i amb alguna cerveseta), i com no temps de festa a la Plaça de la Font!! Albert, definitivament ets la persona més educada de la URV (tu guanyes, quina paciència!!). Lídia (díme corazón), gràcies per les teves abraçades! Les trobaré molt a faltar! Arriba el moment on us he de confessar que entre vosaltres sempre m'he sentit molt *còmode*, doncs plegats m'heu demostrat, en fets i en paraules, que sempre puc comptar amb vosaltres! Gràcies parella!

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en paraules et podria arribar a dir què ha significat i què significa per mi conèixer-te. Gràcies Puxeu, segur que sereu uns pares genials!

Gràcies Pere, company de dinars, sopars i celebracions, de classes d'anglès i d'aventures per terres gal·leses, quan vas ser un dels primers en fer el cim del "*Dinas Bran Câstle*" ja prometies arribar lluny. Ara que estàs com el Gasol i el Rudy amb els "estars" americans, te'n recordes no dels de casa? Thanks Pere!! Gràcies Esther per ser més que una companya una bona amiga que m'ha animat…unes 3 vegades al dia! Gràcies també per fer-me riure tant i per compartir amb mi la teva vida. Saps què penso de tu, Esther? A veure si et sona, jo no ho hagués pogut expressar millor; *"ets una persona increïble, generosa, bona, molt bona, preciosa per dins i per fora..."* Gràcies Marina, podria fer copy-paste de la frase anterior, però et mereixes unes línies personals i intransferibles… i et diré gràcies per ser una de les poques persones que sempre m'ha dit el que pensa, per ser tan maca i per ser una bona amiga. Crec que per fi estàs on et mereixes perquè molta gent encara no ho sap...però ets una CRAKKK!! Gràcies David per fer-me somriure cada dia, per ser tant i tant positiu!! Sense la teva companyia fer *westerns* mai no hagués estat tan divertit! *"Ai Lucía, tierna de noche y tierna de día..."*, com acabava la *Pajuelo's popular cita*? Gràcies Helena i Sabina! He rigut molt amb vosaltres dues, el Congrés a París passarà a la història! Per cert, trobaré molt a faltar el nostre "today is the day?"

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dir-vos que seria un plaer compartir més temps plegats però quan uns comencen...els altres acaben! Forma part del "cicle dels doctorands". Tots vosaltres junt amb doctorands que ja no estan presents com el Gerard, la Safae, el Pep o l'Abuelo heu estat durant tot aquest temps la meva segona família. Gràcies per fer-me sentir com a casa!

No m'oblido dels nostres veïns enòlegs! Doncs m'agradaria donar gràcies especialment al Braulio, per arrencar-me un somriure dia sí dia també i, com no, a la Rosana, amb qui he compartit molts bons moments durant tota aquesta etapa. Ànims "xiqueta" que ja et falta poc!

Però quan has de fer un experiment o unes pràctiques no tots els materials ni reactius sempre estan a mà. Exceptuant, si el Santiago treballa amb tu o prop teu, al qui li agraeixo la seva gran ajuda, eficàcia i eficiència. Gràcies Santiago!!! Gràcies també Toni pel teu suport i la teva ajuda i gràcies Mari per guardar per mi un dels teus simpàtics "Bon dia"! Esther, gràcies per la teva ajuda, per ser tan simpàtica i eixerida com eficient, responsable i resolutiva.

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I després d'aquestes 38 gràcies (estan contades!) alguns possiblement pensaran que per fi s'ha acabat…
Doncs no! Paciència, que ja gairebé estan!

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Fora de la feina i del laboratori, m'agradaria agrair el suport de molta gent de la qual he anat aprenent petites coses. Però com que aquest apartat hauria de tenir també una certa limitació d'espai, voldria donar especialment gràcies a la Noemí i a la Iolanda, per compartir amb mi una altra visió de la tesi. Espero poder presenciar la vostra tesi ben aviat!! A totes dues gràcies! Gràcies també a tu Joana per la teva ajuda.

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Gràcies a tots. Aquesta tesi és una demostració de perseverança, treball, esforç i sacrifici. Però també és un conjunt d'il·lusions, alegries, coneixements i experiències. En definitiva, de recerca.

Ara sí que sí. Tots a punt???
Preparados, listos...
TESI!!!!!!!!

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Als de casa

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Abbreviation List

AKT/PKB: protein kinase B

APS: Adapter protein with a PH and SH2 domain

C/EBP: CCAAT/Enhancer Binding Protein

CAP: Cbl-Associated Protein

CHO: Chinese Hamster Ovary cells

CNS: Central nervous system

CVD: Cardiovascular diseases

DPm: mean degree of polymerization

EGCG: Epigallocatechin gallate

ERK1/2: Extracellular Signal-Regulated Protein Kinase

ER: Endoplasmatic Reticulum

FFA: Free Fatty Acids

GA: Gallic acid

Gab-1: Grb2-Associated Binder-1

GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase

GcK: Glucokinase

Glut-4: Glucose transporter type 4

G6p: Glucose-6-phosphatase

GO: Glucose oxidase

Grb2: Growth-factor-receptor-bound protein 2

GSK3: Glycogen Synthase Kinase-3

GSPE: Grape-Seed Procyanidin Extract

HDL: High Density Lipoprotein

HGP: Hepatic glucose production

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HSL: Hormone-Sensitive Lipase

HOMA index: Homeostasis model assessment index

IL: Interleukin

IDDM: Insulin-dependent diabetes mellitus

IPGTT: Intraperitoneal glucose tolerance test

IR: Insulin Receptor

IRS: Insulin Receptor Substrate

JNK: c-Jun N-Terminal Kinase

LDL: Low Density Lipoprotein

MAPK: Mitogen-Activated Protein Kinase

MEK: MAPK/ Extracellular Signal-Regulated Kinase Kinase

MKP: MAPK phosphatases

mTOR: Mammalian target of rapamycin

NIDDM: Non-insulin-dependent diabetes mellitus

NO: Nitric oxide

PDK-1: Phosphoinositide-Dependent Protein Kinase-1

PEPCK: Phosphoenol Pyruvate Carboxykinase

PI3K: Phosphatidylinositol-3-Kinase

PKA: Protein Kinase A

PKB/Akt: Protein Kinase B

PKC: Protein Kinase C

PPARγ: Peroxisome Proliferator Activated Receptor-γ

PP1: Protein phosphatase 1

Pref-1: Preadipocyte Factor-1

ROS: Reactive oxygen species

RTK: Receptors tyrosine kinase

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Shc: Src Homologous and Collagen-Like Protein

SHP2: Src Homology 2 Domain-Containing Tyrosine Phosphatase

SOCS: Suppressors of cytokine signalling

SREBP1c: Sterol-Regulatory Element-Binding Protein-1c

STZ: Streptozotocin

TAG: Triacylglycerides

T2DM: Type-2 diabetes mellitus

TNF-α: Tumor Necrosis Factor-α

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I. Introduction

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1. Glucose metabolism and insulin resistance

1.1 Glucose homeostasis and insulin action

The maintenance of normal glucose tolerance or glucose homeostasis is controlled by an accurate balance between endogenous glucose production by the liver, glucose absorption by the intestine and glucose uptake and metabolism by peripheral tissues. In a fasting state, glucose disposal is used mainly by insulin-independent tissues, whereas little glucose is metabolized in insulin-dependent tissues. Hepatic glucose production is responsible for maintaining the flux of glucose. This is a necessary step since the brain and other neuronal tissues use glucose at a constant rate [1]. This balance becomes disrupted in a postabsorptive state, thus resulting in an increased glucose plasma concentration that stimulates pancreatic β-cells to synthesise and secrete insulin. Insulin is released into the portal vein and carried to the liver, where its primary function is to suppress hepatic glucose production. Moreover, insulin stimulates glucose uptake by peripheral tissues and promotes the synthesis and storage of glycogen, lipids and proteins in fat, liver and muscle while inhibiting their degradation and release into the circulation [2]. Glucagon is released by pancreatic α-cells during starvation, and its function, initiated by binding to specific receptors, is opposite to that of insulin.

Although quantitatively the uptake and metabolism of glucose take place mainly in muscles, fat tissue plays an important role in glucose homeostasis. Increments in the plasma insulin concentration exert a potent antilipolytic effect, which leads to a marked reduction in the plasma free fatty acid (FFA)

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level. These changes in the plasma FFA level also increase glucose uptake in muscle and contribute to the suppression of hepatic glucose production [1].

The uptake of glucose by tissues is the rate-limiting step in glucose use and storage. In the liver, glucose is phosphorylated by glucokinase and, depending on the cell's requirements, can be stored as glycogen, via the activation of glycogen synthase (glycogenogenesis), or oxidized to generate ATP via the activation of enzymes such as pyruvate kinase or phosphoenolpyruvate carboxykinase (glycolysis).

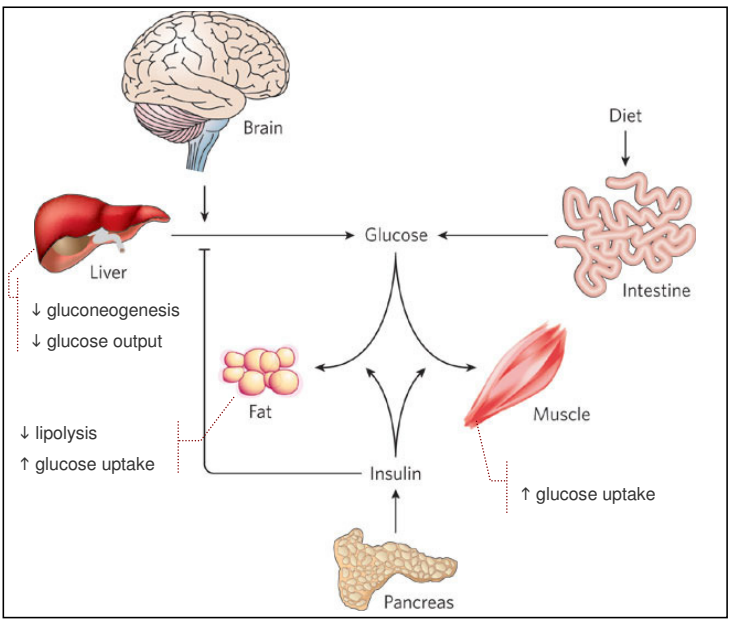

Figure 1. Glucose homeostasis and insulin action. Adapted from Evan D. Rosen and Bruce M. Spiegelman. *Nature,* 2006, 444; p. 847-853.

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Moreover, insulin inhibits the production and release of glucose by a mechanism that involves regulating the expression of genes encoding hepatic enzymes that are responsible for blocking gluconeogenesis and glycogenolysis. In muscle, glycogen synthesis is the main pathway for the insulin-induced glucose metabolism. In adipocytes, on the other hand, after entering the cell, glucose is stored primarily as lipids via the activation of lipid synthetic enzymes [3].

1.2 Insulin signal transduction

Insulin mediates its physiologic functions by binding to the insulin receptor, which is located at the plasma membrane on the liver, skeletal muscle and adipose tissue. Upon insulin binding to the insulin receptor α subunit, the receptor's intrinsic tyrosine kinase located in the β subunit is activated, thus resulting in an increased catalytic activity of the kinase. The autophosphorylation of the insulin receptor catalyses the tyrosine phosphorylation of cellular proteins, such as members of the IRS family, Shc and Cbl. These activated proteins provide docking sites for downstream signalling molecules through their Src homology 2 (SH2) domain, which results in the activation of several signalling pathways, including phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and the TC10 pathway [4,5].

These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation, and

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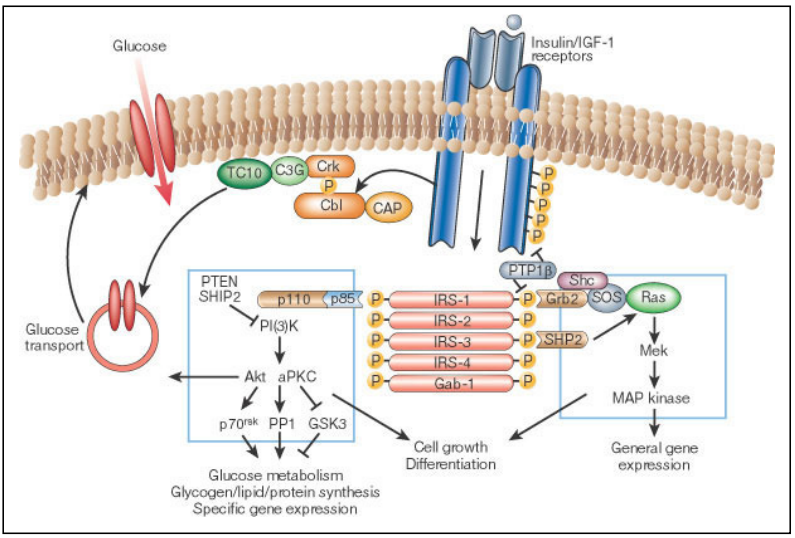
gene expression, which results in the regulation of glucose, lipid and protein metabolism [3].



Figue 2. Insulin interacts with the insulin receptor to activate insulin signalling, leading to physiologic functions of insulin. Adapted from Saltiel et al. *Nature*, 2001, 414(6865): p. 799-806.

The insulin stimulation of glucose uptake into muscle and fat cells requires the movement of Glut-4-containing vesicles from intracellular compartments to the plasma membrane. Tyrosine phosphorylation of the IRS proteins after insulin stimulation leads to an interaction and an activation of PI3-kinase, which in turn recruits protein kinase B (AKT/PKB) and 3-phosphoinositide-dependent protein kinase-1 (PDK1) to the plasma membrane. PDK1 phosphorylates the activation loop of AKT at T308, but a full activation of AKT also requires a phosphorylation on S473 by the mTOR/Rictor complex, though other kinases have also been reported to induce this phosphorylation [6,7]. AKT activation

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induces the translocation of Glut-4-containing vesicles from their intracellular pool to the plasma membrane. The fusion of the vesicles with the plasma membrane leads to the extracellular exposure of Glut-4 proteins, which allows the uptake of glucose into the cell [3].

Despite evidence supporting an important role for the PI3K/AKT pathway, several studies have shown that a separate pool of insulin receptors localized in lipid raft microdomains is also required for Glut-4 translocation [8]. This pool of insulin receptors can phosphorylate the adapter protein APS and result in the activation of the G protein TC10, which resides in lipid rafts. Once activated, TC10 may provide a second signal to the Glut-4 protein. This involves the stabilization of cortical actin which, seems to be important in Glut-4-containing vesicle translocation from their intracellular pool to the plasma membrane [3,9].

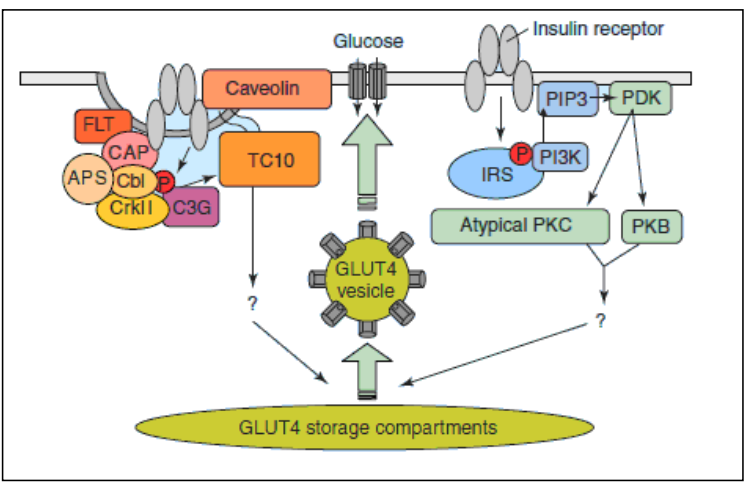

Figure 3. Signalling pathways involved in insulin-induced Glut-4 translocation. Adapted from *Current Biology*, 2003, Vol. 13, p. 574–576.

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Not only is insulin able to control glucose transport and glucose metabolism, but its actions also coordinate glycogen, lipid and protein synthesis. Insulin activates glycogen synthesis by promoting glycogen synthase dephosphorylation through the inhibition of glycogen synthase kinase 3 (GSK-3) or the activation of protein phosphatase 1 (PP1). Once the serine kinase AKT is activated, it phosphorylates and inactivates GSK-3, leading to the activation of glycogen synthase and, therefore, glycogen synthesis. Insulin increases synthesis, blocks the degradation of proteins through the activation of mTOR pathway, and regulates the synthesis of lipids. It also inhibits their degradation, mainly through the activation of transcription factor steroid regulatory element-binding protein (SREBP)-1c, which promotes the expression of genes encoding fatty-acid synthetic enzymes [3].

In addition to the metabolic effects of insulin, the binding of insulin to its receptor also triggers multiple signalling pathways that participate in cellular growth and differentiation. These pathways involve the tyrosine phosphorylation of IRS and/or Shc proteins, which activate the adaptor protein Grb2 and result in the activation of RAS protein, thus leading to the activation of the serine kinase cascade MAPK. Once activated, Ras operates as a molecular switch through the activation of Raf, MEK and ERK proteins. Activated ERK can translocate into the nucleus, where it catalyses the phosphorylation of transcription factors, thus initiating a transcriptional programme that leads to cellular proliferation or differentiation [3-5]. Several studies have shown that MAPK pathway might also be involved in the regulation of glucose uptake. The inhibition of PI3K protein prevents the full activation of p44/p42 MAPK in several cell types and the p38 MAPK inhibitor SB203580

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reduces the glucose uptake induced by insulin in 3T3-L1 adipocytes [10,11]. The p38 MAPK has been proposed as a positive regulator of insulin action because of its ability to increase the uptake of glucose by the plasma-membrane-localised Glut-4 transporter [12,13].

1.3 Insulin resistance

1.3.1 Metabolic basis of insulin resistance

Insulin resistance is defined as a pathologic state of decreased responsiveness of target tissues to normal circulating levels of insulin [14]. In other words, insulin resistance is a clinical state in which a normal or elevated insulin concentration reflects an impaired biological response. The pathophysiology of insulin resistance involves the complex network of signalling pathways, activated by the insulin receptor, which regulates intermediary metabolism [14].

Insulin resistance and insulin deficiency lead to a dysregulation of insulin effects on lowering blood glucose levels and are manifested by a decreased insulin-stimulated glucose uptake and metabolism in adipocytes and skeletal muscle, as well as by an impaired suppression of hepatic glucose production, which eventually causes elevations in fasting and postprandial glucose levels [1,14]. *In vivo* studies have shown that in humans, skeletal muscle is the principal site of glucose uptake under insulin-stimulated conditions (accounting for approximately 75% of glucose disposal in the postprandial state) and that less glucose is metabolized by adipose tissue [14].

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I. Introduction

Insulin resistance in skeletal muscle manifests itself primarily as a reduction in insulin-stimulated glycogen synthesis, which in turn is a consequence of reduced glucose uptake. The main characteristic of insulin resistance in adipose tissue is an uninhibited lipolysis. Accumulating evidence suggests that the disturbed function of adipose tissue in obesity plays a prominent role in the development and/or progression of insulin resistance [15]. The normal function of adipose tissue is to buffer the daily influx of dietary fat. However, when the buffering capacity for lipid storage in adipose tissue is decreased, as in obesity, other tissues are exposed to an excessive influx of fatty acids and TAG. This in turn may result in TAG storage, which interferes with insulin sensitivity (skeletal muscle and liver) and insulin secretion (pancreas). Moreover, in obesity, adipose tissue is characterized by the progressive infiltration of macrophages. Under normal conditions, the integration of inflammatory and metabolic pathways is beneficial for the maintenance of good health: many hormones, cytokines, signalling proteins, transcription factors and bioactive lipids can function in both metabolic and immune roles. However, in an altered metabolic state like obesity, the inflammatory response is modified [16]. Macrophage infiltration in adipose tissue may perpetuate a vicious cycle of macrophage recruitment, which can lead to disturbance in the secretion of adipokines and excessive fat storage in non-adipose tissues, thus resulting in the progressive loss of adipocyte function, insulin resistance and, ultimately, type-2 diabetes [15].

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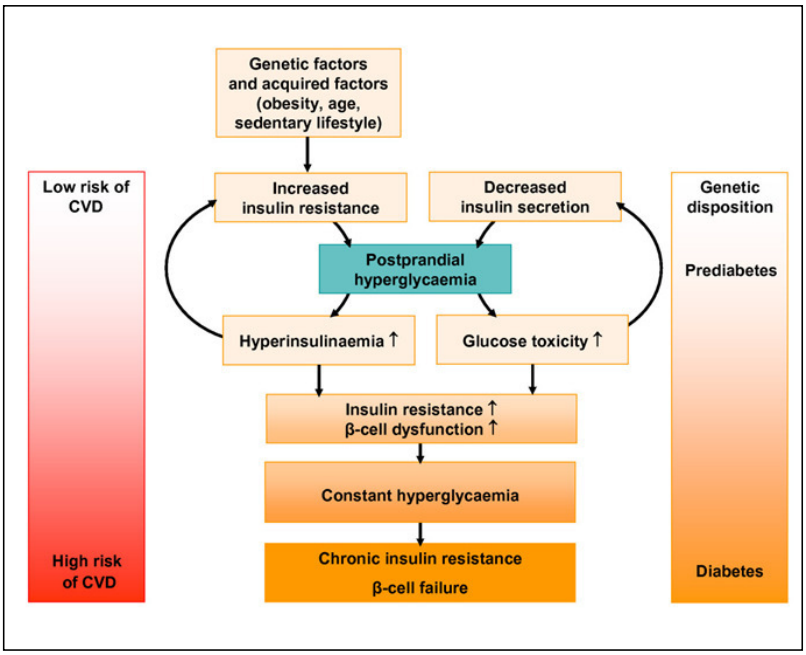

Figure 4. The role of postprandial hyperglycaemia in the development of type 2 diabetes and CDV. Adapted from Hanefeld M., *Cardiovascular Diabetology*, 2007; 6(1):20.

1.3.2 Molecular mechanisms of insulin resistance

The aetiology of insulin resistance includes both genetic and environmental factors [17]. Several circulating factors, such as hormones, free fatty acids, cytokines and adipokines, interleukins and inflammatory markers can also contribute to insulin resistance. Oxidative and cellular stress may also influence normal insulin action by altering the insulin-signalling pathway, which can lead to the development of insulin resistance [17,18]. This section includes several known mechanisms of insulin resistance which have been studied in this thesis.

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1.3.2.1 Hyperinsulinaemia

Hyperinsulinaemia, or chronic insulin exposure, induces peripheral insulin resistance by impairing insulin-signalling proteins. Several studies have shown that hyperinsulinaemia induced insulin resistance by impairing the tyrosine kinase activity of the insulin receptor. However, recent studies have mainly focused on dysregulation at the insulin-postreceptor level [2,17]. IRS-1 protein content is lower in 3T3-L1 adipocytes that have been chronically exposed to insulin and in adipocytes from insulin-resistant subjects and patients with overt Type-2 diabetes [19]. Moreover, hyperinsulinaemia may also induce IRS-1 phosphorylation at Ser307, which is related to the inhibition of IRS function, which deteriorates the insulin-signalling pathway [20,21]. The rapamycin-sensitive pathway (mTOR pathway) is implicated in the serine/threonine phosphorylation and degradation of IRS-1 after chronic insulin treatment, thus causing a reduction in insulin action [21]. Moreover, the inhibition of the mTOR pathway with rapamycin in 3T3-L1 adipocytes can prevent insulin resistance caused by chronic insulin treatment by preventing the reduction of IRS-1 protein levels, the down-regulation of PKB phosphorylation and the down-regulation of insulin-stimulated glucose transport [19]. However, rapamycin had no effect on the decrease in insulin-induced MAPK phosphorylation or Glut-4 protein levels, which suggests that chronic insulin exposure leads to a down-regulation of PKB and MAPK pathways through several mechanisms in adipocytes. Therefore, downstream of IRS-1, other insulin-signalling molecules are deregulated by hyperinsulinaemia. PKB activation was reduced in adipose tissue and skeletal muscle from diabetic db/db mice; Glut-4 expression was lower in insulin-resistant individuals; and both proteins were down-regulated in

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culture adipocytes after chronic insulin exposure [19]. Moreover, chronic insulin treatment may also induce another important group of insulin-resistance factors, the suppressors of cytokine signalling, SOCS-1 and -3, which may compete with IRS-1 for association with insulin receptor or augment proteosomal IRS-1 degradation, thus impairing insulin signalling. Hyperinsulinaemia therefore induces the deterioration of insulin action at several sites along the insulin-signalling pathway.

1.3.2.2 Cytokines and adipocytokines

Adipose tissue, the main site of energy storage, is also an endocrine organ made up of various cell types, including adipocytes, stromal cells, immune cells and vascular endothelial cells, which can produce large numbers of proteins collectively called adipocitokines or adipokines. Adipokines communicate with brain and peripheral tissues to regulate energy homeostasis and metabolism [22]. Many of these adipokines are well-known pro-inflammatory factors, such as TNF-α, leptin and IL-6 and they often exhibit upregulation in obesity. Other adipokines, such as adiponectin and IL-10, are anti-inflammatory adipokines that improve insulin sensitivity [23]. These mediators, synthesized from cells of the immune system as well as by adipose tissue, are critically involved in the regulation of insulin action [16].

Tumour necrosis factor α (TNF-α)

Tumour necrosis factor-α is a cytokine secreted by activated macrophages and a paracrine/autocrine factor highly expressed in the adipose tissue of various rodent obesity models and in the adipose tissue of obese humans. TNF-

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α is also an important mediator of insulin resistance and is considered the first molecular link between obesity, diabetes and chronic inflammation [24].

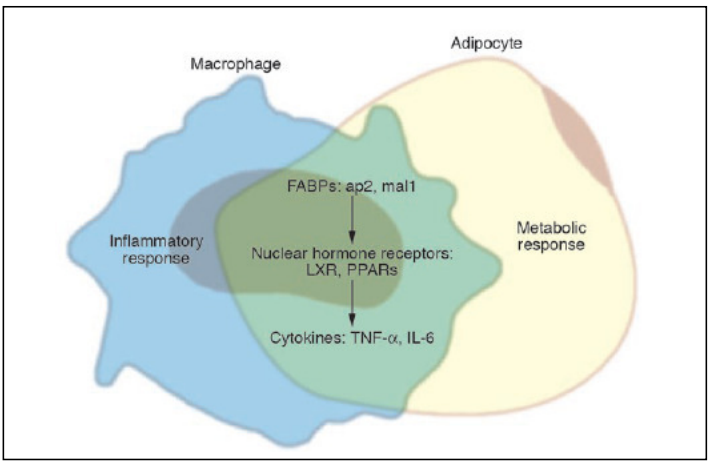

Figure 5. Integration of metabolic and immune responses in adipocytes and macrophages. Adapted from Kathryn E. Wellen and Gökhan S. Hotamisligi, *J Clin Invest*., 2005, Vol. 115(5), p. 1111-1119.

TNF-α induces lipolysis on adipose tissue, which is one of the main and immediate targets of TNF-α, thus resulting in a higher concentration of FFA plasma [25]. This in turn leads to a significant reduction in the intracellular glucose concentration because 1) it impairs the ability of insulin to suppress hepatic glucose output, 2) it impairs the ability of insulin to stimulate glucose uptake in skeletal muscle and adipose tissue and 3) it inhibits insulin secretion from pancreatic β-cells [14,17]. At the molecular level, both TNF-α and FFA can inhibit the insulin-signalling pathway by the induction of several serine kinases which phosphorylate IRS-1 on serine residues. Consequently, IRS-1 activation through tyrosine phosphorylation is impaired, which leads to defects in IR-mediated signalling and subsequent insulin resistance [26,27]. TNF-α has also

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been shown to inhibit the secretion of adiponectin in adipose tissue and to downregulate the expression of important genes such as adiponectin, Glut-4, IRS-1, C/EBP-α and PPAR-γ in 3T3-L1 adipocytes [25].

On the other hand, many genes expressed in adipose tissue that are responsible for inflammation, immune response and energy balance are known to be upregulated by TNF-α. Moreover, TNF-α may induce insulin resistance by increasing ROS levels, which can also activate multiple serine kinase cascades and decrease insulin signalling responses [28].

Adiponectin

Adiponectin is an abundant circulating cytokine, mainly synthesized by adipocytes, that sensitizes liver and skeletal muscle to insulin [25]. Serum levels of adiponectin are reduced in individuals with visceral obesity and states of IR. Also, treatment with adiponectin in obese animals decreases hyperglycaemia and FFA plasma levels and improves insulin sensitivity [18]. In liver, adiponectin induces fatty acid oxidation, decreases lipid synthesis and FFA uptake and represses gluconeogenesis by enzyme downregulation. In muscle, adiponectin favours glucose and FFA oxidation mainly due to AMPkinase activation [17]. Moreover, adiponectin inhibits inflammatory events by decreasing the expression of adhesion molecules on blood vessel walls, inhibiting chemotaxis of macrophages and their conversion to foam cells or inhibiting the proliferation of smooth muscle cells [17]. Adiponectin antagonizes many effects of TNF-α and this in turn suppresses adiponectin production. Furthermore, the synthesis of adiponectin is impaired in states of calorie excess, which may be associated with leptin resistance or leptin deficiency [29].

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Leptin

Leptin is a hormone secreted by adipocytes that plays a pivotal role in regulating energy homeostasis [17]. In normal conditions of weight maintenance, its concentrations are positively correlated with total body fat mass. However, obesity is associated with high circulating levels of leptin, which results in leptin resistance in the central nervous system, where endogenous leptin is no longer effective [18]. This phenomenon has been linked to a reduced uptake of leptin into the central nervous system, though it may be due to high levels of SOCS protein expression, since this protein is also able to bind to the leptin receptor [30]. In addition to its well-defined role in energy balance, several data clearly support a role for leptin in the regulation of glucose homeostasis. Leptin is able to reverse hyperglycaemia in ob/ob mice and improve glucose homeostasis in lipodystrophic mice and in humans [18]. These glucose-lowering effects of leptin are mediated through several organs. Leptin improves insulin sensitivity in muscle and liver by reducing intramyocellular lipid levels and decreasing intracellular hepatic triacylglycerol levels [18,31]. Leptin also inhibits insulin release, so it may have a direct interaction with insulin metabolism [32].

1.3.2.3 Glucocorticoids

Glucocorticoids (such as dexamethasone) are well-known insulin antagonists reported to induce insulin resistance in *in vitro* and *in vivo* experiments as well as in clinical trials [33-35]. Glucocorticoids enhance gluconeogenesis and glucose release in liver and may also impair peripheral glucose uptake by various mechanisms. Dexamethasone treatment can aggravate glucose uptake

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by increasing the availability of FFA, thus promoting lipolysis, which diminishes glucose oxidation and glucose uptake by peripheral tissues. In addition, the insulin-stimulated recruitment of Glut-4 to the cell surface was inhibited by dexamethasone in muscle and adipose tissue. Several studies have suggested that dexamethasone treatment inhibits glucose uptake by inhibiting glucose transport, thus impairing the translocation machinery of Glut-4 rather than impairing insulin signal transduction in 3T3-L1 adipocytes [33,36]. At the molecular level, it has been suggested that dexamethasone induces the attenuation of p38 MAPK signalling by the up-regulation of two MAPK phosphatases (MKP-1 and MKP-4), which reduces the activation of the Glut-4 in the plasma membrane [33]. *In vivo* studies have reported that chronic dexamethasone treatment also impairs insulin-stimulated PKB activation in muscles and adipocytes, though the effects of dexamethasone treatment on GSK-3 activation are contradictory [34,37]. On the other hand, reactive oxygen species levels increased after dexamethasone treatment, which may stimulate inflammatory kinases, thus decreasing the insulin-stimulated phosphorylation of several proteins and impairing the insulin-signalling pathway [28].

1.3.2.4 Oxidative and cellular stress

Reactive oxygen species (ROS), the radical forms of oxygen from products of mitochondrial respiration and enzymatic oxidases, have been shown to participate in normal cellular responses, including signal transduction pathways and gene regulation, thus supporting their potential role as second messengers [18]. However, oxidative stress, measurable as an accelerated ROS production or a decreased scavenging of ROS, induces tissue damage, which is responsible

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for a progressive deterioration in the capacity of insulin secretory beta cells and a lower insulin resistance in target tissues [28]. It has also been shown that oxidative stress leads to the activation of multiple serine kinase cascades, which increases the serine phosphorylation of IRS proteins and impairs insulin signalling. The increase in FFA, hyperglycaemia and hyperinsulinaemia, as well as the ROS accumulation caused by nutrient overload in obesity, could place a strain on endoplasmic reticulum (ER) capacity and trigger ER stress response [23]. Alternatively, an increase in fatty acids and glucose metabolism can lead to higher oxidative stress by mitochondria, which in turn could induce the expression of key immune genes through redox-regulated transcription factors or stress kinases [38]. Therefore, cellular stress caused by obesity and the subsequent activation of inflammatory signalling pathways appear to be central in obesity and insulin resistance.

1.3.3 Diseases associated with insulin resistance: Metabolic Syndrome and Diabetes Mellitus

Insulin resistance, obesity, atherogenic dyslipidemia, high blood pressure and proinflammatory or prothrombotic state are common, interrelated clinical disorders that are associated with a higher risk of cardiovascular diseases (CVD) and Type-2 Diabetes Mellitus and defined as the *Metabolic Syndrome* [39,40]. The prevalence of the Metabolic Syndrome is increasing around the world and, since its aetiology is still unknown, much attention has focused on the treatment of its individual components. Insulin resistance was a main risk factor associated with the Metabolic Syndrome. Though it was first known as the Insulin Resistance Syndrome, it has recently been suggested that the

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functional failure of adipose tissue may be the most important risk factor behind the syndrome [41].

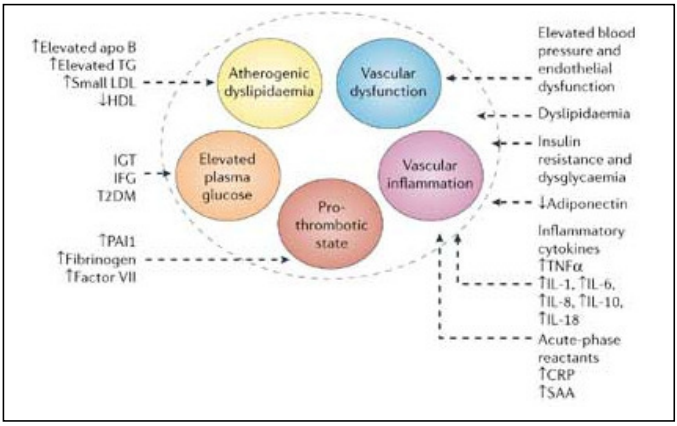

Figure 6. Risk factors of the metabolic syndrome. Adapted from Scott M. Grundy, *Nature Reviews Drug Discovery* 5, 2006, p. 295-309.

Diabetes Mellitus is a metabolic disorder characterized by chronically elevated levels of blood glucose resulting from a reduction in or the absence of insulin activity. Two states are described by the term diabetes mellitus: Type-1, a degenerative disease also known as Insulin-Dependent Diabetes Mellitus (IDDM) and Type-2 or Non-Insulin-Dependent Diabetes Mellitus (NIDDM), a polygenic and a multifactorial disease with a strong risk factor for cardiovascular disease.

The autoimmune reaction of the body to pancreatic beta cells in the islets of Langerhans and the resulting destruction of these beta cells cause an immediate insulin deficiency, resulting in Type-1 Diabetes Mellitus [42]. The administration of insulin or insulin analogues is fundamental to replacing the missing hormone since, due to the deficiency or absence of insulin, blood glucose levels cannot be maintained within the normal range. On the other

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hand, Type-2 Diabetes Mellitus is characterized by a group of disorders: a β-cell dysfunction, an increase in glucose production in the liver and an impaired insulin action or insulin resistance [43]. In Type-2 Diabetes, a dysregulation of the adaptive response of the pancreas, which adjusts insulin secretion in order to maintain glucose homeostasis, leads to a permanent elevation of glucose plasma levels. Although hyperglycaemia is detected in the first state of the disease, pancreatic β-cells can maintain normal glucose tolerance by a progressive increase in insulin secretion. However, further increases in glucose levels are associated with a progressive decline in the fasting insulin concentration. Pancreatic cell deficiency is characterized by an early loss of first-phase insulin response to glucose, followed by a gradual collapse of the later insulin response and a loss in the maximal secretory capacity of the beta cell. Consequently, pancreatic β-cells fail to offset the elevated glucose plasma concentration, thus leading to both a hyperglycaemic and hyperinsulinaemic state [1]. On the other hand, though a genetic predisposition to develop Type-2 Diabetes is known to exist, environmental factors such as obesity and a sedentary lifestyle are considered to be the major causes of this disorder [44]. Reducing weight, increasing physical activity and incorporating healthier eating habits may help to reduce the risk of developing Type-2 Diabetes.

In conclusion, the maintenance of normal glucose homeostasis will help us to prevent pathologies related to impaired glucose metabolism, such as insulin resistance or diabetes. The dramatic increase in these pathologies underlines our interest in finding food factors that may help to prevent these diseases and reduce the risk of developing cardiovascular disease, one of the leading causes of morbidity and mortality in industrialized countries.

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2. Procyanidins in the context of insulin resistance-related diseases

2.1 Flavonoids as bioactive compounds

Epidemiological data strongly suggest that diets rich in plant-derived food are generally associated with a preventive role against the development of chronic diseases [45]. In recent years, therefore, research has focused on identifying specific plant components that provide health as well as nutritional benefits.

Kitts et al. define *bioactive compounds* as extranutritional natural constituents found in small quantities in plant products and lipid-rich foods [46]. Bioactive plant food constituents, "plant bioactives", have been defined more recently as non-nutritive constituents inherent in food plants that, when ingested, have anticipated health promoting or other beneficial effects as well as possible toxic effects [47]. Bioactive compounds, commonly found as a mixture of molecules in plants, are involved in the metabolic processes of plants and may have specific functions, such as to influence structure, colour and flavour, or to participate in the plants' defence systems, protecting them from insects, microbial infections or UV light [48]. Evidence is growing that plant constituents, also termed phytochemicals or phytonutrients, may help reduce the risk of chronic diseases such as cancer or cardiovascular diseases [45]. However, there is little understanding of the precise biological mechanism(s) that lead plant-based bioactive food components to provide health-promoting benefits.

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Plant bioactives can be divided into several groups, including polyphenols, phytosterols, carotenoids, glucosinolates, organosulfur compounds, soluble or insoluble dietary fibre, and plant sterols [48]. Phenolic compounds, commonly known as polyphenols, are the most numerous and widely distributed group of bioactive molecules. Polyphenols are present in all plants, including vegetables, fruits and grains. A diet rich in fruit and vegetables therefore contains high levels of polyphenols [49].

Flavonoids are the most numerous phenolic compounds. They are present in a wide range of plant-derived foods, mainly in the skin of fruits such as grapes and in the epidermis of leaves such as tea leaves. The basic structure of flavonoids comprises two benzene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring (C) in the middle [50,51]. In addition to this diversity, they can be hydroxylated, methoxylated, glycosylated or acetylated. Flavonoids are usually subdivided according to their chemical structure into flavan-3-ols or flavanols, anthocyanins, flavonols, flavones, isoflavones and flavanones.

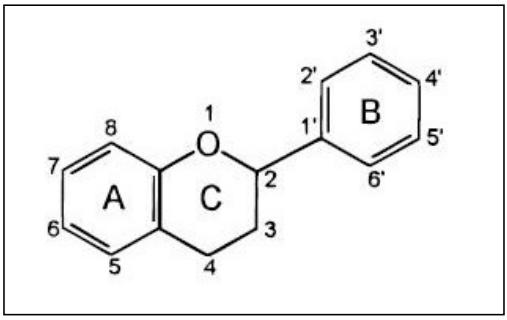

Figure 7. Basic flavonoid structure. Adapted from Patricia M. Aron et al., *Mol. Nutr. Food Res*. 2008, (52): p. 79-104.

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After ingestion, flavonoids must be absorbed. The exact mechanisms involved in flavonoid absorption are not clear. However, it has been suggested that after consumption, the majority of dietary flavonoids (glycosylated flavonoids) are absorbed from the small intestine and pass through the gut wall into the blood stream. Before absorption, the sugars attached to the flavonoid skeleton must be removed. This process is controlled by the action of enzymes in the small intestine, which are responsible for the release of the flavonoid skeleton from its sugar. The location at which the sugar is attached to the flavonoid skeleton affects the mechanism by which glycosylated flavonoids are absorbed [52]. Several pathways for polyphenol absorption, involving a sodium-glucose co-transporter (active transport) or a lymph system transportation, have been proposed [53].

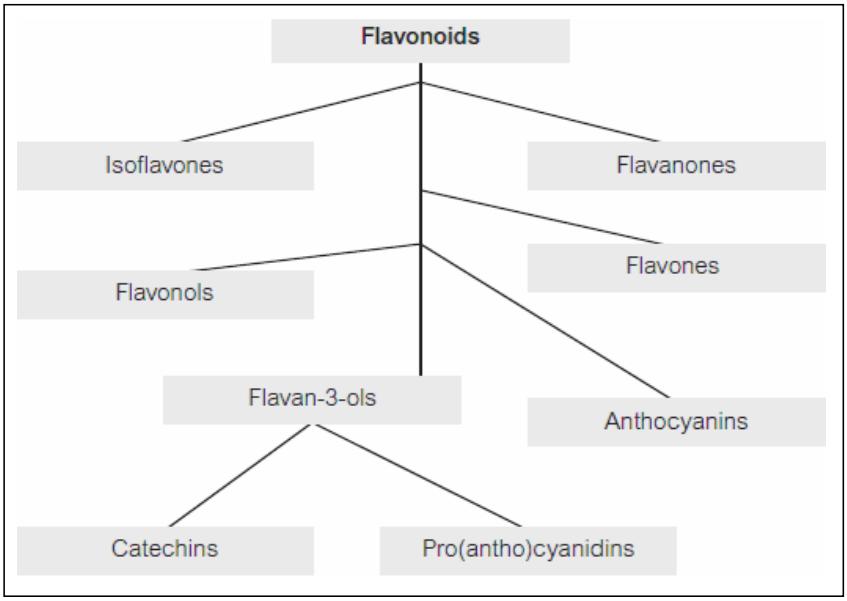

Figure 8. Categories of flavonoids. Adapted from Anna Denny and Professor Judith Buttriss, British Nutrition Foundation, EuroFIR 2004.

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Flavonoids are then further metabolized first in the gut and then in the liver and kidneys to produce flavonoid secondary metabolites, which can be detected in blood and urine after flavonoids are consumed. Flavonoid secondary metabolites enter into circulation. Much evidence suggests that they exert biological effects on the body [54]. However, the detection and quantification of dimers and trimers of procyanidins, which are oligomers of flavan-3-ols, in the rat plasma suggest that free forms of flavonoids can also be absorbed and metabolized as flavonoid secondary metabolites [55].

2.2 Effects of flavonoids on insulin resistance-related diseases

Flavonoids perform a wide array of biochemical and pharmacological actions. Epidemiological studies have shown that moderate red wine intake is inversely associated with the risk of cardiovascular disease and that red-wine flavonoids are mainly responsible for these protective effects through the inhibition of LDL oxidation and/or the inhibition of platelet aggregation and adhesion [56,57]. Flavonoids are also known to possess antioxidant [58], anti-inflammatory [59], antiallergic, antiviral and anticarcenogenic [60] activities. These protective properties have made phenolic compounds the focus of nutrition research that aims to further describe the beneficial effects of flavonoids and better explain their molecular mechanisms of action [45,61].

With regard to the effects of flavonoids on disorders related to insulin resistance, some reports have shown that monomeric flavonoids improve hyperglycaemia in various models of insulin resistance. Some of them act as antihyperglycaemic agents in STZ-induced insulin-resistant animals (a model

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that reproduces Type-1 Diabetes) in genetic models of metabolic syndrome or in genetic models of Type-2 Diabetes [59,62-68]. Other flavonoids, such as cyanidin 3-O-β-D-glucoside, myricetin and epigallocatechin gallate (EGCG), ameliorate the hyperglycaemia induced by a high-fat or high-fructose diet in rodents [69-71]. Quercetin, sakuranetin, kaempferol, kaempferol 3-neohesperidoside (the flavonoid compound glycoside) and EGCG have insulinomimetic effects on stimulating glucose uptake in differentiated 3T3-L1 adipocytes, in rat soleus muscle and in rat skeletal muscle [72-75]. However, not only monomers but also extracts rich in flavonoid structures help to prevent the progression of diabetes by improving hyperglycaemia in streptozotocin-induced diabetic rats as well as in genetic diabetic mice [76,77]. Other studies have also shown the effects of flavonoids on animal models of diet-induced insulin resistance or diabetes. A green tea polyphenol extract regulates the expression of genes involved in glucose uptake and insulin signalling in the muscle of rats fed a high-fructose diet [78]. Green tea supplementation also improves insulin resistance and increases Glut-4 content in the same rat model [60]. Flavones extracted from seed residues of *Hippophae rhamnoides L.* exert antihypertensive effects at least in part by improving insulin sensitivity in rats fed a sucrose-rich diet [79]. Moreover, the administration of soy isoflavones reduces serum insulin concentration and improves insulin resistance in insulin-resistant rats on a high-fat diet [80].

Though considerable caution should be exercised when translating animal findings to humans, several studies suggest that certain flavonoids can help to improve hyperglycaemia in subjects with impaired glucose tolerance. The isoflavones or isoflavonoids found in soy are considered the biologically active

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components of dietary soy that modulate hyperglycaemia and reduce body weight, hyperlipidaemia and insulin resistance in obese and diabetic human subjects [81,82]. Recent studies have also shown the positive effects of flavonoids in slightly decreasing glycaemia in normoglycaemic individuals or improving several blood parameters in subjects with impaired glucose tolerance, even though some of these effects were eventually reversed [83,84]. However, most flavonoid studies in humans have had controversial results, mainly because of the diversity of the phenolic structures assayed. In fact, food contains mixtures of flavonoid structures with different degrees of polymerization. Also, it is known that flavonoid effects are not only due to one molecule but due to synergic effects, which makes it difficult to identify the bioactive molecules [85]. More work is therefore needed in order to better define the effects of each structure.

2.3. Physiological effects of procyanidins and their mechanisms of action

2.3.1 Possible procyanidin activity on insulin resistance-related pathologies

The largest and most ubiquitous class of flavonoids, the flavan-3-ols, comprise the main constitutive units of condensed proanthocyanidins. Homo-oligomeric proanthocyanidins with two (3',4') B-ring hydroxyl groups are termed procyanidins and are one of the most common types of proanthocyanidins found in nature [51]. Procyanidins are oligomeric structures formed by the polymerization of the monomeric flavan-3-ols (+)-catechin and (−)-epicatechin up to 10 subunits, while further polymerized structures are

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classified as condensed tannins. Generally, a nucleophilic attack on the carbocations by epicatechin or catechin could produce dimers and then oligomeric proanthocyanidins linked through C4–C8 or C4–C6 bonds. Both of these linkages are called B-type proanthocyanidins. However, alternative linkages, called A-type proanthocyanidins, can occur between C2 and C7, and this subclass lacks two hydrogens compared to the B-type [51,86]. The flavan-3-ol subunits may carry acyl or glycosyl substituents and the most common substituent bound as an ester is gallic acid to form 3-O-gallates. The high complexity of procyanidins is due to the different configuration of the monomers, the different types of interflavanoid bonds, and the various lengths of the chains, known as the degree of polymerization (DP) [86].

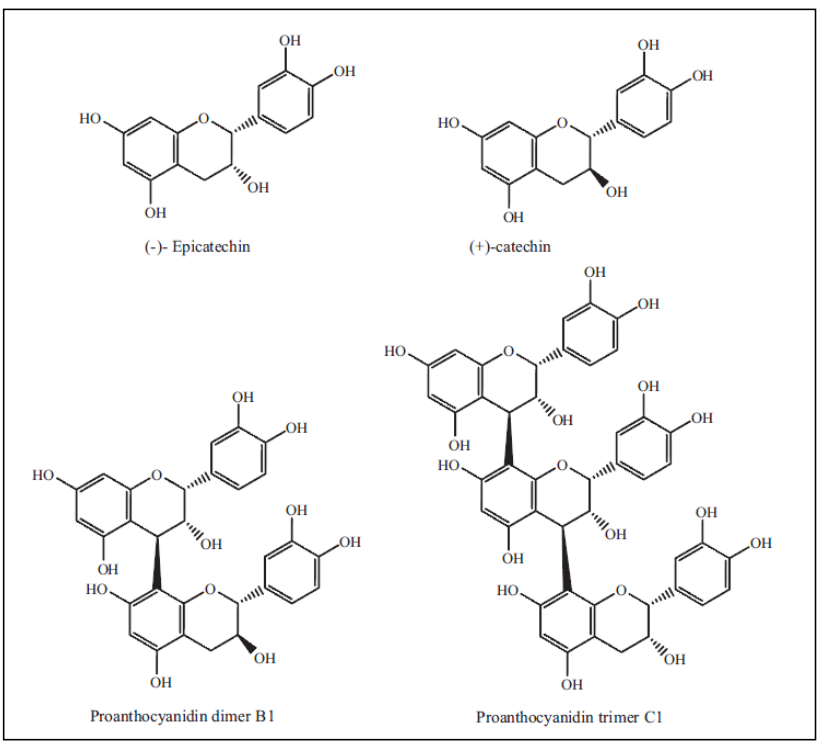

Figure 9. Structures of monomeric flavan-3-ols and dimer and trimer of proanthocyanidin. Adapted from Jeevan K. Prasain, *Phytomedicine,* 2009, 233–243.

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Procyanidins are widely distributed in the human diet. They are present in the fruits, bark, leaves and seeds of many plants and derived foods such as green tea, apples, cocoa, chocolate, grapes, apricots and cherries. They are especially abundant in fruit juices and red wine [50,51,87]. Several authors have studied flavan-3-ol consumption and bioavailability. Arts et al. estimated that the mean intake of flavan-3-ol monomers in the Netherlands was 50 ± 56 mg/day, with tea being the major contributor followed by chocolate and apple. Gu et al., reported that daily proanthocyanidin intake could vary from 10 mg to 0.5 g/day and they estimated that the mean daily proanthocyanidin intake in the United States was 53.6 mg/person/day [88]. The lack of reliable concentration data for proanthocyanidin in foods has made it impossible to accurately evaluate their dietary intake. Most studies have been conducted with total flavonoid intake. These have estimated values of between 5 mg/day and 1 g/day, depending on the country of study, though, most of these studies focused on a limited number of structures, mainly monomers, and investigated only a relatively small number of foods [89,90].

The beneficial effects of procyanidins against coronary heart diseases, atherosclerosis and several metabolic processes associated with their development have been widely studied [57,91,92]. Procyanidins are involved in the modulation of cholesterol and lipid metabolism [93,94], induce changes in vascular events [95], have antigenotoxic and cardiovascular effects [96,97] and improve oxidative or inflammatory states [98-101]. Procyanidins have antiproliferative effects and have been studied as cancer preventive agents [102,103]. However, there is little information about their involvement in glucose metabolic disorders, though several pathologies such as obesity, insulin

resistance and diabetes are directly related to glucose metabolism and insulin action deficiencies [1,14].

Previous *in vivo* and *in vitro* studies suggest that procyanidins may be beneficial against these metabolic disorders thanks to an insulin-like activity, since they mimic some of the physiological effects of insulin [104-106]. Moreover, most of these studies used natural extracts rich in procyanidins with different degrees of polymerization instead of purified procyanidins, partly because of the difficulty in identifying and purifying the various procyanidin structures. Natural extracts rich in procyanidin structures are reported to exert antihyperglycaemic effects in several models of insulin resistance or diabetes. In alloxan or STZ-induced diabetic rats, a model of Type-1 Diabetes Mellitus, procyanidins from persimmon peel decreased serum glucose and glycosylated proteins [99]. The administration of a standardized extract from the bark of the French maritime pine, pycnogenol, significantly reduced blood glucose levels [107] and grape seed extracts, which contain a rich mixture of monomeric and oligomeric procyanidins, have an antihyperglycaemic effect [104-106]. Grape seed extracts also improve hyperglycaemia in a mouse model of Type-2 Diabetes [108], restore glucose levels in high-fructose-fed rats [106], and alleviate insulin resistance in mice and HepG2 cells [109]. Dietary supplementation with cacao liquor proanthocyanidins are also reported to prevent the development of hyperglycaemia in diabetic obese mice [110].

Few studies have focused on humans. These have mainly involved clinical trials with healthy or diabetic patients. Pycnogenol extract showed glucose-lowering effects in clinical trials with diabetic patients [111]. The consumption

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of unsweetened cranberry juice rich in trimer and heptamer proanthocyanidins is also associated with a favourable postpandrial glycaemic response in healthy humans [112], while low-calorie cranberry juice has a favourable glycaemic response in patients with Type-2 Diabetes [113]. Although all these studies suggest that procyanidins are beneficial against insulin resistance and related pathologies by acting as antihyperglycaemic agents, most of them do not fully describe their intracellular mechanisms.

2.3.2 Intracellular mechanisms of procyanidin effects

The antioxidant effects of procyanidins, their ability to influence receptor function and the modulation of cell signalling pathways and gene expression through direct interaction with intracellular components provide a wide base for the physiological effects ascribed to these compounds, which help explain some of the intracellular mechanisms of the procyanidin effects [51,58].

It has recently been suggested that, after procyanidin intake, oligomeric procyanidins exert their hypoglycaemic activity by suppressing digestive enzymes such as α-glucosidase or α-amilase that participate in carbohydrate digestion, thus inhibiting glucose absorption and postpandrial hyperglycaemia [114,115]. However, procyanidins may also act by modulating pancreas function, since it is known that a dysregulation of the pancreas leads to elevated glucose plasma levels. However, few studies have analysed how procyanidins affect the pancreatic tissue [116]. Pancreatic β-cells synthesise and secrete insulin, which is released into the portal vein and carried to the liver, where its primary function is to suppress hepatic glucose production and

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stimulate glucose uptake by muscle and adipose tissue. The antihyperglycaemic action of procyanidins might therefore be partly due to their insulinomimetic effect in suppressing hepatic glucose production by increasing hepatic glucokinase activity [109] and/or decreasing glycogenolysis in the liver [117] or by inducing glucose uptake in peripheral tissues [104,105]. Procyanidins might achieve all of these effects by activating the proteins involved in the insulin-signalling pathway or in the insulin-mediated glucose transport or by modulating the expression of several genes involved in both processes. For instance, the antihyperglycaemic effect of a grape-seed procyanidin extract (GSPE) in streptozotocin-induced diabetic rats (the GSPE extract used by our research group) is explained in part by its insulinomimetic properties in increasing the glucose uptake in L6E9 myotubes and in 3T3-L1 adipocytes by inducing Glut-4 translocation to the plasma membrane that requires PI3K and p38 MAPK proteins [105]. Another extract rich in procyanidin structures stimulates glucose uptake by inducing Glut-4 translocation in 3T3-L1 adipocytes [118]. In addition, type-A procyanidin polymers of cinnamon extract display insulin-like activity by increasing the gene expression and protein levels of tristetraprolin, insulin receptor, and glucose transporter 4 in culture adipocytes [119].

Insulin also promotes the synthesis and storage of glycogen, lipids and proteins in liver and peripheral tissues, while inhibiting their degradation and release into the circulation [1]. In Type-2 diabetic individuals, the ability of insulin to inhibit the lipolysis of adipose tissue and reduce the plasma FFA concentration is markedly reduced. Chronically elevated plasma FFA concentrations can lead to insulin resistance in muscle and liver and impair

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insulin secretion [1]. Moreover, an impaired adipocyte differentiation appears to be a precipitating factor in the development of Type-2 Diabetes [15]. In this sense, long-term GSPE treatment increases the lipolytic rate and downregulates some mature adipocyte markers, such as glycerol-3-phosphate dehydrogenase (G3PDH) activity, PPARγ2 and HSL mRNA in 3T3-L1 adipocytes [120,121]. Moreover, procyanidins limit adipocyte formation by altering the gene expression profile during *in vitro* adipocyte differentiation [122]. Therefore, in culture adipocytes, procyanidins can modulate several metabolic processes, such as the mobilization of lipid stores or the inhibition of adipocyte differentiation [123]. In addition, GSPE mimics the anabolic effects of insulin because activates lipid and glycogen synthesis in 3T3-L1 adipocytes [117]. However, GSPE stimulates glycogen synthesis less than insulin and induces a higher incorporation of glucose into lipid, mainly because more glucose is directed to glycerol synthesis. These results therefore suggest that procyanidins may use a different mechanism of action to those of insulin, though they share some intracellular mediators, such as PI3K or p38 MAPK, or some metabolic process, such as Glut-4 translocation [105].

In summary, the insulinomimetic properties of grape-seed procyanidins and their differences with respect to insulin mechanisms suggest that procyanidins are beneficial against insulin resistance or diseases related to insulin resistance. However, more detailed studies at the molecular and cellular levels as well as in animal models are needed to determine how procyanidins exert their antidiabetic activity.

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II. Objectives

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Objectives

Bioactive compounds are nonessential biomolecules present in food that have the capacity to modulate one or more metabolic processes that may lead to improvements in health. Bioactive food components therefore play an important role in health maintenance. However, the precise biological mechanisms that lead plant-based bioactive food components to exert health-promoting benefits are not fully understood. The discipline that studies the molecular mechanisms underlying the bioactivity of nutrients and explains the basis of nutrient actions on human health is nutrigenomics.

Flavonoids are a group of bioactive molecules that are present in a wide range of fruits and vegetables. One of the most abundant subclasses of flavonoids are the procyanidins—oligomeric forms of flavan-3-ols that are distributed in the human diet. Our research group, which is working in the field of Nutrigenomics, has described some of the beneficial effects of a grape-seed procyanidin extract (GSPE) in preventing and ameliorating several metabolic disorders that are risk factors for the metabolic syndrome. The motivation behind this thesis was the scarce information on the effects of GSPE on glucose metabolism.

GSPE is reported to mimic some of the physiological effects of insulin and so lower hyperglycaemia in streptozotocin-induced diabetic rats. Moreover, GSPE stimulates glucose uptake in insulin-sensitive cell lines by using some of the intracellular mediators of the insulin-signalling pathway and some of the insulin mechanisms. However, GSPE showed some divergences when compared to insulin action. After chronic GSPE treatment, there was little resistance to the effects of insulin. Moreover, some intracellular intermediaries as well as some

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metabolic process were activated differently by insulin and GSPE. These divergences suggested a possible role of GSPE under insulin-resistant conditions.

Most of the work was conducted in cell lines (mainly 3T3-L1 adipocytes), where adipose tissue was highlighted as an important target for procyanidin action. Previous results for the effects of GSPE on glucose metabolism and its involvement in the insulin-signalling pathway, plus the fact that adipose tissue is important in the development of insulin resistance and metabolic syndrome, supported the need for a thesis aimed at increasing our understanding of how procyanidins might prevent *in vivo* insulin resistance pathologies.

All the above studies were conducted with a procyanidin extract that reflects a real food source. Its application in *in vivo* studies is also an approach of human food intake. However, working with an extract has several limitations, mainly at the cellular level, which also make it difficult to identify the molecular mechanisms. Also, since various compounds of the procyanidin extract can exert antagonistic or synergic effects or interact with different targets, determining the molecule(s) responsible for the effects of the extract is more complex.

In view of the above, the main purpose of this thesis is to understand how dietary procyanidins modulate glucose metabolism under a resistant condition, mainly in adipose tissue. To achieve these objectives, our specific aims are:

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1) To evaluate the effectiveness of procyanidins in improving glucose metabolism in different *in vivo* and *in vitro* experimental models of impaired glucose tolerance and/or impaired insulin action.

2) To further describe, at the molecular level, the metabolic targets of procyanidins in insulin-signalling pathways that explain procyanidin insulinomimetic effects.

3) To identify the molecular bioactive components of a grape-seed procyanidin extract in order to determine which compound/s are responsible for the total extract effects.

This research is part of a more general project developed by the Nutrigenomics Research Group of the Department of Biochemistry and Biotechnology of the Rovira i Virgili University in Tarragona, Spain. The author has also completed an international *stage* at the IRIBHM institute, *Institut de Recherche Interdisciplinaire en Biologie Humaine et Moléculaire* of the Faculty of Medicine of the *Université Libre de Bruxelles, Université d'Europe* (ULB) in Brussels, Belgium. This thesis has been supported by a grant from the Rovira i Virgili University.

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III. Results

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(Submitted at Journal of Nutritional Biochemistry)

1.

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Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance

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Running title: GSPE effects under insulin resistance

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Keywords
high-fat diet, adipose tissue, obesity, procyanidins, insulin resistance

Abstract

Flavonoids are beneficial compounds against risk factors for metabolic syndrome but their effects and the mechanisms on glucose homeostasis modulation are not well defined. In the present study we first checked the efficacy of grapeseed procyanidin extract (GSPE) for stimulating glucose uptake in insulin-resistant 3T3-L1 adipocytes.
Results show that when resistance is induced with chronic insulin treatment, GSPE maintain a higher stimulating capacity than insulin. In contrast, when dexamethasone is used as the resistance-inducing agent, GSPE is less effective. Next we evaluated how effective different GSPE treatments are at improving glucose metabolism in hyperinsulinemic animals (fed a cafeteria diet). GSPE reduced plasma insulin levels. The lower dose (25 mg GSPE/kg body weight*day) administered for 30 days improved the HOMA IR index. This was accompanied by down-regulation of Pparγ2, Glut4, and Irs1 in mesenteric white adipose tissue. Similarly, a chronic GSPE treatment of insulinresistant 3T3-L1 adipocytes down-regulated the mRNA levels of those adipocyte markers, although cells were still able to respond to the acute stimulation of glucose uptake.
In summary, 25 mg/kg bw*day of GSPE has a positive long-term effect on glucose homeostasis, and GSPE could be targeted at adipose tissue, where it might directly stimulate glucose uptake. This work also highlights the need to carefully consider the bioactive dose, since a higher dose does not necessarily correlate to a greater positive effect.

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Introduction

Metabolic syndrome and its association with an increased risk of cardiovascular disease and type 2 diabetes is an important public health concern. First-line therapy for managing the metabolic syndrome is to modify the patient's lifestyle. For this reason, the Mediterranean diet, rich in flavonoids, can be considered an effective treatment for improving health in patients with metabolic syndrome [1, 2, 3]. Of all the ways in which flavonoids act on risk factors, the way they modulate glucose homeostasis is the least well defined. Some studies on humans have shown positive flavonoid effects. Taking 30 mg per day of polyphenol for 18 weeks tended to decrease glycaemia in normoglycemic individuals [4]. Ingesting green tea for 32 weeks initially improved several blood parameters (e.g. glucose) though this was reversed by day 87 [5]. In fact, results on humans are controversial, partly due to the diversity of the phenolic structures assayed, that is, flavonoids include hundreds of monomeric structures, oligomers, polymers, and modified forms.

Several studies on rodents have shown that monomeric flavonoids are antihyperglycemic agents in some models of insulin resistance [6, 7, 8, 9, 10 and 11]. Also, extracts containing a mixture of flavonoid structures can have antihyperglycemic effects. In fact, food contains mixtures of flavonoid structures and different degrees of polymerization, and this makes it difficult to establish which of the molecules is the active one. Furthermore, it is likely that the effects are not due to one molecule but to synergic effects [12]. Up to now, most work has been done in streptozotocin (STZ)-diabetic rats [13, 14 ,15, 16]. However, STZ-animals are not an insulin-resistance model. There has been much less work done on insulin resistant models. Al-Awwadi et al. have proven that an extract rich in grape oligomeric procyanidins restores glucose levels in a fructose-induced resistance model [17], and Lee et al. also proved the positive effects of Persimmon peel procyanidins on db/db mice [18].

The mechanisms that exert such effects might vary among flavonoids, though many of the studies on monomerics flavonoids found increased glucokinase (Gck) mRNA expression in liver, highlighting this gene expression as a target for antihyperglycemic monomeric flavonoid effects [7, 8, 9, 19]. Most studies on procyanidin extracts mainly rely on their analysis of antioxidant or antiinflamatory effects [17, 18].

In this paper, we analyze how effectively a grape seed procyanidin extract ameliorates several insulino-resistant situations. Such experimental conditions would be of great importance in the field of functional food design, where the effects of natural compounds must be evaluated in a context of middle/long term administration, and carefully considering the doses that might be administered. In fact, our results highlight the importance of cautiously interpreting results obtained from only one flavonoidtreatment condition.

Materials and Methods

Cell culture and glucose uptake assay

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described [21]. Insulin resistance was induced in mature adipocytes by using different agents: 1 nM insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) for 15 hours, 3 nM TNFα (ProSpec-Tany TechnoGene LTD, Rehovot, Israel) for 5 hours, 25 mU/mL glucose oxidase (SIGMA, Missouri, USA) for 2 hours on depletion media (serum-free supplemented DMEM containing 0.2% BSA) and 100 nM dexamethasone (SIGMA, Missouri, USA) for 24 hours. PBS solution was used as control. During the last 30 minutes of incubation, adipocytes were treated

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with either 100 nM insulin or 100 mg/L GSPE and then glucose uptake was assayed. The effects of chronic GSPE treatment in insulin-induced insulin resistance were analyzed by preincubating 3T3-L1 adipocytes for 2h with 100 mg/L GSPE and maintaining them for a further 15h with 1nM insulin.

GSPE or insulin were acutely stimulated for 30 minutes in these long-term treated cells and glucose uptake was analyzed. Glucose uptake was determined by measuring the uptake of 2-deoxy-d-[3H] glucose as previously described [16]. Each condition was run in triplicate. According to the manufacturer (DRT, Dax, Fance) GSPE essentially contains the following procyanidins: monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5-13 units; 31.7%).

Animal Experimental Procedures

Wistar female rats weighting between 160-175g were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22 ºC with a 12-h light, 12-h dark cycle. After 1 week in quarantine the animals were divided in two groups: a control group (12 animals) fed with a standard diet (Panlab A03) and a cafeteria group (36 animals) fed with a cafeteria diet (bacon, sweets, biscuits with pâté, cheese, muffins, carrots, milk with sugar) and water plus the standard diet. Animals were fed *ad libitum* and the food was renewed daily.

Obesity was induced in the animals with cafeteria diet for 13 weeks. Afterwards, the cafeteria group was divided in three subgroups (12 animals/group): i) cafeteria group: rats treated with a vehicle (sweetened condensed milk), ii) cafeteria + 25: rats treated with 25 mg of GSPE/kg of body weight (bw)*day and iii) cafeteria + 50: rats treated with 50 mg of GSPE/kg of bw*day. Rats were fed either the vehicle or GSPE dissolved in the vehicle by controlled oral intake with a syringe. After 10 days of GSPE treatment, 6 animals from each group were sacrificed (short treatment). After 30 days of GSPE treatment, the remaining 6 animals of each group were sacrificed (long treatment). Schematic diagram of procyanidin treatments is showed in scheme 1. Three days before the sacrifice, an intraperitoneal glucose tolerance test (IPGTT) was carried out (2 g glucose/kg bw). Glucose was measured with a glucometer after blood samples had been collected by tail bleeding (Glucocard, Menarini, Barcelona, Spain).

At 9 a.m. on sacrifice day, the rats were treated with GSPE or vehicle and food was renewed. At 11 a.m. food was withdrawn. After 3h of fasting the animals were sacrificed by beheading. Blood was collected using heparin and animal tissues were excised, frozen immediately in liquid nitrogen and stored at -80°C until analysis. All the procedures were approved by the Experimental Animals Ethics Committee of the Rovira i Virgili University.

Glucose plasma levels were assayed using an enzymatic colorimetric kit (GOD-PAP method from QCA, Spain). Hormone plasma levels were determined using ELISA kits following the manufacturers' instructions (Rat insulin from Mercodia, Uppsala, Sweden and leptin from LINCO, Missouri, USA). Measurements were taken with an ELISA Anthos Zenyth 200 st Microplate Reader.

Quantitative RT-PCR

Total RNA was isolated from adipocytes grown in 6-well plates using the High Pure RNA Isolation Kit (Roche). mRNA levels were measured using real-time RT-PCR as previously described [22]. Amplifications were performed using the following primers, Glut4 CAACGTGGCTGGGTAGGCA (forward) and ACACATCAGCCCAGCCGGT (reverse); Irs1 CCCACAGCAGATCATTAACC (forward) and AGAGACGAAGATGCTGGTGC (reverse); adiponectin

GATGGAGGAGCACAGAGCC (forward) and GGCCGTTCTCTTCACCTACG (reverse); resistin GTACCCACGGGATGAAGAACC (forward) and GCAGACCCACAGGAGCAG (reverse); Pparγ2 CTGTTGACCCAGAGCATGGT (forward) and AGAGGTCCACAGAGCTGATTCC (reverse); C/EBPα GGTGGACAAGAACAGCAACGA (forward) and CGTTGCGTTGTTTGGCTTTATC (reverse). The mRNA levels of each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA detected in each sample (GAPDH; CATGGCCTTCCGTGTTCCT (forward) and CCTGCTTCACCACCTTCTTGA (reverse)).

Total RNA from liver and adipose tissue were extracted using TRYZOL reactive following the manufacturer's instructions. cDNA was generated using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystem).

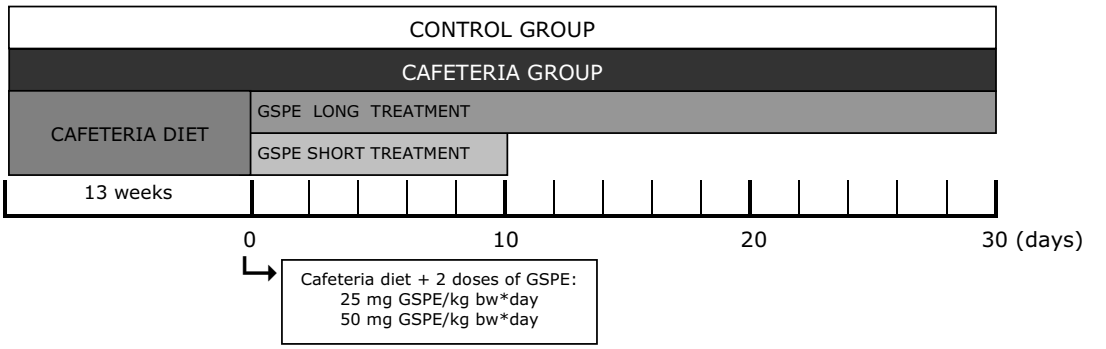
Quantitative PCR amplification and detection were performed by using specific TaqMan Assay-On- Demand probes (Applied Biosystems, Rn00562597-m1 for Glut4, Rn01495763-g1 for Srebp1c, Rn00440945_m1 for Pparγ2, Rn02132493-s1 for Irs1, Rn00565347_m1 for Glucose-6-phosphatase (G6p) and Rn00561265_m1 for Gck).

Cyclophiline was used as the reference gene in quantitative PCR (Applied Biosystems TaqMan Assay-On-Demand probe Rn00690933_m1).

Reactions were run on a quantitative Real-Time PCR 7300 System (Applied Biosystem) according to the manufacturer's instructions. Quantifications were performed in triplicate.

Calculations and statistical analysis

Results are expressed as the mean ± SEM. Effects were assessed using one-way ANOVA or Student's T-test. All calculations were performed using SPSS software. (SPSS, Inc., Chicago, IL).



Scheme 1. Schematic diagram of procyanidin treatments.

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Gemma Montagut Pino
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Results

GSPE acutely stimulate glucose uptake better than insulin does in chronic insulininduced insulin resistance in 3T3-L1 adipocytes

Previous results led us to postulate that GSPE could be effective as a preventive and/or corrective agent against insulin-resistance. We now evaluated this role in 3T3-L1 adipocytes under different metabolic insults. We selected four well-validated experimentally models of insulin resistance, all of them with physiological relevance *in vivo* and involved in diferent cellular response pathways: chronic hyperinsulinemia [23], TNF-α [24], dexamethasone [25], and oxidative stress treatment [26].

Figure 1 shows the stimulation of glucose uptake by acute insulin or GSPE treatment in 3T3-L1 cells preincubated with the insulin resistance-inducing agents. Fifteen hours of 1 nM insulin treatment increases the basal glucose uptake, but limits maximal acute insulin stimulation to 23.6%. Under this resistant situation, an acute stimulation with GSPE (100 mg/L) reaches 47.2 % of its maximal stimulation under a non-resistant situation. GSPE was not observed to be more efficient at acutely stimulating glucose uptake after chronic insulin treatment in any of the other insulin-resistance conditions. After inducing insulin-resistance by treating the 3T3-L1 cells with glucose oxidase (GO) 25 mU/mL, insulin acute stimulation reached 70.6% of its maximal effect. Acute GSPE treatment leads to similar results.

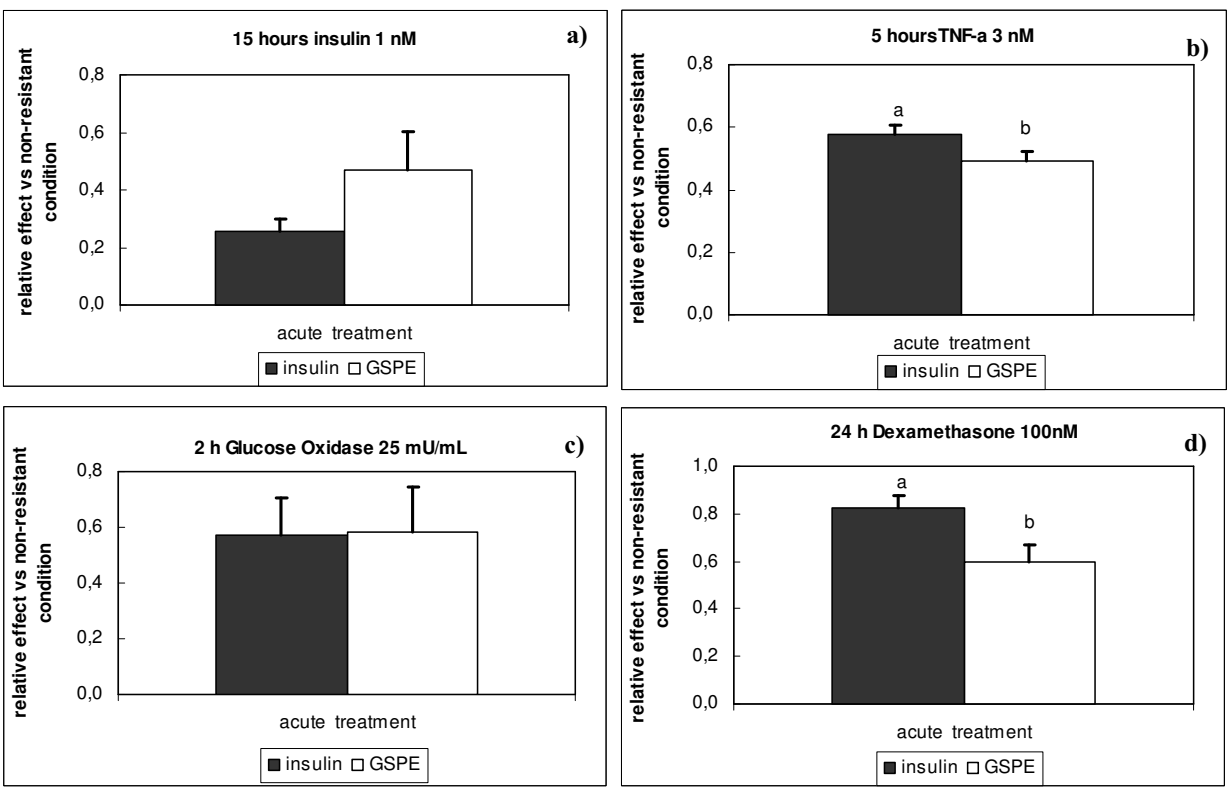

Figure 1. Effects of acute grapeseed procyanidin extract (GSPE) treatment on glucose uptake in insulin resistant 3T3-L1 adipocytes. Fully differentiated adipocytes were incubated with 4 different insulin-resistance inducing agents: a) 1 nM insulin for 15 hours; b) 3 nM TNFα for 5 hours c) 2 hours depletion media + 2 hours 25 mU/mL glucose oxidase on depletion media and d) 100 nM dexamethasone for 24 hours. During the last 30 minutes of incubation, adipocytes were treated with either 100 nM insulin or 100 mg/L GSPE and then 2-deoxy-d-[^{3}H] glucose uptake was measured. Values represented are respectively the insulin or GSPE-induced acute stimulation effects after insulin resistance was induced. Each value represents the mean ± SEM at least of two experiments run in triplicate. Different letters indicate statistically significant differences between treatments ($p<0.05$).

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Following 5 hours of 3 nM TNF-α treatment, an acute dose of insulin only increases glucose uptake to 57.4% of maximal possible stimulation. Similarly GSPE stimulates 49.1% of its maximal acute effect. However, after 24 hours of 100 nM dexamethasone treatment, acute GSPE was less efficient at stimulating glucose uptake, since it only reached 59.7% of its maximal possible effect without resistance, while acute insulin showed 82.5% of its effect without resistance.

Long-term GSPE effects on insulin-induced insulin resistance in 3T3-L1 cells

We next checked whether a long-term treatment with GSPE improved chronic insulininduced resistance in 3T3-L1 adipocytes. **Figure 2** shows that a long-term GSPE treatment starting 2 hours previous to chronic (15h) insulin treatment and maintained until the end of insulin resistance induction tended to slightly improve the acute (30 min) insulin and GSPE stimulation of glucose uptake.

Next, we evaluated the effects of the long-term GSPE treatment on the mRNA levels of some key glucose uptake proteins, insulin signalling, adipokines and differentiation markers. Surprisingly, we found that this long-term (17.5 hours) GSPE treatment caused a strong down-regulation of Glut4, Irs1, adiponectin, resistin, Pparγ2, and Cebpα **(table 1)**, despite the cells maintaining their capacity to acutely respond to insulin and GSPE (shown in figure 2).

GSPE reduces insulinaemia in cafeteria fed rats

We tested *in vivo* the effects of GSPE in hyperinsulinemic animals. We used a mild model of insulino-resistance, provoked by feeding healthy female Wistar rats with a cafeteria diet for 13 weeks. Then we started 4 different GSPE treatments. **Table 2** summarizes the chronic observed effects of these treatments.

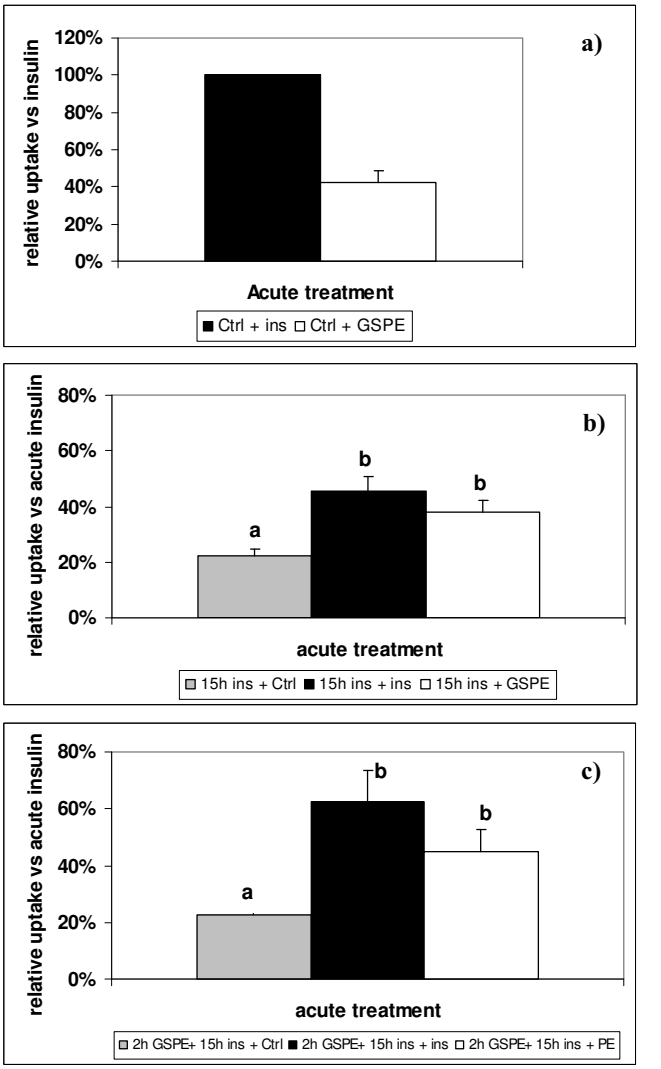

Figure 2. Effects of a long-term treatment of grapeseed procyanidin extract (GSPE) on insulin or GSPE-induced glucose uptake stimulation in resistant 3T3-L1 adipocytes. a: controls without insulin resistance induction; b: fully differentiated 3T3-L1 adipocytes were incubated for 15 hours with 1nM insulin in order to induce insulin resistance; c: prior to the chronic insulin treatment, cells were preincubated for 2h with 100 mg/L GSPE, which was maintained during the insulin chronic treatment. After these treatments, acute stimulation (30 minutes) of GSPE (black columns) or insulin (white columns) was performed and glucose uptake was analyzed. Grey columns indicate basal glucose uptake. Each value represents the mean ± SEM of three experiments run in triplicate. Different letters indicate statistically significant differences between treatments ($p<0.05$).

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Chronic treatment	2 h control + 15 h insulin 1 nM	2 hours GSPE 100 mg/L + 15 hour insulin 1 nM		
Acute treatment	**-**	**-**	**Insulin 100 nM**	**GSPE 100 mg/L**
Glut4	1.17 ± 0.16 **a**	0.54 ± 0.15 **b**	0.44 ± 0.10 **b**	0.57 ± 0.02 **b**
Irs1	1.10 ± 0.18 **a**	0.30 ± 0.02 **b**	0.45 ± 0.08 **b**	0.24 ± 0.02 **b**
Adiponectin	1.02 ± 0.21 **a**	0.36 ± 0.03 **b**	0.31 ± 0.05 **b**	0.31 ± 0.02 **b**
Resistin	1.23 ± 0.44 **a**	0.30 ± 0.10 **b**	0.24 ± 0.12 **b**	0.23 ± 0.10 **b**
Pparγ2	0.52 ± 0.13 **b**	0.02 ± 0.00 **c**	0.02 ± 0.01 **c**	0.02 ± 0.00 **c**
Cebpα	0.36 ± 0.03 **b**	0.01 ± 0.00 **b**	0.01 ± 0.00 **b**	0.01 ± 0.00 **b**

Table 1. Effects of chronic grapeseed procyanidin extract (GSPE) treatment on mRNA levels in insulin-resistant 3T3-L1 adipocytes. Fully differentiated adipocytes were given the chronic treatment indicated in the first row. During the last 30 minutes, each group of adipocytes received the acute treatment indicated at the second row. At the end of treatment, RNA was extracted to quantify gene expression using real time PCR. mRNA gene expression is expressed relative to the control group. Each value represents the mean ± SEM. Different letters indicate the statistically significant differences between treatments ($p<0.05$).

Table 2

10 days	Control	cafeteria	+25	+50
body weight (g)	281.0 ± 9.0 **a**	393.8 ± 27.0 **b**	379.7 ± 21.0 **b**	359.8 ± 17.0 **b**
visceral adipose tissue (g)	20.0 ± 3.0 **a**	57.8 ± 7.1 **b**	54.0 ± 6.5 **b**	51.6 ± 5.7 **b**
Leptin (ng/mL)	8.80 ± 1.47 **a**	35.15 ± 3.18 **b**	28.89 ±3.68 **b**	26.64 ± 4.44 **b**
HOMA IR	0.14 ± 0.02 **a**	6.24 ± 2.18 **b**	3.24 ± 1.40 **a,b**	3.87 ± 1.76 **a,b**
Glucose (mM)	3.32 ± 0.11 **a**	3.45 ± 0.23 **a**	4.79 ± 0.13 **b**	3.66 ± 0.12 **b**
Insulin(ng/mL)	0.04 ± 0.02 **a**	1.42 ± 0.44 **b**	0.61 ± 0.23 **a**	0.95 ± 0.44 **b**

Table 2a. Effects of 10 days of grapeseed procyanidin extract (GSPE)-treatment on weight and plasma parameters

30 days	Control	cafeteria	+25	+50
body weight (g)	274.5 ± 10.0 **a**	430.2 ±18.0 **b**	408.5 ± 9.5 **b**	426.8 ± 27.0 **b**
visceral adipose tissue (g)	16.29 ± 0.5 **a**	67.88 ± 5.3 **b,c**	58.74 ± 3.8 **c**	65.17 ± 6.5 **b**
Leptin (ng/mL)	7.97 ± 0.17 **a**	38.79 ± 2.63 **b**	32.60 ± 2.80 **b**	39.50 ± 2.15 **b**
HOMA IR	0.26 ± 0.6 **a**	9.06 ± 3.8 **b**	4.16 ± 3.9 **a,b**	5.35 ± 2.0 **b**
Glucose (mM)	3.58 ± 0.12 **a**	3.76 ± 0.10 **a**	3.81 ± 0.31 **a**	3.47 ± 0.13 **a**
Insulin(ng/mL)	0.06 ± 0.15 **a**	2.13 ± 0.84 **b**	0.77 ± 0.71 **a,b,c**	1.28 ± 0.51 **c,b**

Table 2b. Effects of 30 days of GSPE-treatment on weight and plasma parameters. Animals were fed a cafeteria diet for 13 weeks, and then two GSPE doses (25 and 50 mg of GSPE/kg bw*day, labelled +25 and +50 respectively) were administered simultaneously to the cafeteria diet for 10 days (a) and 30 days (b). Body weight, WAT weight and leptin levels were obtained on the sacrifice day. Glucose and insulin were measured in fasting condition 3 days before the sacrifice. Each value represents the mean ± SEM. Different letters indicate statistically significant differences between treatments ($p<0.05$).

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GSPE did not modify the increase in body weight induced by the cafeteria diet, and it did not change plasma leptin levels either. Only 25 mg of GSPE/kg bw*day for 30 days significantly reduced the total amount of visceral adipose tissue. A main feature of GSPE treatment was to reduce the fasting plasma insulin levels, the 25 mg of GSPE/kg bw*day dose being the most effective. GSPE at this dose reduced insulinaemia both after 10 and 30 days However, only the animals receiving the longer dose maintained their fasting glycaemia and showed a healthier HOMA IR index. In the 30 day- treated animals, an IPGTT of two hours showed lower insulinaemia with the same profile, although there was no improvement in glycaemia (**figure 3**). We also analyzed the insulin and glucose levels of these animals on the day of sacrifice. Because we sacrificed them 5h after the last GSPE treatment, they reflect the acute GSPE effects after 30 days of treatment. **Table 3** shows that insulinemia was still lower than in cafeteria group, but in this case glycaemia was also reduced by GSPE compared to cafeteria effects.

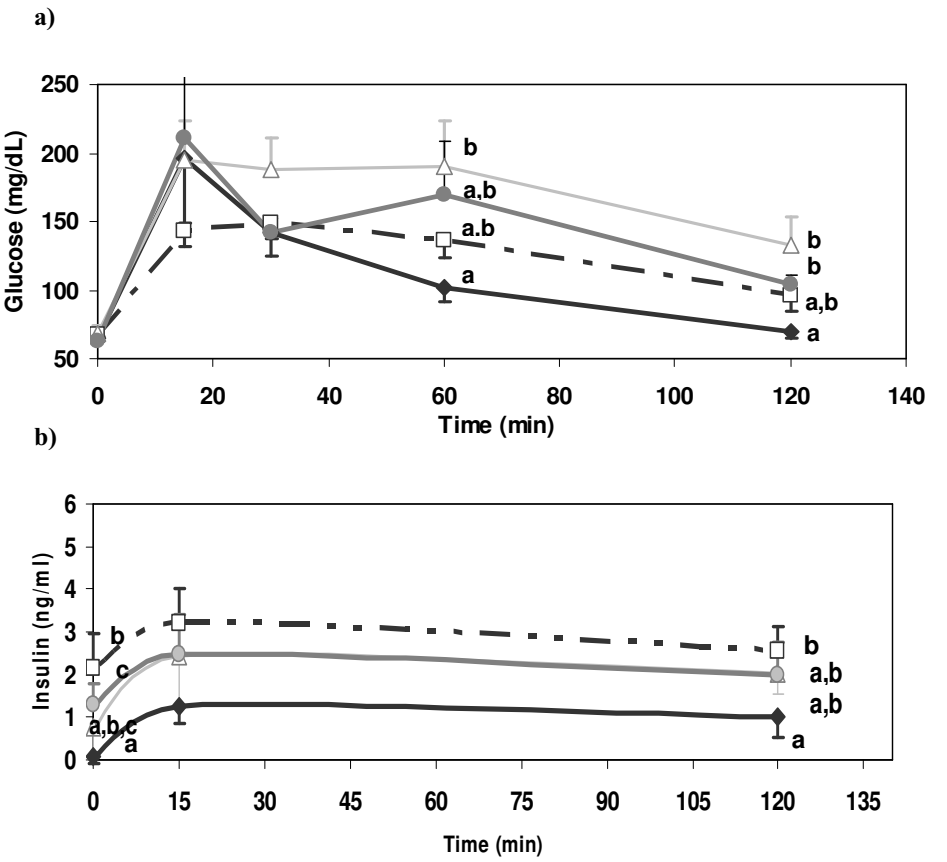

Figure 3. Plasma glucose (a) and insulin (b) profiles after an intraperitoneal glucose tolerance test (IPGTT) on 30 day grapeseed procyanidin extract (GSPE)-treated animals. Rats were fasted overnight, a blood sample was collected by tail bleeding (time zero), and glucose was administered intraperitoneally. Blood samples were taken at the time points indicated in the figure. Glucose levels were quantified using a glucometer and insulin using the ELISA method. Data are the mean ± SEM of six animals. a) Glucose profile. b) Insulin profile. Different letters indicate statistically significant differences between treatments ($p<0.05$)
(♦)Diamond: control group; (□) square: cafeteria group;(△) triangle: cafeteria + 25 mg GSPE/kg bw*day; (●) circle: cafeteria + 50 mg GSPE/kg bw*day

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White adipose tissue is very sensitive to GSPE treatments

Finally we analyzed the modulation of gene expression in the different animal groups on the day of sacrifice. **Figure 4a** shows that in white adipose tissue (WAT), the cafeteria diet downregulated mainly genes involved in initial insulin signaling and insulin-depending glucose uptake (according to the peripheral insulin resistance suggested by plasma parameters). In contrast, Pparγ2 remained unaffected. The liver still seemed to be insulin responsive, since the ratio glucose-6-pase vs glucokinase was lower than that of the controls (Glc6pase: 0.52 ± 0.09; Gck 0.97 ± 0.25, relative expression vs control). Twenty five mg of GSPE/kg bw*day did not have any effect on liver (G6p: 0.63 ± 0.09; Gck: 0.95 ± 0.14 at 30 days) but was very effective on mesenteric WAT. In the latter, the GSPE did not modify any of the first proteins involved in insulin signaling (Irs1) compared to the cafeteria diet. However, we found that GSPE modulated representative markers of mature adipocyte (Glut4, Srebp1c, Pparγ2): after 10 days GSPE seemed to partially revert cafeteria-induced downregulation of such genes, while a longer treatment (30 days) induced a stronger downregulation of all these genes.

The higher dose, 50 mg of GSPE/kg bw*day, showed a similar profile with an even stronger down-regulation of genes after 30 days of treatment. In the liver, this dose was not effective after 10 days of treatment, nor did it favor glucose uptake (Gck: 1.54 ± 0.17; G6p: 0.52 ± 0.02); although it suggested a stimulation of glucose production after 30 days (Gck: 1.67 ± 0.40; G6p: 1.56 ± 0.16).

Discussion

Flavonoids and, more specifically, procyanidins are antihyperglycemic in some experimental conditions, thus their potential value as antidiabetics makes them worth studying further. However, there is no consensus on the chronic effects of procyanidins on the maintenance of whole-body glucose homeostasis, in part due to the relatively scarce work done in this area.

Our *in vitro* results showed different responses to GSPE depending on the assayed model of insulin resistance. Under a chronic insulin insult, GSPE showed a slightly improved efficacy at stimulating glucose uptake compared to insulin. Hoehn et al. [27] used the same insults in 3T3-L1 cells and described their main targets.

Treatment	Control	Cafeteria	+ 25	+ 50
Glucose (mM)	6.93 ± 0.15 **a**	8.46 ± 0.32 **b**	7.74 ± 0.22 **a,b**	8.09 ± 0.37 **a,b**
Insulin (ng/mL)	1.04 ± 0.21 **a**	5.42 ± 0.8 **b**	4.12 ± 0.95 **a,b**	3.63 ± 0.79 **a,b**

Table 3. Acute grape-seed procyanidin extract (GSPE) effects on metabolite and hormone plasma levels after 30 days of chronic GSPE treatment. Animals were fed a cafeteria diet for 13 weeks, and were then administered two GSPE doses (25 and 50 mg of GSPE/kg bw*day, labelled +25 and +50 respectively) simultaneously with the cafeteria diet for 30 days. 5h after the last GSPE treatment, the animals were sacrificed and glucose and insulin plasma levels were measured. Each value represents the mean ± SEM. Different letters indicate statistically significant differences between treatments ($p<0.05$)

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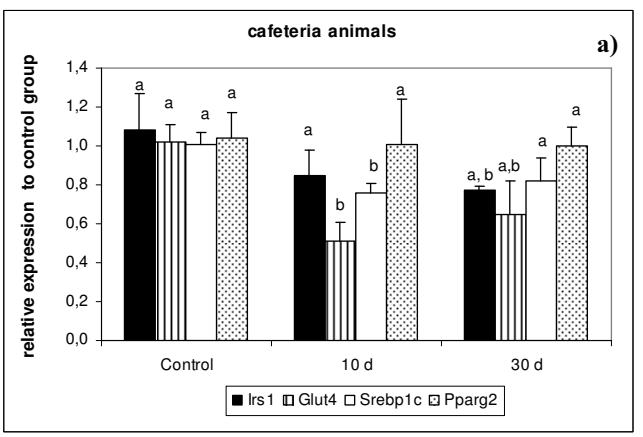
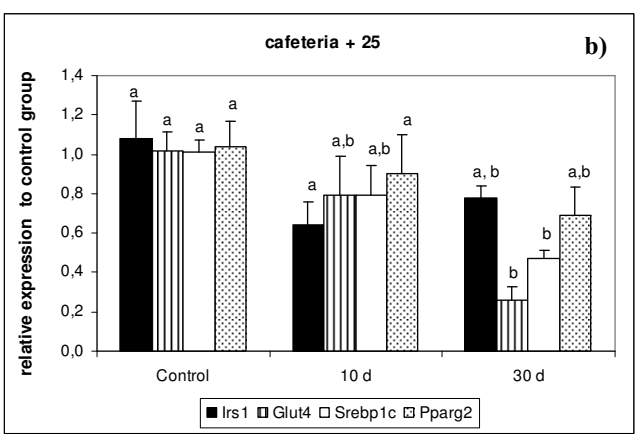
According to their study, chronic insulin was the only insult involving Irs1. The fact that GSPE has a stronger acute effect on this condition suggests that GSPE depends only partially on Irs1 to stimulate glucose uptake. This is in agreement with our previous results showing that GSPE activates insulin receptors [37], however, this is not the only mechanism it has for stimulating glucose uptake [28]. In contrast we found GSPE to be less effective at acutely stimulating glucose uptake under TNFα and especially under dexamethasone treatments. TNFα and dexamethasone induce insulin resistance at Glut4 level [27].

Moreover, dexamethasone induces insulin-resistance through up-regulation of the MAPK phosphatases 1- and 4-, resulting in p38 MAPK dephosphorylation, which limited Glut4 activity [29]. We have shown that GSPE depends on p38 to activate glucose uptake [28]. This evidence suggests GSPE affects the Glut4 transporter itself, which could be by direct interaction or by modulating its activity via p38. In fact, some other flavonoids act directly on glucose transporters, but until present all the studies reported an inhibitory effect [30, 31].





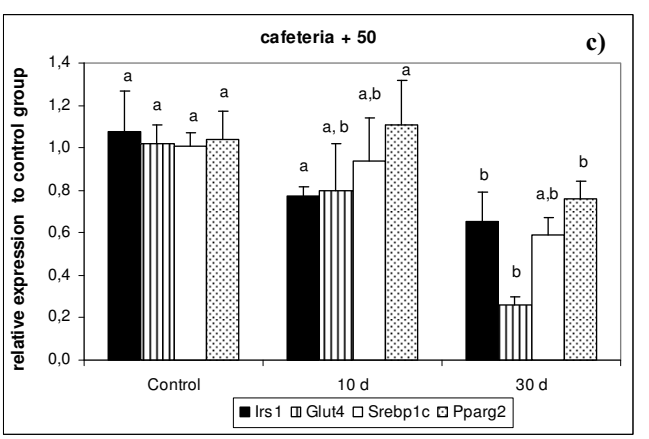


Figure 4. Gene expression in adipose tissue from animals treated with grapeseed procyanidin extract (GSPE). After 10 or 30 days of GSPE treatment (a: effects of the cafeteria diet, b: 25 mg GSPE/kg bw*day; c: 50 mg GSPE/kg bw*day) the animals were sacrificed and adipose tissue samples obtained. mRNA expression of indicated genes was measured using real time PCR. Data are the mean ± SEM of six animals. Different letters indicate statistically significant differences between treatments ($p<0.05$).

We assayed the chronic effects of GSPE *in vivo* on a disturbed metabolic situation that resembled the most prevalent situation in Western societies and where the main insulinresistance factor was hyperinsulinemia. A slight improvement in the plasma insulin resistance parameters was the main result of these chronic GSPE treatments, after longterm (30 days) treatment. This is the first study to evaluate the effects of GSPE on cafeteria diet-induced insulin resistance. Al-Awwadi et al. [17] used a fructose insulinresistant model to assay the effects of the same dose of grape-derived procyanidin extracts that we used (25 mg/kg bw* day) but for a longer period (6 weeks). They found a slight effect on plasma glucose. Persimmon peel procyanidins at a dose of 10 mg/kg of bw* day for 6 weeks on db/db mice only improved glycosylated protein [18]. All these results suggest that procyanidins have a moderate effect on improving plasma insulin resistance, but only after long-term treatment.

These previous studies showed that procyanidins improved oxidative status and had an anti-inflammatory effect [17, 18]. We focused on key points of insulin resistance. Our data single out mesenteric white adipose tissue as an important target for GSPE, although GSPE did not clearly improve insulin resistance in this tissue. GSPE downregulated Glut4. GSPE treatment did not improve glucose uptake after the IPGTT. Interestingly, however, the insulin required to maintain glucose levels was lower than in the cafeteria group. This suggests that GSPE could act as insulinomimetic. GSPE would probably act more slowly than insulin because, although we did not find an improvement in glucaemia 2 hours after the intraperitoneal dose, we did find an improved HOMA IR in the fasting situation. Thus we suggest that GSPE acts on adipose cells despite its lower Glut4 mRNA levels. Glut4 has classically been regarded as marker for insulin resistance [32]. However, we found *in vitro* that a long term GSPE treatment down-regulated Glut4 mRNA, but did not modify the glucose uptake response to acute GSPE or insulin treatment. This and previous studies show that adipocytes can acutely respond to glucose uptake signaling regardless of reduced Glut4 mRNA levels [33, 34]. They also suggest that GSPE does not activate glucose uptake through its effects at transcriptional level, but rather through a different mechanism. As deduced from the *in vitro* studies mentioned above this could involve modulating Glut4 selfactivation. Furthermore, we did not find significant gene expression modulation either in muscle (results not shown) or liver that could help to explain the effects of GSPE, and this reinforces the specificity of white adipose tissue as target of GSPE. The lack of glucose homeostasis by GSPE in liver differs from other closely related studies. Several flavonoids induce expression of hepatic Gck mRNA or a decrease in mRNA of G6p [7, 8, 9, 19]. Previously, we also found increased Gck and decreased G6p mRNA levels after acute GSPE treatment of STZ-animals [35]. The different molecules assayed or different metabolic situations might explain the divergences between these studies and our present results. In the present study we found that GSPE did not affect glucose entrance/output in the liver at the 3 lower doses, but at the highest dose (50 mg/Kg bw* day for 30 days) it did activate G6p, suggesting a negative effect on glucose homeostasis, and highlighting the importance of the dose assayed.

We also found a strong down regulation on markers of mature white adipose tissue, which was not fully matched by a reduction in adipose tissue. We previously found down-regulation of *in vitro* adipose markers that suggested the adipocytes tended to lose their terminally differentiated state after chronic GSPE treatment [22]. In this study we show that this also takes place *in vivo* in an adipogenic milieu (cafeteria diet). Similarly, Cyanidin 3-o-b-D-glucoside reduces WAT and body weight in mice when administered simultaneously to a 30% high-fat diet at a high dose (200 mg/kg bw) for 12 weeks [6]. Mice

fed a high-fat diet (60% energy as fat) and submitted to EGCG treatment (3.2 g/kg diet) also take 16 weeks to reduce the body weight gained, and the visceral fat weight [36]. In the present study GSPE treatment probably needed longer to change the size of adipose depots and thereby influence insulin resistance.

In conclusion, *in vitro* GSPE differed from insulin in how it stimulated glucose uptake in insulin resistant adipocytes. This suggests that GSPE is partly dependent on Irs1 when stimulating glucose uptake but also suggests that GSPE has a direct effect on Glut4 transporter activity. These mechanisms could be responsible for slightly improving plasma insulin resistance parameters (mainly by reducing insulinaemia). This is achieved by a 25 mg /kg*bw*day GSPE dose administered for 30 days on a cafeteria diet-induced impaired glucose tolerance model. This dose did not modify key points of hepatic glucose metabolism (Gck or G6p) but provoked a strong down-regulation on mature adipocyte markers that might be independent of the direct effects of GSPE on Glut4.

Acknowledgments

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PROCEEDINGS

Differential effects of grape-seed derived procyanidins on adipocyte differentiation markers in different in vivo situations

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Published online: 19 September 2007

Keywords Procyanidin · Catechin · Adipocyte · Differentiation · Adipose markers · Adipose tissue

Introduction

Obesity is one of the main problems in industrialized societies and adipose tissue is a key target tissue of this disease . There is therefore much interest in studying the adipose metabolism and the mechanisms of adipocyte differentiation. Procyanidins (oligomeric forms of catechins) are the most abundant polyphenols in red wine, apple and chocolate. Previous studies by our group showed that grape seed derived procyanidins (GSPE) limit adipocyte formation by altering the gene expression profile during in vitro adipocyte differentiation [1]. GSPE also decreased the mRNA levels of PPARγ2 and HSL on fully differentiated 3T3-L1 adipocytes [2]. In this paper we analyze the effects of GSPE on adipocyte differentiation markers under different in vivo physiological conditions.

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Materials and methods

Chemical

Grape seed procyanidin extract was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract essentially contained monomeric (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%), oligomeric procyanidins (5–13 units; 35.7%), and phenolic acids (4.22%).

Animal experimental procedures

For the acute treatment, we worked with 2-month-old male Wistar rats from Charles River Laboratories (Barcelona, Spain). Some of the animals were treated with streptozotocin to obtain animals with Type 1 diabetes mellitus [3]. On the day of the experiment, the rats were fed an oral gavage of GSPE in aqueous solution (250 mg/kg body wt). Five hours after treatment, the rats were killed by beheading and epididymal tissue was frozen immediately. For the chronic treatment, 2-month-old male fa\fa Zucker rats from Charles River (Barcelona, Spain) were chronically administered 19.5 mg/g day of GSPE. The treatment was oral gavage and the GSPE was dissolved in sunflower oil. After 30 days of treatment, the rats were killed by beheading and mesenteric and epididymal adipose tissues were excised and frozen immediately. All procedures were approved by the Animal Ethics Committee of the Rovira i Virgili University.

Quantitative RT-PCR

Changes in the mRNA expression of selected adipocyte markers (PPARγ2, HSL, C/EBPα and Pref-1) [1] were analysed by quantitative PCR. Briefly, 1 mg of total RNA was reverse transcribed by the SuperScript II Rnase H_Reverse Transcriptase (LifeTechnologies). RT product corresponding to 20 ng initial RNA was amplified according to the protocols of Applied Biosystems. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an endogenous control to normalize data. The following primers were used:

GAPDH	CAT GGC CTT CCG TGT TCC T (forward)
	CCTGCT TCA CCA CCT TCT TGA (reverse)
PPARγ2	CTG TTGACC CAG AGC ATG GT (forward)
	AGA GGT CCA CAG AGC TGA TTC C (reverse)
HSL	GGA GCA CTA CAA ACG CAA CGA (forward)
	AAT CGG CCA CCG GTA AAG AG (reverse)
Pref-1	TGC GCC AAC AAT GGA ACT T (forward)
	TGG CAG TCC TTT CCA GAG AAC (reverse)
C/EBPα	GGT GGA CAA GAA CAG CAA CGA (forward)
	CGT TGC GTT GTT TGG CTT TAT C (reverse)

Statistical analysis

Results are expressed as mean ± SEM. Effects were assessed using *t* test. All calculations were performed using SPSS software.

Results and discussion

In healthy Wistar rats, an acute high oral dose of GSPE decreased epididymal mRNA levels of early adipocyte markers such as PPARγ2, C/EBPα and Pref-1 but did not affect late adipocyte markers such as HSL. However, the same acute GSPE treatment induced almost opposite effects on streptozotocin-induced diabetic animals (Fig. 1). In this pathological model we also studied an animal group treated with an effective dose of insulin to evaluate the insulin-like effect of procyanidins. As we showed previously [4] when working with another functions targets of procyanidins, the GSPE did not totally mimic the insulin effect. The effects obtained in the healthy animals under acute treatment were similar to those obtained in the fa/fa Zucker rats after a chronic oral treatment with GSPE i.e. decreased mRNA levels of PPARγ2, C/EBPα and Pref-1, and minimal effect on HSL gene expression (Fig. 2A).

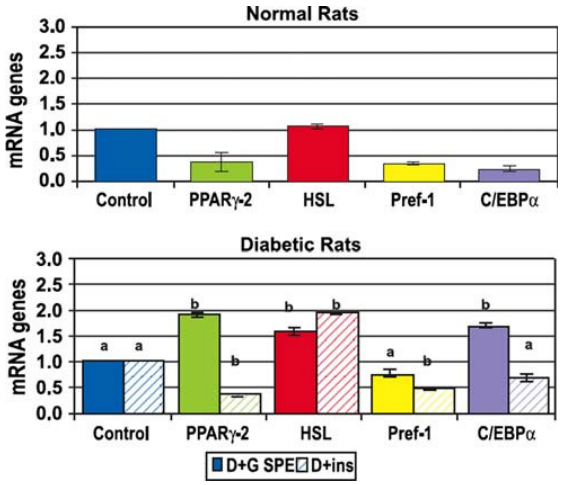

Fig. 1 GSPE effects on mRNA levels of adipose differentiation markers

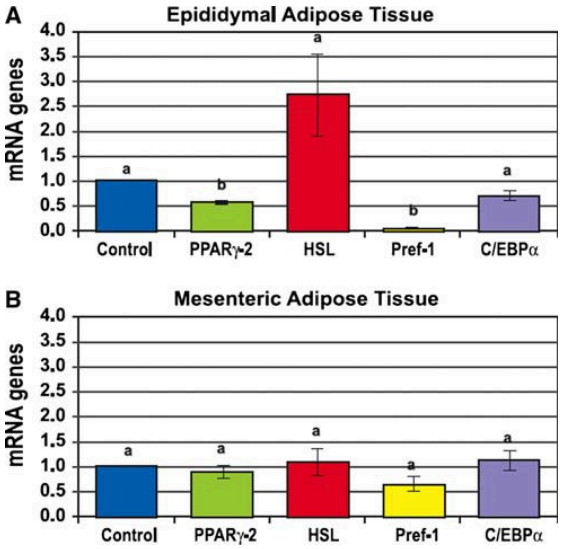

Fig. 2 GSPE effects on mRNA levels of adipocyte markers in in different adipose tissues

However, these effects were only identified in epididymal adipose tissue (Fig. 2B). The same analysis on mesenteric adipose tissue showed a decrease only in Pref-1 expression with no changes in the other adipocyte markers. In conclusion, these results show that GSPE modifies adipose differentiation markers differently depending on the physiological conditions and adipose tissue depot.

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Oligomers of grape-seed procyanidin extract activate the insulin receptor and key targets of the insulin signaling pathway differently from insulin☆

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Abstract

Procyanidins are bioactive flavonoid compounds from fruits and vegetables that possess insulinomimetic properties, decreasing hyperglycaemia in streptozotocin-diabetic rats and stimulating glucose uptake in insulin-sensitive cell lines. Here we show that the oligomeric structures of a grape-seed procyanidin extract (GSPE) interact and induce the autophosphorylation of the insulin receptor in order to stimulate the uptake of glucose. However, their activation differs from insulin activation and results in differences in the downstream signaling. Oligomers of GSPE phosphorylate protein kinase B at Thr308 lower than insulin does, according to the lower insulin receptor activation by procyanidins. On the other hand, they phosphorylate Akt at Ser473 to the same extent as insulin. Moreover, we found that procyanidins phosphorylate p44/p42 and p38 MAPKs much more than insulin does. These results provide further insight into the molecular signaling mechanisms used by procyanidins, pointing to Akt and MAPK proteins as key points for GSPE-activated signaling pathways. Moreover, the differences between GSPE and insulin might help us to understand the wide range of biological effects that procyanidins have.

Keywords: Flavonoids; Glucose uptake; Insulin receptor; Insulin signaling pathway; Procyanidins

1. Introduction

Natural compounds with insulin-like activity (insulin mimetics) have been proposed as potential therapeutic agents in the prevention and/or treatment of metabolic syndrome and diabetes. They would act by promoting glucose transport and glucose metabolism [1]. Given the increasing prevalence of these pathologies, it is important to determine and to understand the possible protective role of the bioactive compounds that are found in our diets or that could be added to our diets.

Flavonoids are bioactive polyphenols from fruits and vegetables that have been described as mimicking the metabolic and mitogenic actions of insulin. Insulin signal transduction is initiated when insulin binds to the insulin receptor which activates its intracellular tyrosine kinase domain. Activation of the insulin receptor leads to phosphorylation of several intracellular protein substrates including insulin receptor substrate 1 (IRS1), IRS2 and Shc. This initiates the two major signaling cascades: (1) the phosphatidylinositol 3-kinase (PI3K) pathway that includes activation of Akt leading to activation of glycogen synthase and the other enzymes/proteins necessary for the acute metabolic effects of insulin and (2) the mitogen-activated protein kinase (MAPK) pathway that includes activation of ERK1 and ERK2 leading to gene transcription, protein translation and cell growth [2–4]. Although PI3K is central to mediating insulin metabolic actions, other kinases such as p42/44 MAPK have also been implicated in insulin-dependent glucose uptake [5].

Flavonoids include a great diversity of structures, and there is accumulating evidence that such different structures might act on different targets and modify the activity of different components of the insulin signaling cascades, such as protein kinase C, PI3K and MAPK pathways [6]. In this context, myricetin acts on IRS-1-associated PI3K and glucose transporter type 4 (Glut4) activity [7]. Glycoside kaempferol 3-neohesperidoside stimulates glucose uptake

Abbreviations: Akt, protein kinase B; CHO-IR cells, Chinese hamster ovaric cells overexpressing the human insulin receptor; EGCG, epigallocatechin-gallate; Glut4, glucose transporter type 4; GSPE, grape-seed procyanidin extract; IR, insulin receptor; IRS, Insulin receptor substrate; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase.

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via the PI3K and protein kinase C pathways and independently of the MEK pathways [8]. Epigallocatechin-gallate (EGCG) has been reported to mimic the metabolic and vasodilator actions of insulin via the PI3K/Akt-dependent pathway [9–12]. However, recent studies have shown that EGCG blocks insulin-dependent Glut4 translocation to the plasma membrane in MC3T3-G2/PA6 adipose cells. It inhibits, as well, hepatic glucose production through an insulin-independent signaling pathway [13,14]. Oligomeric flavan-3-ols have also been reported as the main cause of the beneficial effects of some plant extracts [15,16]. Tannins present in *Cichorium intybus* inhibit protein tyrosine phosphatase, nonreceptor type 1, probably in a PI3K-dependent manner [17]. Moreover, gallotannins such as penta-*O*-galloyl-glucopyranose from banana extract appear to be more potent and efficacious than ellagitannins in IR binding, IR activation and glucose transport induction [18]. The isoflavonoid aglycone-rich fraction of *Chungkookjang* enhances PPAR-γ activity and stimulates the translocation of the glucose transporter Glut4 into the plasma membrane through IRS1 and Akt phosphorylation in 3T3-L1 adipoctyes [19]. Purified cinnamon polyphenols (with a doubly linked procyanidin type A polymer structure) have been identified as insulin mimetic since they increase the expression of the insulin receptor, tristetraprolin and glucose transporter 4 in mouse 3T3-L1 adipocytes [20]. Flavonoids from *Cephalotaxus sinensis* leaves are also the most active compounds at inducing Glut4 translocation in membrane preparations from mice adipocytes [21]. Finally, we previously showed that grape-seed procyanidin extract (GSPE) requires PI3K and p38 MAPK to stimulate glucose uptake in L6E9 myotubes and in 3T3-L1 adipocytes. Furthermore, we showed that GSPE, like insulin, induces Glut4 translocation to the plasma membrane [22]. Here we want to further describe the mechanisms used by GSPE to exert its effects. In the present study, we have investigated whether GSPE is able to interact and/or induce both the autophosphorylation of the insulin receptor and the activation of several protein kinases involved in the insulin signaling pathway. We also checked whether a purified GSPE fraction (fraction VIII), the richest in trimeric structures, was the main cause of these effects.

2. Materials and methods

2.1. Materials and reagents

According to the manufacturer, GSPE (Les Dérives Résiniques et Terpéniques, Dax, France) contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 U; 31.7%) procyanidins. Fraction VIII was obtained by a chromatographic separation of GSPE according to size, where 11 major fractions with an increasing degree of polymerization were identified. These fractions were vacuum dried and kept at −20°C for subsequent use in the biological studies.

We obtained 3T3-L1 cell culture reagents from BioWhittaker (Verviers, Belgium) and Ham's F12, streptomycin/penicillin, fungizone and fetal bovine serum for CHO-IR cells from Gibco (Paisley, UK). Insulin (Actrapid) was purchased from NovoNordisk (Bagsvaerd, Denmark), which also kindly gave us peptid insulin antagonist S459.

Insulin receptor inhibitor HNMPA-$(AM)_3$ (hydroxy-2-naphthalenylmethylphosphonic acid trisacetoxymethyl ester) and Akt inhibitor VIII, an isozyme selective for Akti-1/2, were purchased from Calbiochem (Darmstadt, Germany). 2-Deoxy-D-[^{3}H] glucose was purchased from Amersham Biosciences (Little Chalfont, UK). Bradford protein reagent was obtained from Bio-Rad Laboratories (Hercules, CA, USA). ECL kit was obtained from Amersham Pharmacia Biotech (AT Roosendaal, The Netherlands). Cell Signaling (Leiden, The Netherlands) provided the antibodies anti-IR, anti-phospho-IR (4G10), anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-phospho p44/p42 MAPK and anti-phospho p38 MAPK.

2.2. Cell culture

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described [23]. Briefly, confluent preadipocytes were treated with 0.25 μM dexamethasone, 0.5 mM 3-isobutylmethylxanthine and 5 μg/ml insulin for 2 days in 10%FBS containing DMEM. Cells were switched to 10% FBS/DMEM containing only insulin for 2 days and then to 10% FBS/DMEM without insulin. Ten days after differentiation had been induced, the cells were used for the experiments.

CHO-IR cells (Chinese hamster ovaric cells overexpressing the human insulin receptor) were grown in Ham's F12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 μg/ml fungizone and 0.5% G-418 in 5% CO_2/humidified atmosphere at 37°C. Cells were passaged two to three times a week. Confluent cells were used for the experiments.

2.3. Glucose uptake assay

Glucose transport was determined by measuring the 2-deoxy-D-[^{3}H]glucose uptake of 3T3-L1 adipocytes cultured on 12-well plates as previously described [22]. Briefly, cells were incubated for 7 min in a transport solution containing 0.1 mM 2-deoxy-D-glucose and 1 μCi 2-deoxy-D-[^{3}H]glucose (10 mCi/mmol). Afterwards, glucose uptake was stopped by adding 50 mM glucose and cells were disrupted by adding 0.1 M NaOH/0.1% PBS. Radioactivity incorporated in the cells was determined by a scintillation counter. Protein content was used to normalize the glucose transport values [24]. Each condition was run in triplicate.

2.4. Immunoblotting

CHO-IR cells were deprived of serum for 18 h prior to stimulation with insulin (100 nM) or GSPE (100 mg/L). Cells were then harvested in cell lysis buffer (80 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Brij, 20 mM EDTA, 200 mM NaF, 4 mM sodium vanadate, 10 nM okadaic acid and a complete protease inhibitor cocktail). Proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes as previously described [25]. Individual proteins were detected with the specified antibodies and revealed by horseradish peroxidase-linked secondary antibodies and developed using the ECL kit. The expression level of actin was used to check that equal amounts of protein were loaded. The band's intensity was quantified using the ScionImage software.

2.5. Data analysis

Results are expressed as the mean±S.E.M. Effects were assessed using one-way ANOVA or Student's *T* test. All calculations were performed using SPSS software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. GSPE activates the insulin receptor

We have previously shown that GSPE is an insulinomimetic agent since it requires PI3K and p38 MAPK as mediators to stimulate glucose uptake in 3T3-L1 adipocytes and L6E9 muscle cells [22]. But since we had no evidence of how GSPE could modify these insulin intracellular targets, our first objective was to investigate whether GSPE interacted with the insulin receptor.

First, we incubated 3T3-L1 adipocytes with two different doses of a synthetic peptide, which binds specifically at two discrete hotspots on the insulin receptor showing an antagonist activity [26]. Then we analyzed 2-deoxyglucose uptake after insulin or GSPE stimulation. The GSPE dose (100 mg/L) was chosen because it was previously described to lead to maximal glucose uptake stimulation [22]. Fig. 1A shows that GSPE, like insulin, competes with the specific peptide to interact with the insulin receptor. However, there are some differences between the effects of GSPE and insulin. Pretreatment with the lower dose of peptide inhibited insulin-induced glucose uptake more strongly than GSPE-induced glucose uptake. However, the high dose of peptide inhibited both insulin and GSPE-stimulated glucose uptake to the same extent (approx. 80%).

Once we had demonstrated that GSPE interacted with the insulin receptor, we then evaluated whether GSPE activated insulin receptor intracellular tyrosine kinases. To find out whether GSPE induces the insulin receptor autophosphorylation, we incubated 3T3-L1 adipocytes with the tyrosine kinase inhibitor HNMPA-$(AM)_3$ [27]. Fig. 1B shows that GSPE requires phosphorylation of the insulin receptor to stimulate the uptake of glucose, similarly to insulin. We also used Western blot to demonstrate the GSPE-induced phosphorylation of the insulin receptor. To do so, we used CHO-IR, a cell line that overexpresses the human insulin receptor. Fig. 1C shows that GSPE induced insulin receptor tyrosine phosphorylation, although this phosphorylation was less intense and emerged later than insulin-induced phosphorylation.

3.2. Akt and MAPK are mediators of GSPE effects

Previous results from our group using specific inhibitors showed that PI3K and p38 MAPK are involved in the mechanisms that GSPE uses to stimulate glucose uptake in insulin-sensitive cell lines [22]. We then went further downstream and focused our studies on Akt protein, since Akt activation is necessary for insulin to stimulate glucose uptake. We incubated 3T3-L1 adipocytes with Akt inhibitor VIII, an isozyme selective for Akti-1/2 isoforms. As shown in Fig. 2A, GSPE-stimulated glucose uptake was inhibited by this compound. We also evaluated how GSPE phosphorylated Ser473 Akt in CHO-IR cells. Fig. 2B shows that 100 mg/L of GSPE induced Akt serine phosphorylation to a similar extent as insulin, after 2 min of treatment.

We also studied the MAPK pathway to get a clearer evidence of MAPK involvement in the effects of GSPE. Fig. 2C shows that GSPE activated p44/42 and p38 MAPKs. In this case, we found that GSPE phosphorylated both kinases more strongly than insulin did.

3.3. A fraction enriched with trimeric structures (fraction VIII) reproduces the effects described for the total extract

GSPE contains mainly procyanidins but it also contains to a lesser degree several other structures such as monomeric flavonoids and

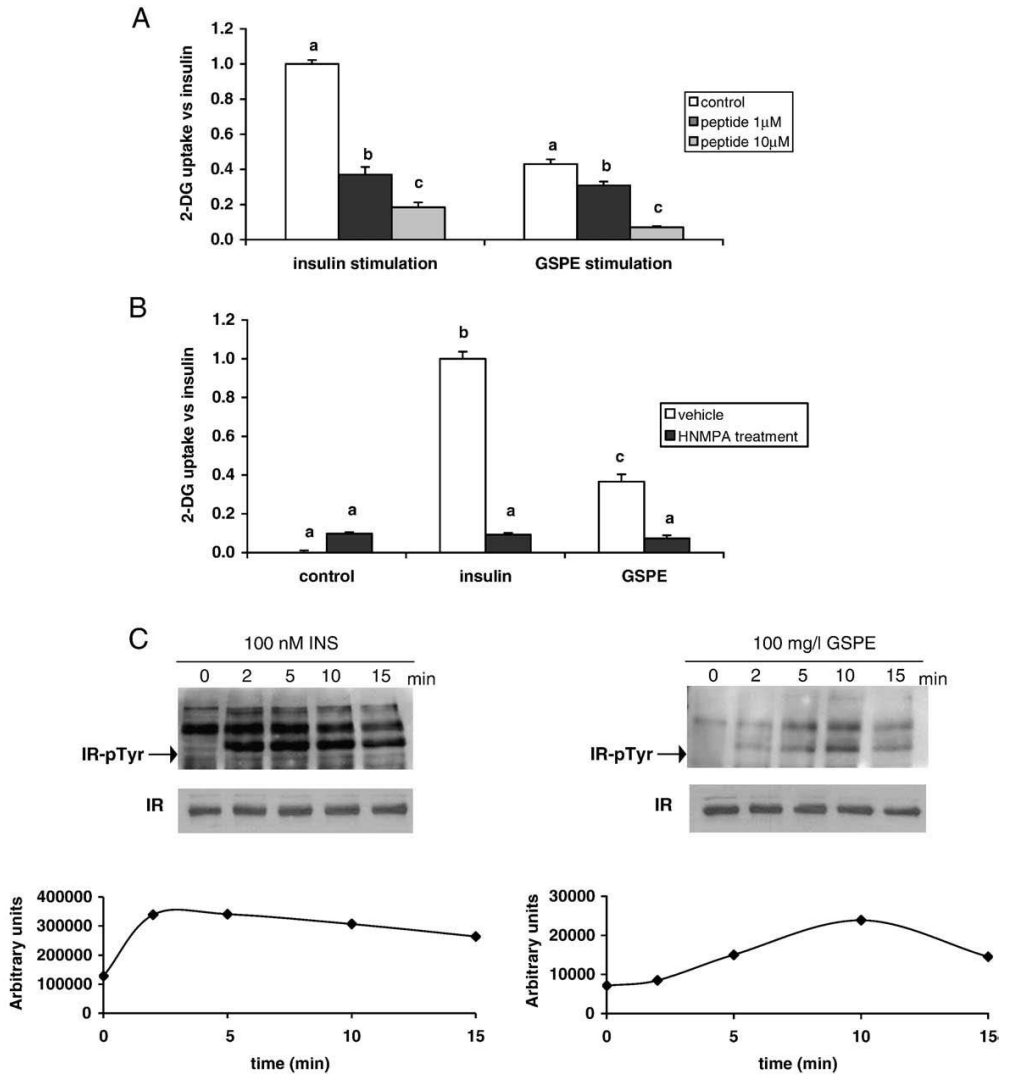

Fig. 1. Effects of GSPE on insulin receptor activation. Fully differentiated 3T3-L1 adipocytes were depleted with serum-free medium supplemented with Dulbecco's Modified Eagle's Medium containing 0.2% BSA for 2 h. Cells were preincubated for 1 h with (A) an insulin antagonist peptide at 1 and 10 μM, respectively. (B) The specific insulin receptor tyrosine kinase inhibitor HNMPA-$(AM)_3$ at 200 μM. 2-Deoxy-D-[^{3}H]glucose incorporated into the cells was determined after stimulating glucose uptake with 100 nM insulin or 100 mg/L GSPE for 30 min. Protein content was used to normalize glucose uptake values. Data shown are means±S.E.M. of two experiments each performed in triplicate. a, b and c indicate significantly different groups with $P<.05$ after insulin or GSPE stimulation, respectively. (C) Phosphorylation of the insulin receptor was immunodetected after stimulating CHO-IR cells with 100 nM insulin or 100 mg/L GSPE for 0, 2, 5, 10 and 15 min. Then, cells were lysed in lysis buffer supplemented with protease and phosphatase inhibitors. Proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes and detected with a specific phospho-tyrosine antibody as previously described in Materials and Methods. Insulin was used as a positive control in all the experiments. Immunoblots and quantification of the bands by ScionImage program are representative of at least two independent experiments.

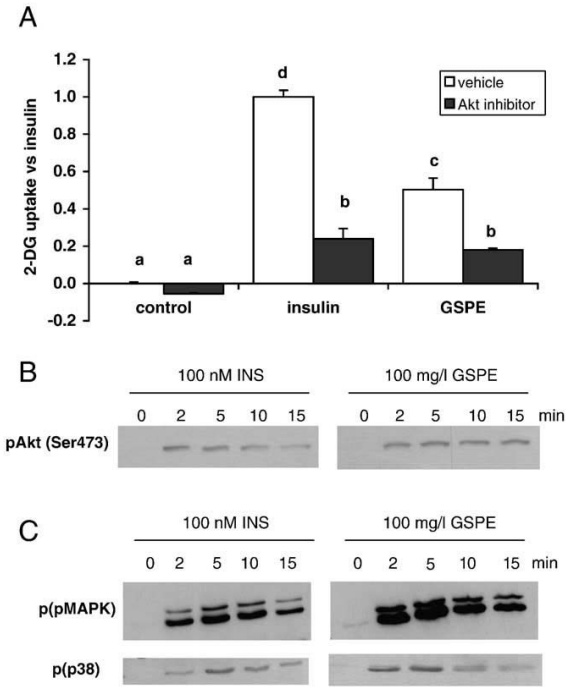

Fig. 2. Effects of GSPE on the downstream signaling insulin pathway. (A) Depleted 3T3-L1 adipocytes were incubated with 15 μM of Akt inhibitor VIII for 1 h. After treatment, cells were stimulated with 100 nM insulin or 100 mg/L GSPE for 30 min and 2-deoxyglucose uptake was assayed. Protein content was used to normalize the glucose uptake values. Data shown are means±S.E.M. of two experiments each performed in triplicate. a, b, c and d indicate significantly different groups with $P<.05$. Phosphorylation of Akt at Ser473 (B) and phosphorylation of p44/42 and p38 MAPKs (C) were immunodetected after stimulating CHO-IR cells with 100 mg/L GSPE or 100 nM insulin. Total protein was isolated and cell lysates were subjected to Western blot analysis using antibodies specific to the target proteins. Immunoblots and quantification of the bands by ScionImage program are representative of at least two independent experiments.

phenolic acids. In order to identify which component was responsible for the healthy effects of GSPE, we first fractionated GSPE by chromatographic separation and selected the fraction with bioactivities similar to or higher than the total extract. This bioactive fraction, known as fraction VIII, was the richest fraction in trimeric structures (mean degree of polymerization [mDP]=2.71±0.09) [28]. We then assessed whether this fraction reproduces the molecular activation of the insulin receptor as well as phosphorylation of the insulin signaling proteins in CHO-IR cells. Fig. 3 shows that fraction VIII phosphorylated the insulin receptor and Akt at the serine and threonine residues similarly to GSPE. Moreover, although MAPK was less phosphorylated by fraction VIII than by GSPE, it was still significantly higher than the phosphorylation induced by insulin (Fig. 3). Table 1 shows the different activation of these insulin signaling proteins after GSPE or fraction VIII treatments vs. the insulin effects.

4. Discussion

Natural compounds that activate the IR and IR-mediated signaling pathway might be beneficial for treating metabolic syndrome and diabetes [29]. We previously reported that a grape seed procyanidin extract (GSPE) possesses insulinomimetic properties, since it decreases hyperglycaemia in streptozotocin-diabetic rats. Furthermore, it stimulates glucose uptake in insulin-sensitive cell lines by using two of the intracellular mediators of the insulin signaling pathway, PI3K and p38 MAPK, and some of the insulin mechanisms such as Glut4 translocation [22]. Moreover, GSPE enhances glycogen and triacylglyceride synthesis in adipocytes after either a long or acute treatment [22,30,31]. In the present study, we show that GSPE activates the insulin receptor and some of the post-receptor signaling mechanisms. Furthermore, we have found that fraction VIII, which was reported as the main cause of the healthy effects of GSPE, mimics the effects of the whole extract.

We show that oligomeric structures of GSPE activate the insulin receptor by interacting with and inducing the tyrosine phosphorylation of the insulin receptor. There are few studies concerning the direct interaction between IR and natural compounds. Studies on the effects of flavonoids on glucose transport strongly suggest that the biological activities of flavonoids highly depend on their chemical structure and the relative orientation of various substitutions in the molecule. Li et al. [27] suggested that the differences between β-penta-*O*-galloyl-glucopyranose and its natural anomer α- penta-*O*-galloyl-glucopyranose when activating insulin-mediated glucose transport after binding to the insulin receptor might be accounted for by structural differences around the anomeric carbon of the glucose core of the molecules. Nomura et al. reported a relationship between the structure of flavonoids and the way they inhibit glucose uptake in an adipose cell line [13]. They showed that some flavonoids (mainly flavones and flavonols) inhibited glucose uptake by inhibiting insulin-stimulated phosphorylation of the insulin receptor, Akt activation and GLUT4 translocation to the plasma membrane, while other flavonoids inhibited glucose uptake by blocking GLUT4 translocation with no effects on IR and Akt phosphorylation. Previous results from our group suggested that oligomeric forms of flavan-3-ol are the main cause of the insulinomimetic effects of GSPE, whereas neither monomeric forms nor phenolic acids showed any effects on glucose uptake stimulation. Here we show that both the whole extract and fraction VIII, a fraction rich in trimeric structures, phosphorylate the IR to a similar extent. Such IR phosphorylation is, however, weaker than that induced by insulin (see Table 1). The lower phosphorylation of the insulin receptor correlates to the lower degree of GSPE-induced glucose uptake, approximately 40%, vs. insulin. Similarly, Wada et al., studying the insulin analogues *detemir* and *glargine*, reported that detemir-induced phosphorylation of the insulin receptor was lower than phosphorylation induced by glargine and human insulin, since detemir had 50% lower affinity for IR, resulting in a less intense signal transduction [32]. Not only is the degree of IR phosphorylation induced by insulin and GSPE different, but also GSPE is less sensitive to 1 μM of an antagonist synthetic peptide. Such a peptide is a heterodimer built with two monomer subunits, which binds to two discrete hotspots on the insulin receptor (designated Site 1 and Site 2) that appear to correspond to the two contact sites involved in insulin binding [26]. Monomer subunits with C–N linkage and Site 1–Site 2 orientation make the peptide antagonistic. Since agonist and antagonist peptide activity depends on the orientation and linkage of the constituent monomer subunits [26], we suggest that oligomers of GSPE might interact with the insulin receptor in different spots than insulin or in the same spots but with a different orientation, resulting in a lower degree of insulin receptor phosphorylation and, consequently, a lower signal transduction and other effects.

Several studies support the idea that a different interaction with the insulin receptor induces a reduction of the receptor phosphorylation resulting in differences in the downstream signaling [33]. Here we show several differences concerning the activation of some downstream IR molecules that reinforce our hypothesis (see Table 1). Continuing with the analysis of how GSPE modulates the insulin signaling pathway, we checked how Akt is involved in the effects of GSPE. Using an Akt inhibitor, we confirmed that Akt is

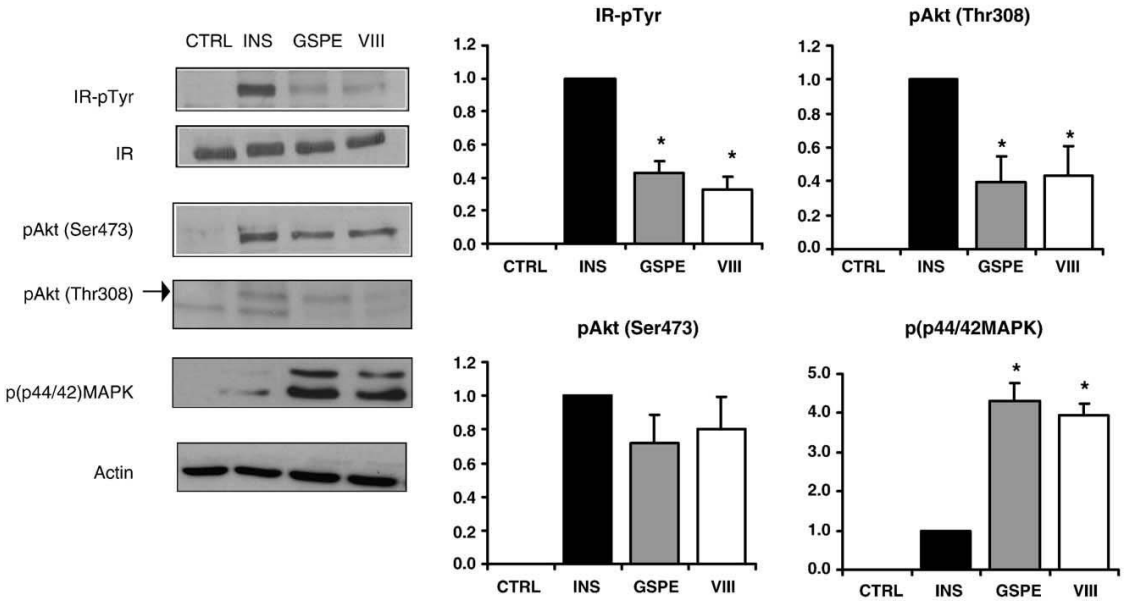

Fig. 3. Effects of fraction VIII on insulin receptor and on insulin signaling pathway. CHO-IR cells were treated with 100 nM insulin, 100 mg/L GSPE and 100 mg/L of fraction VIII for 5 min. Cells were lysed and proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes. Insulin receptor (pTyr), AktSer473, AktThr308 and p44/42MAPK phosphorylation was immunodetected using specific antibodies for their active forms. The expression level of actin was used as a loading control. Band intensity was quantified using the ScionImage software and referred to insulin treatment values for calculations. Blots and data shown are means±S.E.M. of three independent experiments. * indicates significant differences from insulin treatment (100 nM for 5 min) at $P<.05$.

required for GSPE-induced glucose transport. Phosphorylation of Akt at Thr308 by GSPE and fraction VIII was lower than insulin phosphorylation, according to the lower affinity of GSPE to interact with the insulin receptor and the resulting weaker signal transduction. However, both GSPE and fraction VIII stimulated Ser473Akt phosphorylation to the same extent as insulin. Moreover, we also found that p44/p42 and p38 MAPKs were much more phosphorylated by GSPE than by insulin. Other studies have shown different effects of flavonoids on Akt and MAPK phosphorylation. Nomura et al. reported that while kaempferol or quercetin inhibited insulin-stimulated activation of Akt, other flavonoids such as genistein, silbin, theaflavins or EGCG did not have any effects on its phosphorylation [13]. Resveratrol also inhibited the insulin-induced Akt and MAPK activation in rat hepatocytes [34]. However, myricetin increased basal phosphorylation of IR, IRS-1 and Akt, and reversed the reduced level of insulin action on the phosphorylation of these proteins in the soleus muscle of fructose chow-fed rats [7]. From our present results, we cannot establish to what extent the differential phosphorylation of p44/42, p38 and Akt between insulin and GSPE contributes to the different degree of glucose uptake stimulation. However, previously we had found differences between GSPE and insulin in activating glucose metabolism, such as a reduced proportion of uptaken glucose driven to synthesize glycogen in GSPE-treated adipocytes [31]. Insulin enhances glycogen synthesis by inhibiting kinases such as protein kinase A or glycogen synthase kinase-3 and by activating protein phosphatase 1 which in turn promotes glycogen synthase dephosphorylation and activation. Akt mediates many of these events, i.e., it phosphorylates and inactivates glycogen synthase kinase-3 [2]. In other tissues, p38 can directly phosphorylate glycogen synthase kinase-3β [35]. Also, an essential component of mTORC2 (a complex that phosphorylates Akt at Ser473) negatively regulates glycogen synthase activity [36]. Thus, it is likely that the differential activation of signaling components between insulin and GSPE might play an important role in the metabolic effects of GSPE. Further experiments are required to fully understand the exact mechanisms by which GSPE-induced kinase phosphorylation might regulate glucose metabolism. Although our main hypothesis is focused on the possible different insulin receptor sites to which GSPE and insulin bind and/or are oriented, another hypothesis could help us to explain how they activate the downstream pathways differently. GSPE might dissociate slower than insulin from the insulin receptor, since there is a correlation between the mitogenic potential and the occupancy time at the insulin receptor [37,38]. Which pathway gets activated might also depend on whether the insulin receptor is located on the cell surface or in the endosomal compartment. Therefore, GSPE and insulin could interact with receptors located in different compartments since the spatial segregation allows simultaneous and selective signaling via the same receptor isoform in the same cell [39]. Internalization of the insulin receptor appears to be required to phosphorylate and activate the Shc/MAPK pathway. Since GSPE or fraction VIII activated p44/p42 MAPK four times as much as insulin, another hypothesis is that GSPE could induce insulin receptor internalization [33,40]. Finally, we cannot rule out the possibility that GSPE might interact with other receptors to induce the phosphorylation of these kinases. However, more studies must be

Table 1
Summary of the protein activation values obtained by GSPE and fraction VIII

Proteins	% GSPE	% Fraction VIII
IR	43±7%*	39±7%*
Akt (Ser473)	72±20%	80±7%
Akt (Thr308)	39±7%*	44±7%*
p44/42MAPK	430±46%*	395±13%*

Protein phosphorylation values are expressed in % compared to maximal stimulation by insulin. Data shown are means±S.E.M. of at least two independent experiments.
* Indicates significant differences from insulin at $P<.05$.

carried out to clarify the detailed mechanism underlying GSPE-activated signaling mechanisms.

In conclusion, we have found that oligomeric procyanidins of GSPE activate the insulin receptor by interacting with and inducing the phosphorylation of the insulin receptor and that this interaction leads to increased glucose uptake. We have also found that Akt is required for GSPE-induced glucose uptake. However, we have shown that GSPE phosphorylates proteins of the insulin signaling pathway differently than insulin does. Our results point to Akt, p44/42 and p38 MAPKs as key points for GSPE-activated signaling mechanisms. These results might help us to understand the wide range of biological effects of procyanidins and provide further insight into the molecular signaling mechanisms used by procyanidins derived from grape seed.

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A trimer plus a dimer-gallate reproduce the bioactivity described for an extract of grape seed procyanidins

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ABSTRACT

The relationship between grape seed-derived procyanidin extract components and their bioactivity was explored. The monomeric and dimeric structures only acted as anti-inflammatory agents. Similarly, pure C1 trimer was highly effective on LPS-activated macrophages. To reproduce all of the bioactivities of the total extract, a fraction enriched with trimeric structures was needed. This trimeric-enriched fraction was divided into subfractions, the most bioactive of which contained two compounds with a molecular weight equal to a trimer (865) and a dimer-gallate (729), according to spectrometric analysis. Thus, it may be concluded that a mixture of both molecules reproduces the bioactivity in glucose metabolism (3T3-L1), lipid metabolism (HepG2) and macrophage functionality (RAW 264.6).

1. Introduction

The "French paradox" has attracted the attention of researchers worldwide for more than a decade (Renaud & De Lorgeril, 1992). Many studies have proved several positive and healthy effects of grape seed-derived procyanidins (GSPE) (Aron & Kennedy, 2008; Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005). Most of them, however, have examined phenolic extracts derived from grape seed, because they are waste products of the winery and grape juice industry—a rich source of polyphenols—and because it is difficult to find individual compounds as pure structures in these extracts.

The composition of GSPE is known to consist largely of gallic acid, catechin, epicatechin and procyanidin dimers and trimers composed of flavan-3-ol units with C4–C8 or C4–C6 interflavan linkages (Agarwal et al., 2007). These compounds are also present as esters linked to gallic acid in the aliphatic 3-hydroxyl group in the C ring. Some attempts have been made to evaluate how effective the different components of these extracts are at improving some of the well-described properties of the whole extract, mainly growth inhibition and apoptotic death (Agarwal et al., 2007; Faria, Calhau, deFreitas, & Mateus, 2006; Lizarraga et al., 2007). Guo et al. (2007) proved that oligomeric and polymeric grape seed procyanidins are effective at protecting and treating ailments in the central nervous system induced by alcohol abuse.

Grape seed-derived procyanidin extracts have several healthful properties: for example, they act as antioxidants (Puiggròs et al., 2005), they improve lipid metabolism (del Bas et al., 2005), they limit adipogenesis (Pinent et al., 2005), and they function as insulinomimetic agents (Pinent et al., 2004) and anti-inflammatory agents (Terra et al., 2007). This study aims to identify the structure(s) responsible for these healthful effects. This was achieved by two fractionation steps of the initial extract according to its

Abbreviations: GSPE, grape seed-derived procyanidin extract; GA, gallic acid; EGCG, epigallocatechin-gallate; NO, nitric oxide; PGE2, prostaglandin E2; LPS, lipopolysaccharide; PBS, phosphate buffered saline; BSA, bovine seric albumin; EC, epicatechin; TAG, triacylglycerol; DMEM, Dulbecco's modified Eagle medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; HPLC-ESI-MS, high performance liquid chromatography-electrospray ionisation-mass spectrometry; MALDI-TOF, matrix-assisted laser-desorption ionisation-time-of-flight mass spectrometer.

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effectiveness at improving several functions in three different cell lines. Once a highly effective fraction had been identified, its structures were characterised using HPLC-ESI-MS and the molecular weights of individual peaks were confirmed using MALDI-TOF.

2. Materials and methods

2.1. Cells, reagents and materials

The procyanidin extract contained 76% procyanidin with the following composition: 1.63% phenolic acids (mainly gallic acid), 20.92% monomers (mainly catechin + epicatechin), 20.71% dimers + epigallocatechin-gallate (EGCG), 17.33% trimers and 39.41% oligomeric forms of four units or more. Pure molecules were mostly obtained from SIGMA (Madrid, Spain). These were hippuric acid, ferulic acid, 3-hydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, *p*-hydroxyphenylacetic acid, vanillic acid, 3-hydroxyphenylacetic acid, *p*-coumaric acid, epigallocatechin, catechin gallate, epicatechin, gallocatechin gallate, epicatechin gallate, catechin hydrate and epigallocatechin gallate. Procyanidin B1–B4 came from APIN Chemicals (Abingdon, Oxon, UK). All procyanidin extracts, fractions, subfractions and pure molecules were prepared in absolute ethanol. Appropriate dilutions were made in order to obtain a 0.1% (v/v) ethanol concentration in all control and treated wells.

Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium). Insulin (Actrapid®) was from Novo Nordisk Pharma SA (Madrid, Spain). Bradford protein reagent was from Bio-Rad Laboratories (Life Science Group, Hercules, CA, USA), 2-deoxy-[1-^{3}H]-glucose and ^{14}C-acetate was from Amersham Biosciences (Buckinghamshire, England).

2.2. Chromatographic separation of procyanidin extract

GSPE (0.5 g) was subjected to normal-phase chromatography column (35-70 mesh, Interchim, Montluçon, France) preconditioned with solvent A (acetone/hexane, 65:35) as follows: GSPE components were separated according to size using an increasing gradient of solvent B (acetone/hexane, 80:20). First, low molecular weight compounds were eluted with solvent A, then the proportion of solvent B was gradually increased until it reached 100% after 1 h. Finally, an additional volume of solvent B was added, and 10 mL fractions were collected using a fraction collector. The fractions collected were monitored using TLC on PolyGram silica gel 0.2 mm with fluorescent indicator UV_{254} (Macherey-Nagel, Hoerdt, France), with the mixture toluene/acetone/acetic acid (3:3:1, v/v/v). The TLC plates were visualised following spraying with anisaldehyde reagent. Eleven major fractions with increasing degrees of polymerisation were identified, according to their retention time, R_f (Terra et al., 2007). These fractions were vacuum-dried and kept at −20 °C for subsequent use in the biological studies.

The most effective fraction (VIII) was further subfractionated by semipreparative HPLC (Varian, Model 210 Walnut Creek, CA, USA) with a 4 × 250 mm Ultrasep RP18 column (4 μm) (Bischoff, Leonberg, Germany) at room temperature using the following solvents: water/formic acid (95.5:4.5, v/v) (A) and acetonitrile/solvent A (10:90, v/v) (B) with the following gradient system: 0–40% B (0–10 min), 40–60% B (10–35 min), 60–100% B (35–50 min) and 100% B (50–60 min). Detection was carried out at 286 and 306 nm, with a UV–vis detector (Varian, Model 345, Walnut Creek, CA, USA).

An initial approach for determining molecular weight was to use HPLC-ESI-MS. A Platform II (Micromass, Manchester, UK) with electrospray injection (ESI) was used, coupled to the LC apparatus (reversed phase LC on a Waters TM system 600 E, Saint-Quentin, France). Procyanidins can easily shed a proton, generating intense negative ions $[M–H]^{-}$, so detection was performed in the negative ion mode. A low voltage was used to avoid fragmentation; the products were identified by their molecular peaks.

The chromatogram peaks isolated by semipreparative HPLC were also characterised by MALDI-TOF. MALDI-MS spectra were obtained using a matrix-assisted laser-desorption ionisation-time-of-flight mass spectrometer (TofSpec MALDI-TOF) from Micromass (Manchester, UK). This instrument has a pulsed nitrogen laser (337 nm, 4 ns pulse width) and a time-delayed extracted ion source. Spectra were recorded in the positive-ion mode using the reflectron and a 20 kV accelerating voltage.

2.3. Cell culture and measurements

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (Ardévol, Bladé, Salvadó, & Arola, 2000). Ten days after differentiation, fully differentiated adipocytes were washed twice with phosphate buffered saline (PBS) and incubated at 37 °C with serum-free supplemented Dulbecco's modified Eagle medium (DMEM) containing 0.2% bovine serum albumin (BSA) (depletion medium) for 2 h. During the last 30 min of this depletion treatment, the cells were treated with GSPE or insulin. Afterwards, glucose transport was determined by measuring the uptake of 2-deoxy-D-[^{3}H] glucose, as previously described by Pinent et al. (2004). Each condition was run in triplicate.

Table 1
Summary of the bioactivity of the monomeric pure forms. Values in italics mean a statistically significant positive effect ($p < 0.05$).

Monomeric structures	Stimulation of glucose uptake (3T3-L1 adipocytes) 150 mg/L compound (stimulation vs insulin effect 1.04 ± 0.023)	PGE-2 production (RAW macrophages) 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 1.2)	NO production RAW macrophages 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 6.2)
Vanilic acid	0.055 ± 0.018	*60.63 ± 9.61*	*69.69 ± 0.56*
Epicatechin	0.024 ± 0.014	*46.73 ± 0.73*	*64.77 ± 0.70*
Epicatechin gallate	NA	*48.18 ± 6.66*	*32.87 ± 8.72*
EGCG	NA	*53.72 ± 5.63*	*6.67 ± 3.56*
Catechin	0.051 ± 0.018	57.98 ± 5.99	103.71 ± 1.35
Gallic acid	0.048 ± 0.010	*46.63 ± 2.51*	93.4 ± 0.8
p-Hydroxiphenylacetic acid	0.054 ± 0.014	*55.31 ± 4.00*	111.79 ± 5.28
p-Coumaric	0.058 ± 0.020	NA	NA
3-Hydroxybenzoic acid	0.058 ± 0.008	NA	NA
Protocatequic acid	0.073 ± 0.049	NA	NA
Ferulic acid	0.092 ± 0.068	NA	NA
Hypuric acid	0.085 ± 0.047	NA	NA

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Table 2
Summary of the bioactivity of the oligomeric pure compounds. Values in italics mean a statistically significant positive effect ($p < 0.05$).

Oligomeric pure structures	Stimulation of glucose uptake 150 mg/L compound (stimulation vs insulin effect 1.037 ± 0.023)	PGE-2 production 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 1.2)	NO production 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 6.2)	Total cholesterol secretion 25 mg/L compound (fold change vs control 1.00 ± 0.02)	Triacylglycerol secretion 25 mg/L compound (fold change vs control 1.01 ± 0.01)
B3	0.09 ± 0.02	69.92 ± 11.14	83.98 ± 4.56	1.26 ± 0.16	1.18 ± 0.11
B1	0.08 ± 0.07	70.78 ± 15.85	42.56 ± 3.52	1.29 ± 0.12	1.05 ± 0.11
B2	0.09 ± 0.05	79.86 ± 0.17	60.12 ± 7.49	1.15 ± 0.11	1.07 ± 0.09
B4	0.07 ± 0.03	*68.92 ± 0.04*	*46.58 ± 3.90*	1.22 ± 0.13	1.20 ± 0.16
C1	*0.13 ± 0.00 100 mg/L*	*44.38 ± 1.42*	*20.96 ± 5.85*	1.05 ± 0.11	*0.82 ± 0.04*

Table 3
Summary of the bioactivity of the fractions obtained from the grape seed-derived extract. Values in italics mean a statistically significant positive effect ($p < 0.05$). In the first column, the intensity of the shade of grey correlates to the increasing degree of polymerisation.

GSPE fractions (amount obtained)	Stimulation glucose uptake 150 mg/L compound (vs insulin effect 1.04 ± 0.02)	PGE-2 production 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 1.2)	NO production 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 6.2)	Triacylglycerol secretion 25 mg/L compound (fold change vs control 1.01 ± 0.01)	Total cholesterol secretion 25 mg/L compound (fold change vs control 1.0 ± 0.02)	ApoB protein secretion 10 mg/L compound vs control (100.3 ± 0.3)
I (25 mg)	NA	NA	NA	1.07 ± 0.07	0.96 ± 0.06	NA
II (66 mg)	−0.04 ± 0.01	NA	NA	1.01 ± 0.12	1.03 ± 0.10	80.0 ± 12.8
III (6 mg)	−0.05 ± 0.01	NA	NA	0.95 ± 0.09	0.87 ± 0.08	*81.0 ± 3.9*
IV (4.5 mg)	−0.04 ± 0.02	NA	NA	0.85 ± 0.07	0.82 ± 0.05	NA
V (11.5 mg)	−0.09 ± 0.09	*51.91 ± 17.74*	*51.24 ± 14.63*	0.96 ± 0.06	1.03 ± 0.12	*78.5 ± 1.4*
VI (26 mg)	−0.02 ± 0.09	NA	*52.56 ± 9.67*	0.83 ± 0.05	0.97 ± 0.11	79.3 ± 9.1
VII (18 mg)	0.11 ± 0.03	*63.35 ± 14.12*	*23.03 ± 9.05*	*0.43 ± 0.08*	0.71 ± 0.18	NA
VIII (33 mg)	*0.40 ± 0.03*	NA	*44.60 ± 8.39*	*0.36 ± 0.03*	*0.59 ± 0.05*	*46.0 ± 4.9*
IX (23 mg)	*0.49 ± 0.02*	NA	*20.52 ± 5.85*	*0.32 ± 0.01*	*0.58 ± 0.06*	NA
X (16 mg)	*0.35 ± 0.04*	*63.34 ± 11.59*	*23.22 ± 5.76*	*0.41 ± 0.05*	*0.77 ± 0.01*	NA
XI (15 mg)	*0.40 ± 0.04*	NA	48.73 ± 0.77	*0.36 ± 0.03*	*0.81 ± 0.03*	NA
XII (75.4 mg)	NA	NA	*22.48 ± 5.67*	*0.45 ± 0.00*	*0.72 ± 0.03*	NA
XIII (81.6 mg)	NA	*53.85 ± 10.57*	*22.58 ± 3.96*	*0.34 ± 0.01*	*0.68 ± 0.06*	NA
XIV (70.5 mg)	NA	NA	49.44 ± 1.90	*0.45 ± 0.03*	0.77 ± 0.06	NA
XV (33.5 mg)	NA	NA	*26.58 ± 4.13*	0.78 ± 0.09	0.98 ± 0.07	NA

HepG2 cells (ATCC code HB-8065, Manassas, VA, USA) were propagated in DMEM and cultured as previously described (Puiggròs et al., 2005). The only modification was the addition of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (SIGMA, Madrid, Spain) to the culture media. For the experiments, HepG2 cells were seeded at 750,000 cell/well in 12-well-plates and left to grow for two days (80% confluence) in a propagation medium. The medium was replaced 16 h before treatment. Then, lipid synthesis was measured. Procyanidins and ^{14}C-acetate (0.6 μCi/mL) were added to the cell media and 6 h after treatment the media and cells were collected and the lipids were extracted using 3 volumes and 0.5 ml of hexane/isopropanol (3:2, v/v) respectively. Thin layer chromatography was performed with petroleum either: diethyl ether: NH_3 (40:10:0.1) and an additional separation using a hexane/methyl tert-butyl ether (MTBE)/NH_4OH (30:20:01, v/v/v) solvent to obtain the free cholesterol, cholesterol esther and triacylglycerol (TAG) fractions. Each fraction was scraped and determined by scintillation counting. Values were corrected per milligram of protein, determined using the Bradford methodology (Bradford, 1976). The medium was collected after 24 h treatment and apolipoprotein B was detected as described in del Bas et al. (2008).

Murine macrophage cell line RAW 264.7 (European Tissue Culture Collection, ECACC, Ref. 91062702, London, UK) was cultured as previously described (Terra et al., 2007) and used for experiments between passages 5 and 14. At 80% of confluence, adherent monocyte-RAW 264.7 cells were incubated with different compounds and with 1 μg/mL LPS simultaneously for 19 h. The culture medium for control and treated cells was collected and tested for nitric oxide (NO) and prostaglandin E_2 (PGE_2) production. The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction (Terra et al., 2007). The level of PGE_2 released into culture medium was quantified and normalised as previously described (Terra et al., 2007).

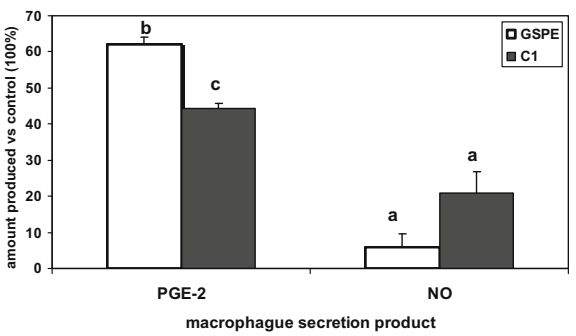

Fig. 1. Inhibition of PGE_2 and NO production by C1 in LPS induced RAW 264.7 macrophages. RAW 264.7 macrophages were simultaneously stimulated with LPS for 19 h and incubated with C1 (trimeric procyanidin) at 5 mg/L and GSPE (grape seed procyanidin extract) at 45 mg/L as reference. PGE_2 and NO production were measured after treatment. Results were normalised to control levels (100.0% ± 6.2). Each bar represents mean ± SEM of nine biological experiments. Different letters mean $p < 0.05$ as compared to GSPE treatment.

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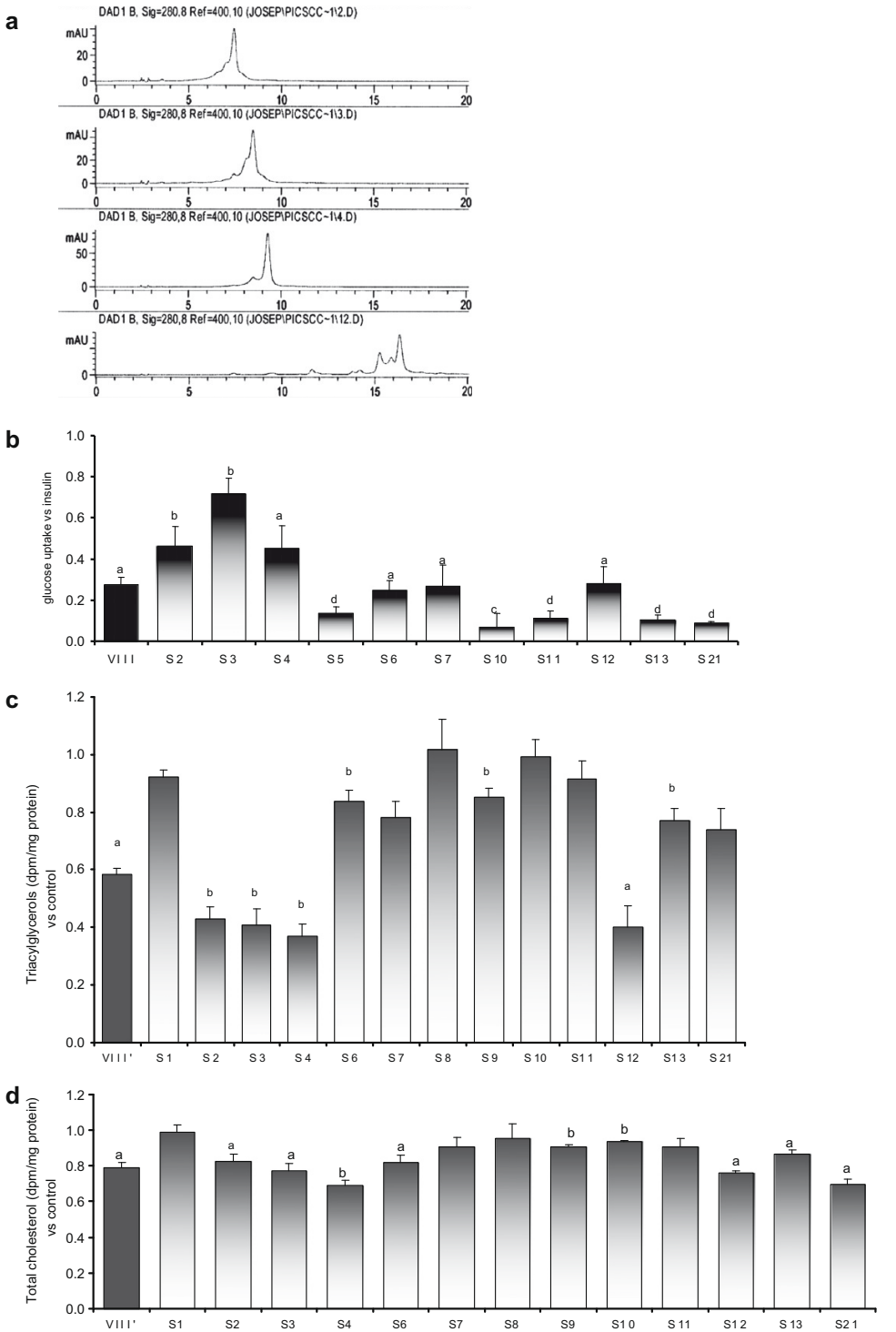

Fig. 2. Results from the second fractionation step. (a) Chromatograms of the most active subfractions. In correlative order: S2, S3, S4 and S12. (b) Stimulation of glucose uptake done by each of the subfractions obtained. After 2 h of depletion, differentiated 3T3-L1 adipocytes were treated for 30 min with 100 mg/L of total fraction VIII or each subfraction. Afterwards, glucose uptake was measured. Results are related to the maximum stimulation achieved by insulin (1.037 ± 0.023). (c and d) Effect of fraction VIII and its subfractions in *de novo* synthesised triacylglycerols (c) and total cholesterol (d) secretion to the extracellular media. HepG2 cultures were incubated for 6 h with fraction VIII or its subfractions at 25 mg/L and ^{14}C-acetate. Afterwards, lipid fractions secreted to the cell culture media were quantified. Media lipid fraction levels (dpm/mg protein) were normalised to the control levels set at one. Each bar represents mean ± SEM. Letters above the bars mean $p < 0.05$ as compared to control. Different letters mean $p < 0.05$ as compared to fraction VIII treatment.

2.4. Calculations and statistical analysis

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

3. Results and discussion

3.1. Monomeric and dimeric components of grape seed-derived extract act mainly as anti-inflammatory agents

The main objective of this work was to identify the molecule(s) responsible for the bioactivity of a grape seed procyanidin extract that act in vivo as (a) an antihyperglycemic (Pinent et al., 2004), (b) an antiatherogenic (del Bas et al., 2005, 2008) and (c) an anti-inflammatory (Terra et al., 2008) product. To meet this objective, three cell lines in which GSPE has been identified as being highly active, namely adipocytes (Pinent et al., 2005; Pinent, Bladé, Salvadó, Arola, & Ardévol, 2005a, 2005b), hepatocytes (del Bas et al., 2005; Puiggròs et al., 2005), and macrophage cells (Terra et al., 2007) were used. The effects that pure compounds have on these cells and the two fractionation steps have on the whole extract were evaluated.

The monomeric components of the extract have clearly been proven to reach body fluids and some of them have been modified in the body (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Thus, we hypothesise that these molecules or their modified forms are the main cause of the effects described. However, as Table 1 summarises, none of these pure monomeric compounds stimulated glucose uptake in the adipocytes, and HepG2 hepatocytes had no effect on TAG or total cholesterol secreted to the cell culture media while 25 mg/L GSPE inhibited TAG and TC secretion 45% and 25% respectively (results not shown). Neither did pure dimeric compounds act on the functions tested (Table 2). Both results agree with those found in the first fractionation step (Terra et al., 2007). Table 3 indicates that the fractions enriched with monomeric and dimeric compounds did not seem to affect adipocytes or hepatocytes. Monomeric components were previously reported not to induce lipolysis in adipocytes (Ardévol et al., 2000) or to protect against oxidative stress in Fao hepatocytes (Roig, Cascón, Arola, Bladé, & Salvadó, 2002). In both situations the total extract was highly effective. Only fractions III and V showed a slight but significant decrease in the amount of ApoB secreted to the cell culture media of HepG2 cells. Both fractions, together with fraction IV, share a peak at the end of the chromatogram that could be epicatechin gallate. In this respect, Yee et al. (2002) also found that, unlike EC, EGCG is a potent inhibitor of ApoB secretion, suggesting that the gallate moiety has a beneficial effect on the catechin molecule and that this is beneficial for lipid metabolism in terms of ApoB secretion.

Also, a common trait in the different approaches was that almost all monomeric (Table 1) and dimeric (Table 2) structures showed anti-inflammatory properties equal to the total grape seed procyanidin extract (Fig. 1) and in some cases had a stronger effect (i.e., EGCG limited NO production). These results agree with previously published works showing the anti-inflammatory effect of procyanidin B2 (Chen, Cai, Kwik-Uribe, Zeng, & Zhu, 2006; Zhang et al., 2006).

3.2. Oligomeric fractions of the extract justify its complete bioactivity

Park, Rimbach, Saliou, Valacchi, and Packer (2000) have shown that trimeric procyanidin C2 do not act as an anti-inflammatory compound. The trimer C1 has now been examined and it has been shown not only to have a considerable anti-inflammatory effect (Fig. 1) but also to be active on hepatocytes and adipocytes (Table 2). C1 reproduces most of the bioactive effects of the total extract. We evaluated it as a pure compound at the same concentration of the total extract and found a much lower effect (GSPE inhibited 45% TAG secretion in HepG2). However, this is the first time a C1 procyanidin has been described as having a bioactivity that is different from its antioxidant activity, which was previously shown to be higher than the antioxidant activity of other smaller oligomeric structures (da Silva Porto, Laranjinha, & de Freitas, 2003).

Examination of the trimeric-enriched fractions in the extract examined here showed that fractions VIII–XIII positively activate all the biological functions (see Table 3). Fraction VIII almost completely lacks monomers and dimers, but has the greatest bioactivity in the extract tested. Another fractionation step was carried out on fraction VIII to reach the objective. Fig. 2a shows a chromatogram of those subfractions whose bioactivity was closer to the total of fraction VIII (S2, S3, S4 and S12) for both cell lines. Fig. 2b–d summarises all the results obtained with these subfractions. Several bioactive subfractions (S2, S3, S4, S6, S12 and S13) can be found effective for both cell lines. However, it should be taken into account that the bioactivity of each one of these subfractions was evaluated at the same concentration as the total initial extract in each cell line. This facilitates the comparison of the effects between subfractions, but also distorts the truth because the amount of each subfraction obtained was in the order of S13 (3.6 mg) > S12 > S11 > S10 > S7 > S6 > S8 > S4 > S5–S9 > S2 > S3 (0.5 mg). S12 was the biggest subfraction and it had the greatest effect, so it was selected for further analysis. Two complementary analyses were carried out: the fraction VIII was analysed with HPLC-ESI-MS and the isolated S12 subfraction with MALDI-TOF analysis. The results showed that S12 has two molecular weight components: a dimer-gallate (729) and a trimer (865), the two peaks found in the HPLC chromatogram (Fig. 2a). The trimer component has been assigned to C1 procyanidin by comparing retention times and because it is the most abundant form in grape seed (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000). However, the dimer-gallate is probably not the B2-gallate, which was identified at S13, a fraction that did not share a single peak with S12 and which was barely active on the three assayed functions. Similarly, Schäfer and Högger (2007) have demonstrated that the inhibitory action of Pycnogenol© on α-glucosidase, in vitro, was stronger in extract fractions with higher procyanidin oligomers. Working with apple procyanidins, Sugiyama et al. (2007) also described an inhibitory effect on pancreatic lipase activity which depended on size. Mao, Van De Water, Keen, Schmitz, and Gershwin (2003) found that the effects on cytokine expression in peripheral blood mononuclear cells depended on the cocoa procyanidin fraction evaluated. Faria et al. (2006) showed that the simpler procyanidin structures, including catechin, have a higher antioxidant activity, which correlates with their antiproliferative effect on the cell lines of breast cancer. Agarwal et al. (2007) identified B2-3,3′-di-*O*-gallate as a major active constituent against growth inhibition and apoptosis, relaying most of its power to its galloyl group because B2 was barely active. Similarly, Lizarraga et al. (2007) observed that the fractions that were most efficient at inhibiting cell proliferation, arresting the cell cycle in the G(2) phase and inducing apoptosis, were the grape fractions with the highest percentage of galloylation and mean degree of polymerisation.

All these results together will be very helpful for understanding some of the differential and/or sometimes contradictory effects described for complete extracts of natural origin. Simple procyanidin structures have higher antioxidant properties. Short structures with higher galloylation seem to be more active as antiproliferatives. At least one trimer and one dimer-gallate are needed to have metabolic effects.

4. Conclusions

Monomeric structures and dimers (mainly fraction VI) of grape seed extract were the only effective as anti-inflammatory agents. Procyanidin C1 was also very active as an anti-inflammatory compound. Subfraction 12 was the most effective in all the parameters examined. As HPLC-ESI-MS and MALDI-TOF analysis showed, this fraction contains a trimer (865) and a dimer-gallate (729). Therefore, a mix of both molecules reproduces the bioactivity in glucose metabolism, lipid metabolism and macrophage functionality which has previously been described for the total grape seed extract.

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IV. General Discussion

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General Discussion

The aim of this thesis was to describe how dietary procyanidins modulate glucose metabolism *in vivo* (mainly in adipose tissue), study *in vitro* the mechanism they use to exert these effects, and identify the molecular bioactive components responsible for the effects of the grape-seed procyanidin extract (GSPE). To achieve these objectives, non-toxic doses of GSPE [1] were used to study the bioactivity and molecular mechanisms of GSPE using *in vitro* models, 3T3-L1 mouse adipocytes and Chinese Hamster Ovary cells that overexpress the human Insulin Receptor (CHO-IR cells) and an *in vivo* model of cafeteria-diet-fed rats.

Grape-seed procyanidin extract (GSPE) was reported to mimic some of the physiological effects of insulin, i.e. to improve hyperglycaemia in streptozotocin-induced diabetic rats and stimulate glucose uptake in insulin-sensitive cell lines [2]. However, procyanidins stimulated glucose uptake in 3T3-L1 adipocytes less than insulin, while the anabolic effects of GSPE for stimulated glycogen synthesis and stimulated glycerol synthesis were also different with respect to insulin action [3]. These differences indicate that GSPE could use different mechanisms from insulin, which suggests that procyanidins can prevent or correct a state of impaired glucose tolerance and/or a state of impaired insulin action by acting mainly on adipose tissue, which is a suggested target for the effects of procyanidins [4].

To evaluate the role of procyanidins in a state of insulin resistance, we generated several models of experimental insulin resistance. Our results showed that GSPE was able to overcome an insulin-resistant condition induced

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by chronic insulin treatment since the procyanidins maintained a higher capacity than insulin to stimulate glucose uptake after resistance was induced.

On the other hand, GSPE was less effective after insulin resistance was induced with glucose oxidase, TNF-α or dexamethasone agents. Since the impairment of insulin action involves defects at different signalling transduction molecules depending on which agent is the insulin-resistant inductor [5,6], these results were useful for identifying which molecules may be involved in the insulin-resistance condition where procyanidins are effective. Insulin resistance induced by chronic insulin treatment involves IRS1 protein, while dexamethasone treatment induced insulin resistance at the Glut-4 level, whose activity is limited mainly due to p38 MAPK dephosphorylation [7,8]. We therefore hypothesized that procyanidins overcome the insulin-induced insulin resistance state by activating a different signalling pathway that was not impaired by insulin resistance. Previous results had shown not only that GSPE required PI3K to stimulate glucose uptake in 3T3-L1 adipocytes, but also that p38 MAPK protein was involved in procyanidin action [2]. As several studies have shown that the MAPK pathway may also be involved in the regulation of glucose uptake [8,9], we suggest that this pathway may be used by GSPE to improve the insulin-induced insulin resistance state through Glut-4 activity modulation, though a direct interaction with the Glut-4 transporter is also possible. In agreement, other flavonoids are reported to act directly on glucose transporters, though they are also reported to have an inhibitory effect [10,11].

After observing the various GSPE behaviours in the insulin-resistant conditions assayed, we wanted to better understand the procyanidin action

mechanism at the molecular level. Activation of the insulin receptor is a necessary prior step for the activation of molecules of the insulin-signalling pathway such as the PI3K or p38 MAPK proteins [12,13]. We therefore analyzed whether procyanidins interact with the insulin receptor and whether they induce insulin receptor autophosphorylation. Our results showed that GSPE activates the insulin receptor by interacting with and inducing the tyrosine-phosphorylation of the insulin receptor. GSPE activation was weaker than insulin-induced activation, probably because GSPE has a lower affinity to interact with the insulin receptor than insulin. We analysed this hypothesis by studying glucose uptake induced by GSPE or insulin after using an insulin antagonist synthetic peptide that bound specifically to the insulin receptor [14]. We found that procyanidins were indeed less sensitive than insulin to the insulin antagonist synthetic peptide. Moreover, the lower affinity of GSPE to interact with the insulin receptor correlated with the lower degree of GSPE-induced glucose uptake. GSPE may therefore interact with the insulin receptor in some of the different contact sites involved in insulin binding or in the same sites as insulin but with a different spatial orientation and resulting in a lower degree of insulin receptor phosphorylation, a lower signal transduction and, accordingly, a lower stimulation of glucose uptake. In agreement with these findings, several studies have shown that a lower affinity to interact with the insulin receptor resulted in lower insulin receptor phosphorylation and consequently a less intense signal transduction [15]. GSPE activates the insulin receptor. Therefore, to provide further insight into the molecular mechanisms of procyanidin action, we investigated how GSPE affects several protein kinases involved in the insulin-signalling pathway. First we analysed whether GSPE activates Akt protein because our previous results suggested that this protein may explain

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the metabolic divergences observed between insulin and GSPE [3]. The lower phosphorylation of the insulin receptor induced by GSPE was transduced downstream because the Akt protein at threonine 308 was also less phosphorylated, thus resulting in a lower degree of glucose uptake stimulation. Unexpectedly, GSPE phosphorylated Akt at serine 473 to the same extent as insulin, which indicates that this serine phosphorylation of the Akt protein may be involved in the molecular mechanisms of procyanidin action. Akt S473 phosphorylation may act as a regulatory switch that could determine Akt substrate specificity [16,17]. Moreover, recent observations have suggested that the phosphorylation of Akt at S473 is required to phosphorylate FOXO proteins that activate cell survival pathways, though the phosphorylation of this serine does not appear to be required for the Akt-mediated phosphorylation on GSK3, which is involved in insulin-induced glycogen synthesis [12,17]. Our findings are therefore in agreement with previous results in which GSPE induced a lower proportion of uptaken glucose driven to synthesize glycogen than insulin [3], as procyanidins may activate other signalling pathways by activating Akt at S473. Considering all the issues we discussed earlier, we suggest that the GSPE-signalling pathway may differ from the insulin-signalling pathway at the Akt level, where GSPE would have more affinity to phosphorylate Akt at S473, thus GSPE might activate more cell survival pathways rather than metabolic pathways. Previous results by our research group also suggest that the p38 MAPK protein is an important GSPE target for stimulating glucose uptake in insulin-sensitive cell lines [2]. Moreover, our results with insulin-resistant adipocytes suggested that procyanidins may overcome the insulin-resistant state by acting via the MAPK pathway. We therefore have more proof of GSPE involvement in cellular growth and

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differentiation pathways. We then analysed how GSPE affects p38 and p42/p44 MAPKs proteins. Surprisingly, p44/42 and p38 MAPKs were phosphorylated approximately four times as much as insulin, though the GSPE initial signal was significantly lower than insulin. In agreement with our previous hypothesis in which we suggested that insulin and GSPE interact differently with the insulin receptor, several studies support the idea that a different interaction to the insulin receptor leads to divergent metabolic- and mitogenic-signalling responses [18].

Furthermore, it has been shown that the disruption of the MAPK pathway adversely affected Glut-4-mediated glucose uptake in response to insulin, though it did not affect the docking or fusion of Glut-4 with the plasma membrane [9]. Several studies have also suggested that p44/42 and p38 MAPK activation is required to increase the intrinsic activity of the Glut-4 glucose transporter [19,20]. Our results therefore suggest a role for p44/42 and p38 MAPKs in the GSPE-stimulated glucose uptake, possibly in the activation of the Glut-4 glucose transporter since GSPE stimulates a four-fold increase in the activation of both protein kinases.

From our results we could hypothesize that not only does GSPE stimulate Glut-4 translocation but that it also may increase the intrinsic activity of the Glut-4 glucose transporter to stimulate glucose uptake. The procyanidin action mechanism could involve different insulin signalling pathways. We suggest that GSPE may stimulate Glut-4 translocation by activating the PI3K-Akt-dependent signalling pathway, while Glut-4 activation may be regulated by two signalling pathways—the p38 MAPK pathway and the p44/p42 MAPK pathway. Therefore,

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Akt, p38 and p44/42 may be important targets for the effects of GSPE and their differential activation between insulin and procyanidins may explain the different degrees of insulin and GSPE-glucose uptake stimulation. Akt, p38 and p44/42 may therefore play an important role in the metabolic effects of dietary procyanidins.

The mechanistic differences between insulin and procyanidins and their capacity to stimulate glucose uptake in adipocytes even when insulin resistance was induced suggested that procyanidins could improve glucose metabolism in a disturbed metabolic situation. Also, it is important to evaluate the effects of GSPE *in vivo* in order to extend our knowledge of GSPE effects in experimental models such as a mild model of insulin resistance. Considering the high incidence of insulin resistance in the general population, this may be a suitable model of human disorders.

To analyse this hypothesis, we evaluated the effectiveness of several GSPE treatments on a mild model of insulin resistance induced by feeding healthy female Wistar rats a cafeteria diet for 13 weeks. We found that the lowest GSPE dose, 25 mg of GSPE/kg body weight*day, administered to cafeteria fed rats for 30 days (long-term treatment) was the most effective treatment since it improved many of the parameters analyzed.

This treatment significantly reduced plasma insulin levels, which increased in the cafeteria group. Also, the HOMA-IR index improved, which indicates that administering 25 mg of GSPE to cafeteria-fed-rats for 30 days exerted a positive long-term effect on glucose homeostasis by improving insulin

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resistance. In agreement with these results, several studies have suggested that procyanidins moderately improve plasma insulin resistance after long-term treatment [21,22]. Zhang et al. showed that grape seed-derived procyanidins sligtly improve fasting blood glucose and the HOMA-IR index in mice fed a high-fat diet and administered fructose for 6 weeks [23]. Though using different experimental methods than ours, they found that procyanidin treatment also improved insulin action in insulin-induced insulin-resistant HepG2 cells, similarly to our results showing that GSPE improved insulin resistance in 3T3-L1 adipocytes after chronic insulin treatment.

Our results also show that, after an IPGTT of two hours, the most effective GSPE treatment maintained glucose levels with a lower insulin secretion than the cafeteria group, even though glucose tolerance did not improve overall. These results suggest that GSPE may act as an insulinomimetic agent, whereby procyanidins replace insulin activity and therefore help to maintain normal glucose homeostasis.

With regard to adiposity, long-term GSPE treatment at 25 mg/kg body weight*day significantly reduced visceral adipose tissue weight and tended to decrease plasma leptin levels in rats fed a cafeteria diet. In agreement with our results, dietary procyanidins administered simultaneously to rats on a high-fat diet tended to reduce plasma leptin and plasma insulin levels compared to rats fed high-fat diet alone [24]. Osada et al. showed that, after 9 weeks, dietary procyanidins also significantly reduced total white adipose tissue weight compared to rats fed high-fat diet. However, in our study procyanidins were administered to normal Wistar rats after obesity was induced with cafeteria diet

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for 13 weeks. Similarly, other flavonoid treatments reduced adipose tissue and body weight in mice fed a high-fat diet [25,26].

Our research group previously tried to evaluate the effectiveness of procyanidins on adipose differentiation. Not only GSPE limited adipocytes differentiation, but also a chronic GSPE treatment impeded the formation of adipose cells [27]. In this thesis we found that a high acute GSPE treatment (250 mg/kg body wt administered for 5h) decreased mRNA levels of PPARγ2, C/EBPα and Pref-1 in healthy Wistar rats and that a chronic GSPE treatment (19.5 mg/g day administered for 30 days) had a similar effect in decreasing these adipocyte differentiation markers in the epididymal adipose tissue of fa/fa Zucker rats. We also foud that GSPE modified adipose differentiation markers differently depending on the physiological conditions; *i.e.* the previous high acute GSPE treatment induced almost opposite effects to STZ-induced diabetic Wistar rats. To study the insulin-like effect of procyanidins we administered an effective dose of insulin to a diabetic animal group. In this diabetic condition, where a destruction of pancreatic β-cells results in insulin deficiency, GSPE did not totally mimic the effects of insulin.

In the cafeteria study, GSPE probably needed to be administered longer in order to change the size of adipose depots and thus influence insulin resistance. In agreement, Lee et al. showed that administration of proanthocyanidins from persimmon peel for 6 weeks have protective effects against hyperlipidemia in db/db mice [21,22].

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Finally, the administration of 25 mg/kg of GSPE for 30 days induced a strong down-regulation on markers of mature white adipose tissue. Glut-4 mRNA, a glucose transporter classically considered a marker for insulin resistance [28], was the most down-regulated marker after this GSPE treatment. Despite the lower Glut-4 mRNA levels in white adipose tissue, we hypothesized that GSPE affects glucose transport in adipose cells. In agreement with this hypothesis, several studies have shown that adipocytes can acutely respond to glucose uptake signalling regardless of reduced Glut-4 mRNA levels [29,30]. These GSPE results suggest that white adipose tissue is an important target for procyanidin effects, even though no reduction in mesenteric white adipose tissue weight was observed after GSPE treatments. The specificity of adipose tissue as a target of GSPE was reinforced because GSPE treatment showed no significant gene expression modulation in either muscle or liver. However, in a db/db Type-2 Diabetes model, the administration of proanthocyanidins from persimmon peel down-regulated expressions of sterol regulatory element binding proteins in the liver [22]. A different metabolic situation, different molecules or different doses assayed may explain the divergences between the results in [22] and ours. Identifying the GSPE bioactive components will help to explain the different and sometimes contradictory effects described for procyanidin extracts.

To identify the molecular bioactive components responsible for the beneficial effects of procyanidins, our research group decided to fractionate GSPE by Chromatographic Separation. First we did an initial fractionation step of our initial extract to obtain several fractions with different compositions. Eleven fractions were obtained and numbered from fraction I to fraction XI. To

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evaluate the effectiveness of each fraction in glucose metabolism, we stimulated glucose uptake in 3T3-L1 adipocytes. Fraction VIII was the first fraction that significantly stimulated the uptake of glucose in adipose culture cells. This fraction was the richest in oligomeric structures, a molecular size that has recently been detected in plasma and urine after procyanidins administration in rats [31,32]. The detection and quantification of trimers is relevant because their presence could suggest that these oligomeric forms are absorbed and metabolized in the same way as the monomeric forms.

In agreement with these results, oligomeric procyanidins are also reported to be mainly responsible for the beneficial effects of several plant extracts [33,34]. Also, the oligomeric fraction of procyanidins reproduced the GSPE molecular activation of the insulin receptor, Akt and MAPK proteins in CHO-IR cells. Because of its effectiveness in improving several functions in different cell lines, our research group decided to perform a further fractionation step and we identified the most bioactive subfraction, S12. Mass Spectrometry analysis showed that the S12 subfraction contains a mix of a trimer and a dimer-gallate. Our results therefore suggest that a mixture of the two molecules reproduces the bioactivity described for the total GSPE extract. These results will help us to optimize the effectiveness of the molecules identified and avoid the possible antagonistic effects of other extract compounds.

In summary, GSPE has a positive long-term effect on a cafeteria-diet-induced impaired-glucose-tolerance model by slightly improving plasma insulin resistance parameters. Moreover, white adipose tissue, where glucose uptake might be directly stimulated by GSPE, is an important target for procyanidins

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effects. The molecular mechanism of action of procyanidins differs from the insulin mechanism since GSPE activates the insulin receptor and protein kinases involved in the insulin signalling pathway differently from insulin activation. This different molecular mechanism between GSPE and insulin could explain why GSPE had a higher stimulating capacity than insulin in insulin-resistant adipocytes after chronic insulin treatment, where insulin action was impaired. Moreover, oligomeric structures of GSPE were the molecular bioactive components responsible for total GSPE effects. These results may help us understand how procyanidins affect an impaired glucose metabolism and provide a further insight into procyanidin signalling in adipose cells.

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V. Conclusions

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Conclusions

1. Grape-seed procyanidin extract (GSPE) induces higher glucose uptake stimulation than insulin in insulin-resistant adipocytes.

2. These results help to better describe the GSPE signalling pathway: the activation of IR by procyanidins leads to the activation of Akt via PI3K and the activation of p38 and p44/p42 MAPK proteins. The activation of both pathways is required for GSPE to stimulate the uptake of glucose in adipose cells.

3. An oral dose of 25 mg/kg bw per day of GSPE has a positive long-term effect on glucose homeostasis by improving insulin resistance parameters on cafeteria-diet-fed rats.

4. *In vivo* results suggest that adipose tissue is an important target for GSPE effects where procyanidins modulate metabolic parameters and adipose markers gene expression.

5. Oligomeric structures of GSPE reproduce the bioactivity and molecular activation of proteins of the insulin-signalling pathway of the total grape-seed extract. A trimer combined with a dimer-gallate has been identified as the molecular bioactive components of the grape-seed procyanidins extract.

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