UNIVERSITAT ROVIRA I VIRGILI
Departament de Bioquímica i Biotecnologia

Adipose Cell Metabolism Modulation

by Red Wine Procyanidins

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Biotecnologia

La interessada

Montserrat Pinent Armengol

A la meva família i a l'Òscar

Que el teu aliment sigui la teva única medecina

Hipócrates

Agraïments

Uf, em toca escriure la part més difícil de la tesi, els agraïments. He estat temptada de fer-los anònims, ja que si he d'escriure els noms de tothom a qui he d'agrair el seu suport durant el procés d'aquesta tesi no acabaria mai. De totes maneres em sembla just intentar donar-vos les gràcies més personalment, així que d'avançat demano disculpes i us dono també les gràcies a tots aquells que em descuidi d'esmentar a continuació.

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Nomenclature

AMPK : AMP-Activated Protein Kinase

AP-1: Activator Protein 1

aP2 (=FABP4): Adipocyte-Specific Fatty Acid Binding Protein

ASP: Acylation-Stimulating Protein

C/EBP: CCAAT/Enhancer Binding Protein

cAMP: Cyclic Adenosine Monophosphate

CAP: Cbl-Associated Protein

CDK: Cyclin Dependent Kinases

CKI: CDK Inhibitors

CREB: cAMP Responsive Element Binding Protein

DHAP: Dihydroxyacetone Phosphate

DHAPAT: Dihydroxyacetone Phosphate Acyltransferase

ERK1/2: Extracellular Signal-Regulated Protein Kinase

FABP4 (= aP2): Fatty Acid Binding Protein-4

FATP-1: Fatty Acid Transport Protein-1

FBS: Foetal Bovine Serum

FFA: Free Fatty Acids

G3PDH: Glycerol-3-phosphate Dehydrogenase

Gab-1: Grb2-Associated Binder-1

GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase

GSK3β: Glycogen Synthase Kinase-3β

GSPE, PE: Grape-Seed Procyanidin Extract

HDL: High Density Lipoprotein

HSL: Hormone-Sensitive Lipase

IBMX: Isobutyl-Methylxanthine

IFNγ: Interferon-γ

IGF-I: Insulin-Like Growth Factor-I

IL: Interleukin

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

NEFA: Non-Esterified Fatty Acid

NFκB: Nuclear Factor Kappa B

PDE3B: Phosphodiesterase-3-B

PDGF: Platelet-Derived Growth Factor

βPDGFR: β-Patelet-Derived Growth Factor Receptor

PDK-1: Phosphoinositide-Dependent Protein Kinase-1

PE, GSPE: Grape-Seed Procyanidin Extract

PEPCK: Phosphoenol Pyruvate Carboxykinase

PI3K: Phosphatidylinositol-3-Kinase

PIP2: Phosphatidylinositol-4,5-bisphosphate

PIP3: Phosphatidylinositol-trisphosphate

PKA: Protein Kinase A

PKB/Akt: Protein Kinase B

PKC: Protein Kinase C

PLK: Polo-Like Kinase

PPARγ: Peroxisome Proliferator Activated Receptor-γ

PPRE: Peroxisome Proliferator Response Element

pRb/ Rb: Retinoblastoma Protein

Pref-1: Preadipocyte Factor-1

RXR: Retinoid X Receptor

Shc: Src Homologous and Collagen-Like Protein

SHP2: Src Homology 2 Domain-Containing Tyrosine Phosphatase

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

STAT: Signal Transducers and Activators of the Transcription

STZ: Streptozotocin

TAG: Triacylglycerides

TGH: Triglyceride Hydrolase

TNFα : Tumor Necrosis Factor-α

TZD: Thiazolidinedione

VLDL: Very Low Density Lipoprotein

Index

I~ Justification and Objectives

This is the age of functional food, in which science devotes efforts to show the nutritional values of food containing factors that provides it with qualities beneficial for health. In this context, we must keep on with the study of the food in our daily intake that is rich in beneficial and protective factors. Red wine, an important component of the Mediterranean diet, can be considered a functional food that, if consumed moderately, has a protective role on human health as it can decrease the risk of suffering some pathologies. The study of the effects of wine on health was triggered by the discovery of the French paradox, i.e. the low cardiovascular mortality rate observed in Mediterranean populations in association with moderate red wine consumption and a high saturated fat intake. The flavonoids, natural phenolic compounds in red wine, are responsible, at least in part, for this protective action against coronary heart disease. This protective action of flavonoids is extended to some other metabolic anomalies that are also risk factors for cardiovascular disease such as insulin resistance, dislipemia, arterial hypertension and obesity (collectively termed the metabolic syndrome). In fact, obesity is one of the main problems in industrialized societies, even being described as the 'epidemic of the 21st C'. However, the relationship between red wine consumption and obesity remains barely studied and shows unclear results. Obesity is a major cause of insulin resistance, and red wine flavonoids are postulated to act against insulin resistance. The antioxidant properties of flavonoids are claimed to mediate these effects against insulin resistance, but direct effects on adipocytes, a target of insulin action, remain uninvestigated. Adipose tissue plays an important role in the regulation and pathological dysregulation of metabolic homeostasis: it is a key target in obesity, since it is a fat storage organ as well as an endocrine organ that secretes adipocytokines, and it is also an insulin-sensitive

tissue and consequently largely implicated in insulin resistance. Owing to this, basic research regarding the effects of red wine flavonoids on the adipocyte, a largely unexplored field at present, is of major importance to further learn and understand their potential beneficial roles in these pathologies.

Obesity is defined as an excess of white adipose tissue. Changes in adipose tissue mass may be associated with a change in adipocyte volume (hypertrophy) and /or a change in adipocyte number (hyperplasia). On the one hand, the process of lipogenesis, lipolysis, fatty acid and glucose uptake may alter adipocyte volume. On the other hand, changes in adipocyte number are governed by changes in preadipocyte maturation by adipogenesis, preadipocyte replication and cell deletion by apoptosis. Therefore, studies of the effects of red wine flavonoids on adipocytes should include research on both aspects, the fully differentiated adipocyte metabolism, and the effects on the differentiation program of preadipocytes.

Previous studies of our group addressed the effects of red wine procyanidins (a class of flavonoids) on adipocyte metabolism. Results demonstrated that a grape seed procyanidin extract (containing the main red wine flavonoids), modified the lipid metabolism by increasing lipolytic rate. Procyanidins are oligomers of catechin or epicatechin, and the study also showed that the monomeric forms did not have this lipolytic activity. As those results pointed out the adipocyte as a target organ for the catechin oligomers, it was decided to focus on the effects of a procyanidin extract on adipocytes. Therefore, the main objective of this thesis is to improve our knowledge of the action of red wine procyanidins on the adipocyte. This basic research would help to understand the protective effects of red wine flavonoids against some pathologies.

To reach the objective of this thesis, it has been divided into the following parts, with specific aims:

Study of metabolism

1. Describe intracellular mechanisms involved in the already demonstrated lipolytic effect of grape seed procyanidins.
2. Evaluate the possible hypoglycemic role of GSPE and describe the molecular mechanisms of this effect on the adipose cell.
3. Analyze the effects of grape seed procyanidins on the depot of triglycerides and glycogen.

Study of differentiation

4. Investigate whether grape seed procyanidins alter the process of differentiation of the adipose cell.
5. If so, find out molecular mechanisms through which they affect adipogenesis.

II~ Introduction

1 The adipocyte

1.1 Model of study: 3T3-L1

Given that this thesis aims to perform a screening or first approximation to the effects of procyanidins on the adipocyte, it was decided to carry it out mainly with an *in vitro* model. The model of study chosen to perform most of the experiments of this thesis was the 3T3-L1 cell line. This is a preadipocyte cell line that derives from disaggregated Swiss 3T3 mouse embrio cells (**1**). After hormonal treatment they can differentiate and acquire morphological and biochemical characteristics of adipocytes (**2-5**). This preadipocyte cell line has the advantage over primary preadipocytes in that it provides a homogenous population and can be carried in culture indefinitely. It is one of the most used cell lines for the study of white adipocytes, together with 3T3-F442A (**2, 4, 6**), and that confers an advantage for the design of experiments and the interpretation of the results, as it is highly characterized. Therefore, 3T3-L1 preadipocytes constitute a reliable and suitable model for analysing the proliferation stage, commitment of preadipocytes to adipocyte (induction of differentiation), and hypertrophy (lipid accumulation) during adipocyte differentiation (**4, 7**).

However, the extrapolation of results obtained with this cell line has to be carried out with caution, as it shows some differences with primary murine and human adipose cells, and also because it is an *in vitro* model, obviously different from the *in vivo* situation (**8**).

1.2 Brief introduction to adipose tissue

Adipose tissue is the main energy storage organ of animals. It has the ability to retain fat following food intake, and release fatty acids and glycerol into the blood during starvation. There are two phenotypes of adipose tissue: brown adipose tissue, and white adipose tissue. The former is scarcer, is rich in mithocondria, and is highly related with thermogenesis, while the white adipose tissue is abundant and widely dispersed (**9**). This work concentrates on white adipose tissue, though no specific mention of this will be made later in the paper.

Its role as energy storage starts after feeding, when fat, in form of monoacylglycerides and fatty acids, is taken up by enterocytes. Those intestinal cells re-synthesize triacylglycerides (TAG), incorporate them into chilomicrons, secrete them into the lymphatic circulation and later to the systemic blood circulation that will reach the tissues. Instead, glucose and aminoacids from the diet are brought directly to the liver (through the portal circulation), and in the liver they will be metabolised to acetyl-CoA and subsequently to fatty acids. Those fatty acids, as long-chain fatty acyl-CoA, are readily esterified to TAG and incorporated into the VLDL secretory product of liver. Both chilomicrons and VLDL transport TAG through the organism, and TAG will be hydrolysed by lipoprotein lipases in order to release fatty acids that will be taken as metabolic fuel by many tissues. In the adipose tissue, the fatty acids are re-esterified to glycerol in the formation of storage TAG. Fatty acids are also synthesized *de novo* from glucose in adipose tissue but this source, under a western human diet, contributes less to adipose TAG storage than fatty acids released from circulating lipoproteins (**10-12**).

On the other hand, during starvation adipose tissue contributes to maintaining the blood caloric homeostasis; TAG are hydrolyzed into fatty acids and glycerol, which are released into the circulation. Fatty acids are the preferred fuel during starvation for many tissues. Instead, glycerol is mainly used by the liver for gluconeogenesis, through the synthesis of glycerol-3-phosphate catalysed by glycerol kinase (**10**).

The storage pool of TAG is constantly renewed: there is a turnover and TAG are synthesized and hydrolyzed at a steady rate (**12**). At the same time as TAG are hydrolysed to produce fatty acids, adipocytes re-esterify part of these fatty acids back to TAG, leading to a recycling process (**13**). Upon nutritional or hormonal stimulation, the rate of synthesis or hydrolysis increases leading to a net increase/ decrease of the TAG pool.

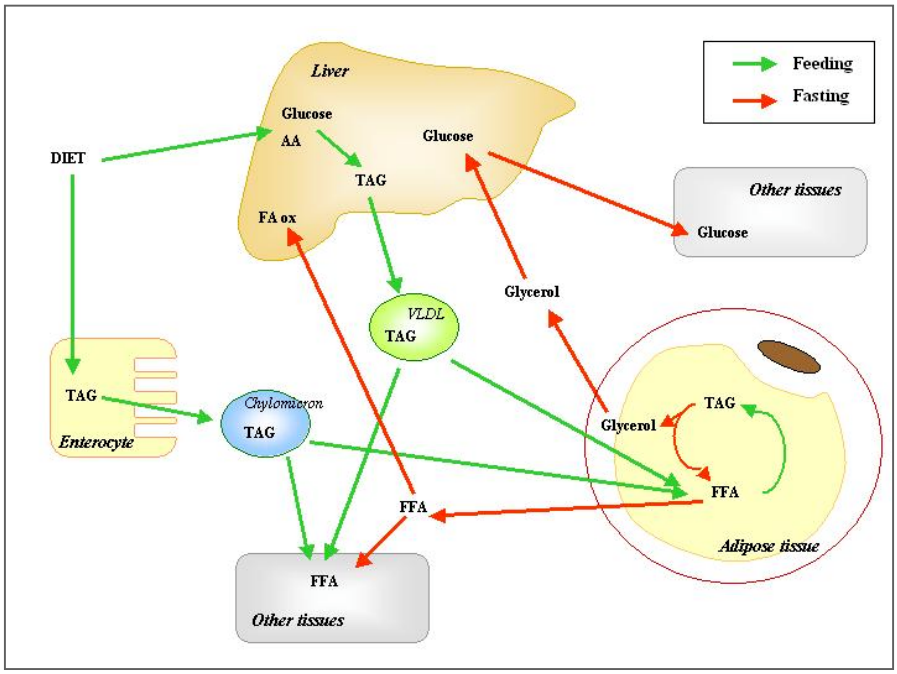

Figure 1. Metabolism of triglicerides within the organism
TAG: triacylglycerides, AA: aminoacids, FFA: free fatty acids, FA ox: fatty acids oxidation.

1.3 Regulation of fat stores

Regulation of metabolism is not only achieved through nutritional control, but it is to a great extent under hormonal control. Insulin and glucagon are the hormones that mainly control the adipose metabolism. After feeding, insulin is secreted from the β cells of the pancreas, facilitating the synthesis of TAG in adipose tissue. In adipocytes, insulin binding to its receptor provokes several anabolic responses: a short term response, in which insulin stimulates glucose uptake and induces changes in the phosphorylation state of key enzymes (e.g. it blocks activity of hormone-sensitive lipase (HSL), the enzyme that catalyzes TAG hydrolysis); and a late control, in which insulin stimulates intracellular signalling cascades which, through modulation of transcription factors, activate the synthesis of lipogenesis-related enzymes, therefore increasing TAG synthesis capacity (**10**).

Instead, glucagon is released by α-cells during starvation, and its function, initiated by binding to specific receptors, is opposite to that of insulin. Glucagon activates HSL enhancing TAG hydrolysis, and represses a set of TAG-synthesizing enzymes, consequently diminishing the flux of glucose to TAG, so glucose can be used by other tissues that require it as fuel (**14**).

Classically, adipocytes have been regarded exclusively as energy storage organs. However, following the discovery of adipocyte-secreted factors with roles in appetite regulation, glucose homeostasis, body adiposity and insulin sensitivity, a more dynamic function of adipocytes has emerged. During last decade several molecules have been identified as adipocyte-secreted, such as leptin, resistin, adiponectin, adipsin and ASP. Other proteins secreted by adipose tissue are cytokines TNFα and IL-6. Together, all those factors are called adipocytokines. Adipocytokines act in concert with the autonomic

nervous system, liver, and muscle in the coordination of energy homeostasis and fuel metabolism. Hence, the adipocyte can act in an endocrine and paracrine way and modulate its own metabolic activities and at the same time signal to other tissues to regulate their energy metabolism in accordance with the body's nutritional state (11, 15-19).

1.3.1 Catabolism (Lipolysis)

As already mentioned, a main function of adipose metabolism is to serve as energy storage, so, when energy is required a series of mechanisms is activated in order to release fuel for other tissues, through lipolysis. The control of lipolysis is complex and involves lipolytic an anti-lipolytic effectors, as well as its receptors and signalling pathways, HSL, other lipases, and proteins such as perilipins.

The lipolytic response of fat cells depends on the balance between stimulatory and inhibitory pathways. Short-term lipolysis has been largely studied. Stimulation/inhibition by several factors triggers signalling cascades that lead to the activation/inhibition of lipolysis by de/phosphorylation of key enzymes. Therefore, kinases play an important role in this sequence of signalling events. Protein kinase A (PKA) is a kinase largely involved in the activation of lipolysis (20). PKA is regulated by intracellular levels of cAMP. This molecule acts as second messenger: an increase in cAMP drives the cell to lipolysis, while a reduction in cAMP levels inhibits TAG hydrolysis. Regulation of cAMP is carried out both through the activation/inhibition of adenylyl cyclase, the enzyme that catalyses the synthesis of cAMP, or by the regulation of phosphodiesterase-3-B (PDE3B), the enzyme that controls cAMP

degradation. Among the factors that regulate lipolysis through adenylyl cyclase control, the best characterized are catecholamines. Adrenalin and noradrenalin are adrenergic agonists that acutely regulate lipolysis through binding to β-adrenergic receptors. There are several adrenergic receptors: the β-adrenoreceptors (including 4 subtypes), the α1-adrenoreceptors and the α2-adrenoreceptors (each one including 3 subtypes), all members of the superfamily of G-protein-coupled receptors (21, 22). Differences between adrenergic receptors include the different affinity of each receptor for ligand, the variable receptor composition between species, and the different resistance to desensitisation. G-proteins mediate the transduction of signals from hormones to adenylyl cyclase: β-receptors are coupled to G_s-proteins, which activate adenylyl cyclase; instead, adenylyl cyclase in negatively regulated by G_i-proteins, to which α2–receptors are coupled, providing a dual regulation of cyclase activity. Adenosin is another acute regulator of lipolysis, by binding to the A_1 adenosine receptor. This receptor is coupled to Gi and therefore inhibits adenyl cyclase activity. Among the agents able to stimulate phosphodiesterase-3-B, and therefore reduce PKA activity and lipolysis, is insulin, the physiologically important antilipolytic hormone. The insulin signalling pathway leading to the activation of phosphodiesterase-3-B involves the insulin receptor, insulin receptor substrates, phosphatidyl inositol-3-kinase and probably protein kinase B (PKB/Akt) (20, 23, 24).

PKA phosphorilates a variety of intracellular substrates, among them HSL, the rate-limiting enzyme of lipolysis: HSL catalyses the hydrolyzation of TAG to diacylglycerides and monoacylglycerides (25-27). Then, monoacylglycerides are hydrolysed to fatty acids and glycerol through monoacyl-lipase.

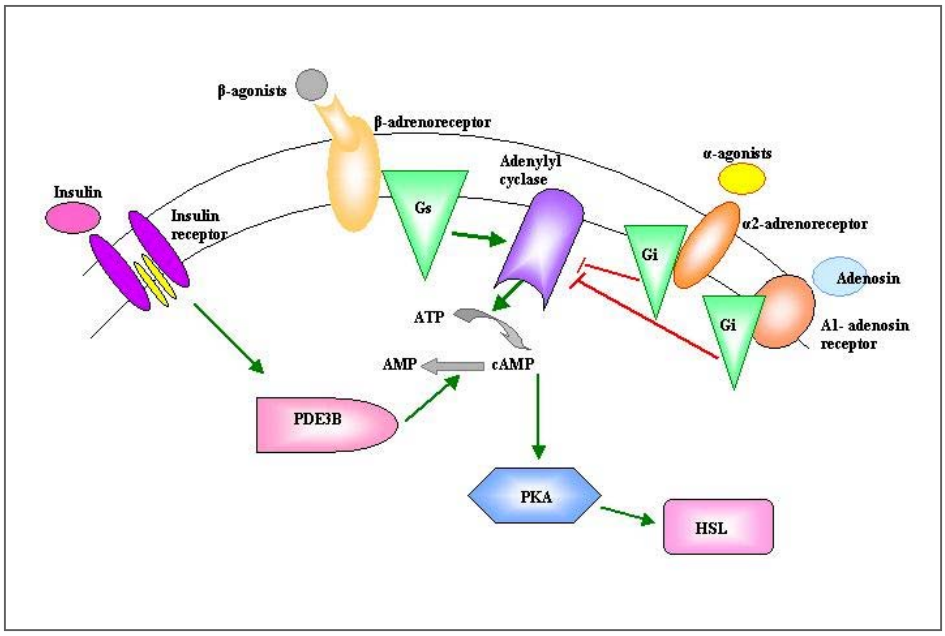

Figure 2. Short-term hormonal stimulation of lipolysis
PKA: protein kinase A, HSL: hormone sensitive lipase, PDE3B, phosphodiesterase 3B
Adapted from Carey (1998), *Adv Exp Med Biol* 441: 157-170

Other PKA phosphorilation targets are perilipins. Perilipins are proteins located on the surface of the lipid droplets, where they seem to act as supressors of basal lipolysis and as a necessary component for full lipolytic stimulation to occur (25, 28). Recent discoveries not only document HSL phosphorilation, but also its translocation from the cytosol to the lipid droplets as being necessary for lipolysis to occur (29). Perilipins are required for PKA-mediated translocation of HSL (30) and phosphorylation of perilipins is necessary to allow HSL to interact with the lipid droplet (26). Other proteins are likely to play a role in the phenomenon of HSL translocation. For instance,

HSL has been shown to bind fatty acid binding protein-4 (FABP4) leading to a modest increase in HSL activity: this stimulation is fatty acid dependent and may be related to the ability of FABP4 to bind fatty acids preventing the feedback HSL inhibition by its products (25).

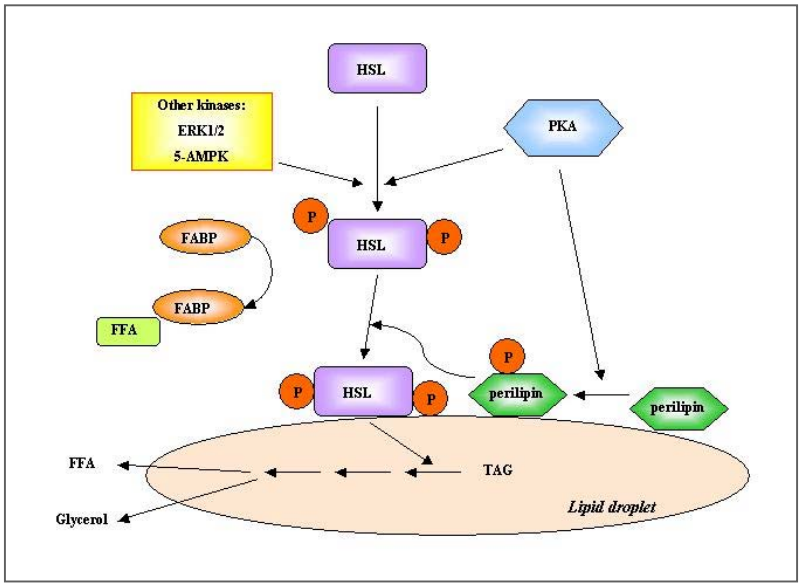

Figure 3. Regulation of Hormone Sensitive Lipase (HSL) activity
PKA: protein kinase A, FABP: fatty acid binding protein, FFA: free fatty acids, TAG: triacylglycerides.
Adapted from Holm (2003*), Biochem Soc Trans* 31: 1120-1124

Further studies regarding the control of lipolysis seem to give evidence of additional non-HSL lipases that also regulate TAG hydrolysis. One such lipase generally known as triglyceride hydrolase (TGH) was cloned, and despite its role in adipocytes is not very well described, current studies suggest that it has a role in basal lipolysis. This non HSL-induced lipolysis is also regulated through PKA (31, 32).

Regarding signal transduction of lipolytic signals, some theories support that not only PKA but additional complementary pathways stimulate lipolysis. Early evidence was the observation of discrepancies between PKA-activated HSL and maximal lipolytic rate, already suggesting the existence of PKA-independent lipolysis (23, 24). Former discoveries regarding this phenomenon are that catecholamines have been suggested to activate not only PKA but ERK1/2 MAPK pathways via β-adrenergic receptors: β_3AR is simultaneously coupled to Gs and Gi, with the consequent activation of the PKA and the MAPK pathways respectively (33), both pathways probably acting in concert to activate lipolysis (26). Actually, several different kinases other than PKA (ERK MAPK, glycogen synthase kinase-4, Ca2+/calmodulin-dependent protein kinase II and AMP-activated protein kinase (AMPK)) phosphorylate HSL at unique serines within the regulatory module and some might modulate HSL activity (26, 27). As MAPK pathways regulate, among others, several transcription factors that can be involved in lipid metabolism (eg. ERK phopshorilates the lipogenic peroxisome proliferator activated receptor-γ (PPARγ), inhibiting its transcriptional activity (34)), this still unexplored net regulating lipolysis could link short term lipolytic control with the highly unknown long term lipolytic regulation. In fact, several factors have been shown to modify long-term lipolysis, despite the mechanisms that they use not being fully described. cAMP, phorbol esthers and dexamethasone modify HSL expression (23). TNFα is one of the most well described long-term lipolytic factors discovered so far. It is a cytokine that can be secreted by adipocytes. Despite the number of studies regarding the mechanism of TNFα to exert its lipolytic effect, this is not still fully elucidated (35-39). Results obtained so far suggest that mechanisms used by TNFα may vary among species. In 3T3-L1,

different mechanisms have been postulated as being used by TNFα to stimulate lipolysis, as is the down-regulation of perilipins via ERK MAPK (40), or the down-regulation of phosphodiesterase-3-B and the subsequent increase in cAMP and the activation of PKA (41). Also long-term exposure of insulin has attracted interest as a lipolytic regulator; actually, results found were quite surprising due to the fact that insulin, an acute inhibitor of lipolysis, increased lipolytic rate when added for long periods (39, 42, 43). The mechanisms used by insulin are still unknown; in primary cell culture insulin treatment was demonstrated to increase basal and maximal lipolysis when combined with high glucose levels, while HSL protein levels were found to have decreased (42); in human adipocytes chronic insulin treatment stimulates lipolysis, lipoprotein lipase expression and TNFα release (43). In 3T3-L1, at the same time as it induced lipolysis, insulin increased the re-esterification rate of fatty acids, which, as a consequence were not released to the medium (39). Finally, it has also been suggested that insulin could act through an alternative lipase, non-HSL (39).

1.3.2 Anabolism

As an energy storage cell, adipocyte has the ability to maintain TAG as lipid droplets. Those TAG stored derive from the already mentioned intake of TAG from lipoproteins, as well as newly synthezised TAG. To synthesize TAG requires the uptake of glucose that will be transformed into fatty acids (lipogenesis) and the uptake of fatty acids. It is worth noting the difference between the TAG synthesis that takes place within the fully differentiated adipocytes, as described above, and adipogenesis, which refers to the process of

differentiation in which precursors acquire adipose morphology, also involving lipid synthesis.

A key step for the synthesis of storage reserves is glucose uptake, which is the main rate-limiting step for glucose metabolism. The key regulator of this process is insulin because adipose tissue is insulin-sensitive. Insulin acts through binding to its receptor: the insulin receptor is a transmembrane receptor with a cytosolic domain that has intrinsic tyrosine protein kinase activity. Following insulin binding to the external alpha chains, it suffers a conformational change in the cytosolic beta chains that activates autophosphorylation. This modification inititiates a network of complex signal transduction pathways which culminate in diverse cell-specific functions (**44**). There are several substrates for their kinase, among which the insulin receptor substrate (IRS) isoforms are found (**44**). The IRS proteins bind to the insulin receptor and become activated. IRS-1 is involved in insulin-induced glucose transport by binding to phosphatidylinositol-3-kinase (PI3K). PI3K enzyme is a nodal point in several signal pathways depending on cell type. On the insulin transduction pathway, it catalyses the phosphorylation of PIP_2 to PIP_3, that acts as a second messenger stimulating the following steps: activation of phosphoinositide-dependent protein kinase-1 (PDK-1) and protein kinase B (PKB/Akt). Final events of this signal transduction cascade are responsible for the activation of glucose uptake, such as the translocation of glucose transporter GLUT4 to the plasma membrane, and for other metabolic effects of insulin (**44, 45**). GLUT4 is a highly insulin-sensitive member of a family of glucose transporters, expressed in adipocytes and muscle cells. They are found in specialized intracellular vesicles, and upon insulin activation they translocate to the plasma membrane where they will exert their function

allowing glucose to enter the cell. In addition to PI3K activity, other signals seem to be required for GLUT4 translocation. This PI3K-independent pathway may involve remodelling of the cortical cytoskeleton, through the adapter protein CAP and G protein TC10 (**45**). GLUT4 translocation is necessary for insulin-stimulated glucose uptake. However there are several studies suggesting that also the regulation of GLUT4 activity is required for glucose transport. Those studies are based on the observation that GLUT4 recruitment to the cell surface in response to acute insulin stimulation is less than the extent of the increase in glucose uptake. This third possible pathway involved in GLUT4 activation and insulin-stimulated glucose uptake have been suggested to require p38 MAPK (**46, 47**); nevertheless, this p38 MAPK function is not fully accepted and there are controversial opinions regarding it (**48**).

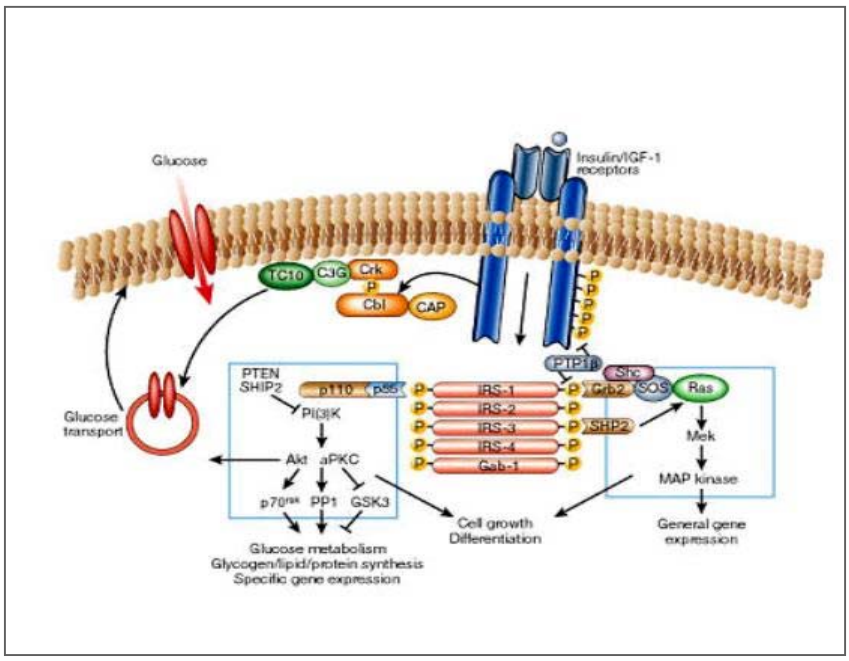

Figure 4. Insulin signalling pathways
From Saltiel (2001), *Nature* 414: 799-806

At the same time as insulin stimulates glucose entry to the adipocyte, the hormone activates compartmentalized enzymes to promote the storage of the incoming glucose as either glycogen, triglyceride or protein (**49**). In adipocytes, glucose uptake drives glucose mainly to TAG synthesis through the activation of lipogenesis and the inhibition of lipolysis. Insulin activates lipid synthetic enzymes, including pyruvate dehydrogenase, fatty acid synthase and acetyl-CoA carboxilase. The inhibition of lipolysis is mediated through the activation of phosphodiesterase-3-B, that causes a reduction in cAMP levels and consequently inhibits HSL. Insulin stimulation of glycogen synthesis is carried out through the activation of the enzyme glycogen synthase, the rate-limiting enzyme in glycogen synthesis. Glycogen synthase is activated by dephosphorylation, and can be regulated at several levels: activation of protein-phosphatase-1; inactivation of glycogen synthase kinase-3β (GSK3β); allosteric activation by glucose-6-phosphate; or translocation of cytosolic glycogen synthase to glycogen-containing fractions. The pathway used by insulin to activate glycogen synthase may vary within cell types (**50**). In 3T3-L1, the requirement of GSK3β inhibition remains controversial. Some authors support its role in insulin stimulated glycogen synthase activation (**51**), at least in part via PI3K and PKB/Akt (**52**); others instead demonstrate that the PI3K-PKB-GSK3β signalling pathway is not sufficient for insulin-stimulated glycogen synthesis (**53-55**) as GSK3β levels decrease during differentiation (**56**) and in mature adipocytes it is barely expressed (**54, 55**). Other major signalling pathways may exist in 3T3-L1 adipocytes. Alternative mechanisms used by insulin in 3T3-L1 to activate glycogen synthase is the activation of protein-phosphatase-1 (activated by phophorylation of its G subunit) via PI3K, and independent of PKB/Akt (**55**). Also the translocation of cytosolic glycogen

synthase to denser glycogen containing fractions may play an important role in the regulation of glycogen synthase activity in this cell type (**57, 58**).

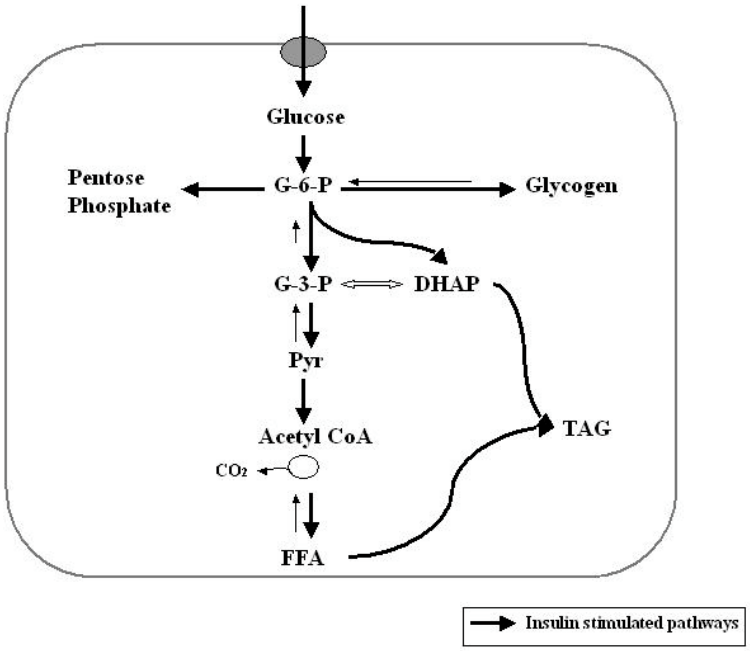

Figure 5. Overview of the major metabolic pathways of glucose in adipose cells
G-6-P: glucose-6-phosphate, G-3-P: glyceraldehyde-3-phosphate, Pyr: pyruvate, DHAP: dihydroxyacetone phosphate, TAG: triacylglycerides, FFA: free fatty acids.

Insulin effects are not only mediated by regulating the activity of enzymes involved in glucose and lipid metabolism, but also controlling the gene expression of proteins involved in such metabolism. Control of gene expression involves the already described PI3K/PKB pathway, and another transduction pathway, mediated by Raf-1, MEK and ERK MAPK (10, 44). The Raf-1/MEK/MAPK pathway, also initiated by the binding of insulin to its receptor, is activated through different proteins. Firstly some insulin receptor

substrates are recruited, such as IRS and Shc, which, after tyrosine phosphorilation act as 'docking sites' for other proteins (Gab-1, SHP2) that lead to the activation of the Ras/MAPK signalling pathway. This sequence of events terminates in the nucleus with the induction or repression of a set of genes involved in late control of metabolic pathways (10, 44).

One of the transcription factors involved in insulin regulation of gene expression is sterol-regulatory element-binding protein-1c (SREBP1c). In 3T3-L1 adipocytes, insulin induces SREBP1c via PI3K and PKB/Akt (**59**). SREBP1c is a member of the family of SREBP transcription factors. It plays a role in activating insulin-responsive elements in promoter regions of insulin responsive genes. Upon insulin activation, SREBP1c induces the expression of several lipogenic genes involved in fatty acid and TAG synthesis (**60-62**). In liver the role of SREBP1c as mediator of insulin's induction of lipogenic enzymes is widely accepted; but its role in 3T3-L1 adipocytes, supported by some studies (**59, 61**), is not well established (**63**). On the contrary, SREBP1c in adipocytes has a clear role as a participant in adipose differentiation (**7, 64**).

1.4 Adipose differentiation

Differentiation is the name given to the process in which precursor cells, named preadipocytes, become adipocytes while suffering changes at molecular and morphological levels. Fully differentiated adipocytes do not undergo cell division and lose their ability to propagate, therefore any increase in adipocyte number reflects the process of differentiation of preadipocytes into mature adipocytes (**6**).

Adipose cells derive from mesenchymal precursor cells with the potential to differentiate along mesodermal lineages (such as myoblasts, chondroblasts, osteoblasts and adipocytes) (65). Preadipocytes represent a stage of adipocyte development in which multipotent precursors have been determined to adipocyte lineage (65). Therefore immortal preadipose cell lines (including 3T3-L1) are already determined to adipocyte lineage. Preadipocytes maintain the capacity for growth, but following commitment to differentiate, they have to withdraw cell cycle before adipose conversion (64, 65).

Preadipose cell lines can commit to differentiate into adipocytes by treatment with adipose inducers (66, 67). During this process, adipocytes suffer molecular processes, intracellular transduction-signalling pathways are activated, and the gene expression profile is highly changed (68-70). Subsequently proteins and enzymes are activated to allow the differentiation to progress. Morphological changes are also suffered during this process as adipocytes adopt a spherical shape, changing from their fibroblastic precursor and they acquire adipose phenotype, characterized by lipid droplet accumulation (2, 4).

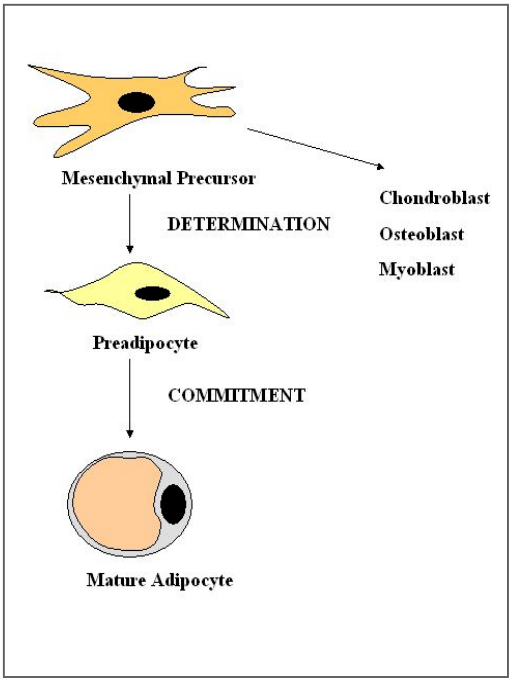

Figure 6. The developmental stages from precursors to mature adipocytes
Adapted from Ntambi (2000), *J Nutr* 130: 3122S-3126S

1.4.1 Induction of adipose differentiation

The ordered and coordinated expression of cell-specific proteins during differentiation is believed to reflect the activation of networks of functionally related genes. The temporal and functional integration of such gene networks can be controlled by a limited number of hormones, growth factors and differentiating factors acting in a combinatorial manner. There are various adipogenic inducers able to activate the differentiation of preadipocytes. The most common is a hormonal cocktail that includes insulin, glucocorticoids (dexamethasone) and cAMP generating agents (IBMX) (71). The exact metabolic pathways used by those agents to induce differentiation remain obscure. Insulin induces differentiation through binding to insulin-like growth factor-I (IGF-I) receptor (72, 73); the subsequent activation cascade may involve IRS-1 and IRS-2 (74), and diverse intracellular signalling pathways have also been related with insulin's induction of differentiation, such as pathways involving PI3K (**75**), PKB/Akt (76), Shc, Ras-MAPK (**10**), and CREB (77). The mechanisms of action of dexamethasone have been postulated to act by inhibiting the transcription of preadipocyte factor-1 (Pref-1), a differentiation inhibitor (78). IBMX, on the other hand, might trigger differentiation because of its ability to increase cAMP.

There are other agents shown to be able to induce differentiation. For instance, thiazolidinediones (79) are used to trigger the differentiation of preadipocytes. Not only do those hormonal factors serve as inducers of differentiation, but foetal bovine serum is widely used in differentiating mediums, suggesting that there are other still undefined factors, such as growth factors, that might have a role in 3T3-L1 differentiation (80).

Following hormonal induction, arrested preadipocytes are stimulated to reenter cell cycle, then arrest proliferation again and undergo terminal adipose differentiation.

1.4.2 Mitotic clonal expansion

Post-confluent 3T3-L1 preadipocytes, when induced to differentiate, undergo one or two rounds of DNA replication and cell doubling (an event known as mitotic clonal expansion). This requirement for the cell to reenter cell cycle has been long questioned (81). In fact, in other cell types mitotic clonal expansion is not a requisite for differentiation (82, 83), but recent works support the hypothesis of a cell cycle reentry which is prior to and indispensable for differentiation of 3T3-L1 (**4, 7, 84, 85**).

Cell cycle refers to the process by which a cell divides into two identical daughters. It is divided into four phases: S (DNA Synthesis to duplicate genetic material), M (Mitosis, cell division), G1 and G2 (Gaps) (86). Cells lacking proliferative signals, cells under antimitogenic signals or at a terminally differentiated stage withdraw from the cell cycle at the early G1 into a resting stage termed G0 (86). Post confluent cells are then at the G0 stage (87). After induction of quiescent cells, a coordinated activation/inhibition of several factors, including enzymes, protein complexes, transcription factors and chromatin reorganization, leads the cells through the cell cycle progression. Cell proliferation is also regulated by distinct machineries called “cell-cycle checkpoints” that ensure the proper progression of the cell cycle (88).

Key proteins in the transition through the distinct phases of the cell cycle are cyclins and cyclin dependent kinases (CDKs). CDKs are Serine/Threonine kinases that need to be activated by binding to cyclin partners. Cyclin-CDKs work in conjunction controlling stage-dependent functions, they are activated sequentially through the different steps of the cell cycle and their regulation is determined by gene transcription and targeted destruction (89). CDKs are also regulated by the binding of CDK inhibitors (CKIs). CKIs represent a superfamily of negative regulators that help to ensure temporary arrest or permanent withdrawal from the cell cycle in response to anti-mitogenic or genotoxic stimuli, mitogen deprivation or as parts of programmes of terminal differentiation (89, 90).

In the G1 phase, the retinoblastoma protein (pRb), a key piece in the regulation of cell cycle, remains unphosphorilated and bound to E2F (a term that includes a family of related transcription factors) preventing its transcriptional activity (91, 92). E2F target genes are related to DNA replication and cell division, necessary for progression through cell cycle, and for cell differentiation (93, 94). Therefore, pRb acts as general repressor of cell proliferation. In the absence of mitogenic signals or in the presence of anti-proliferative signals, cell-cycle progression is prevented by two families of CKIs: the INK4 (inhibitors of CDK4); and the Cip/Kip family of inhibitors ($p21^{Cip1}$, $p27^{Kip1}$and $p57^{Kip2}$). The latter have a broader set of targets, as they inhibit activities of various cyclin-dependent kinases serving in all the four cell-cycle phases (95). Upon proliferative stimuli, CKI levels and activity change, Cyclin D accumulates and in conjunction with CDK4/6 phosphorylate pRB, which releases E2F. Free E2F activates promoters for several cyclins and other factors (e.g. Cyclin E and Cyclin A) (93) . And E2F

is feedback-regulated by complexes of cyclins and CDKs. CyclinE/CDK2 facilitates progression to the S phase, then Cyclin A starts to accumulate. Finally, entry into mitosis is induced by the increased activity of Cyclin B/CDK1 (86).

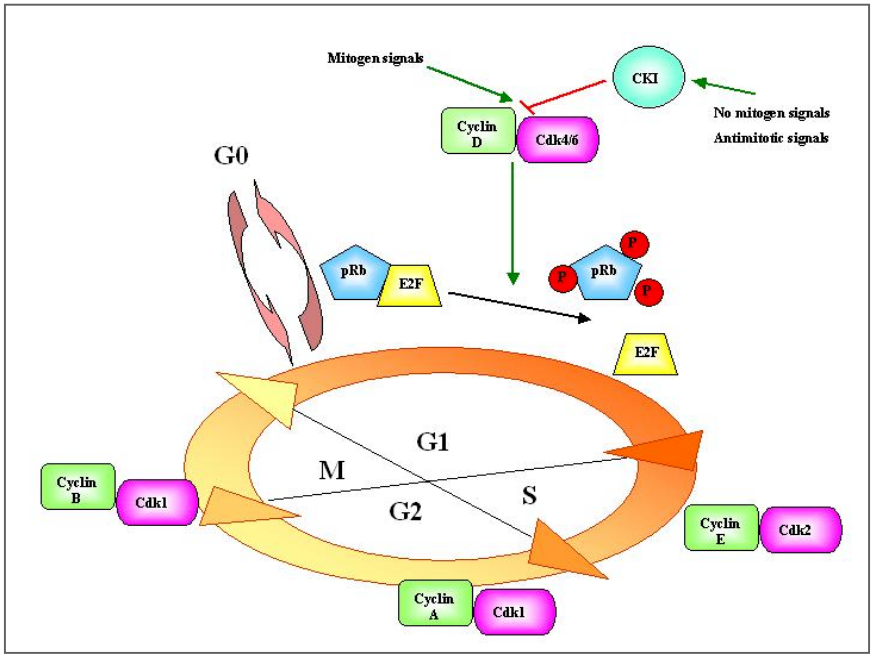

Figure 7. The cell cycle
Adapted from Tessema (2004), *Virchows Arch* 444, 313-323

Cell proliferation precedes differentiation, but a cross talk might exist between the factors that control cell cycle and the regulators of differentiation (96). The relationship between the mechanisms controlling both processes remains unclear, but numerous works highlight the participation of cell cycle-related factors in differentiation (92, 96); for instance, E2F and Cyclin D modulate the expression of the adipogenic nuclear receptor PPARγ (93, 97, 98) and pRb regulates C/EBPβ-DNA binding activity (99). Further study of these discoveries would be able to link

the requirement of mitotic clonal expansion for 3T3-L1 differentiation.

1.4.3 Regulation of differentiation

Adipose differentiation progresses as a consequence of a cascade of genetic events in which sequential activation of a set of transcription factors induces transcription of its target genes, and drives the process until the acquisition of the adipogenic profile. By the time the cell has reentered cell cycle, adipogenic transcription factors start to be expressed; afterwards they acquire transcriptional activity and subsequently activate other factors with the ability not only to induce adipose physiology but also to arrest cells acting as antimitogenic agents (**64, 85, 90, 100**), linking cell proliferation and differentiation. Among the transcription factors that regulate adipogenesis, PPARγ, the CCAAT/enhancer binding protein (C/EBP) family of transcription factors and SREBP1c play a central role (**7, 64**). C/EBPβ and C/EBPδ are members of the C/EBP family of trancription factors, and they are expressed immediately after the induction of 3T3-L1 differentiation (101). At this point, C/EBPβ/δ have been demonstrated to lack DNA-binding activity and remain inactive (102). About 24 h. later, C/EBPβ undergoes phosphorilation and gains DNA-binding activity starting its transcriptional activity (85). C/EBPβ/δ induce the expression of C/EBPα and PPARγ (**64**). Both C/EBPα and PPARγ transcription factors are also regulated by each other in a feedback loop (103), and show antimitotic properties (85, 90, 100, 104). By day 2/3 C/EBPα and PPARγ have acquired DNA-binding activity and they co-ordinately induce the expression of a large group of adipocyte

genes that lead to the acquisition of adipocyte characteristics (**7, 64, 85**). Late events of differentiation are the induction of genes involved in lipid metabolism (**69**), and therefore an increase in lipogenesis, and the acquisition of insulin sensitivity (**65, 103, 105**); terminal differentiation is reached after 6 days post induction of differentiation.

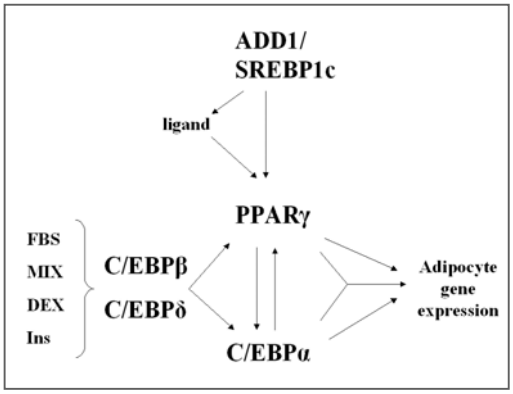

Figure 8-A. Sequential events during adipocyte differentiation.
Adapted from Tong (2001) *Rev Endocr Metab Disord* 2: 349-355

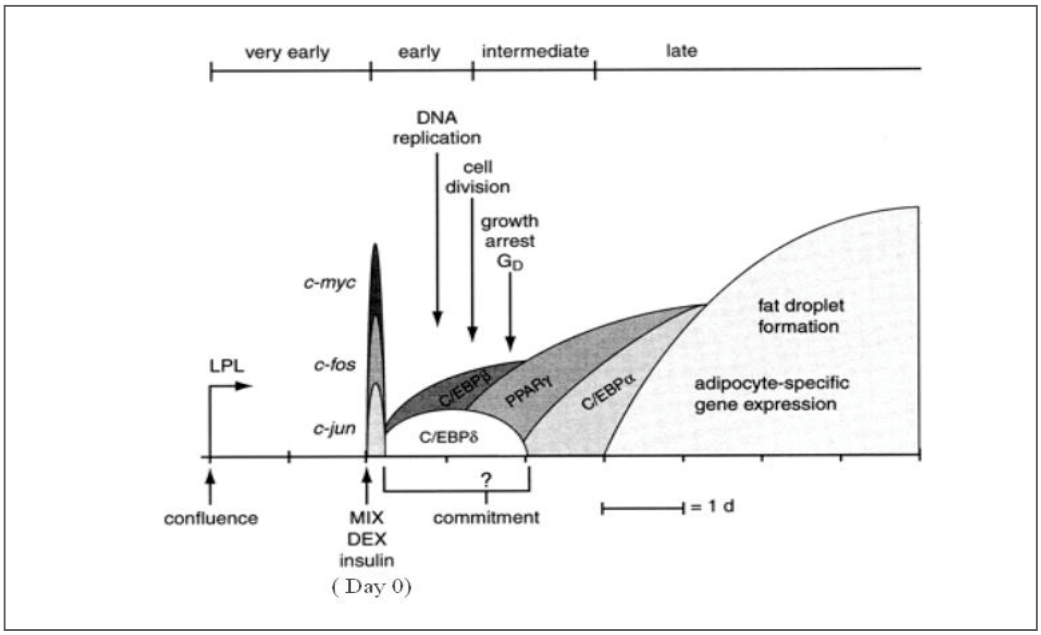

Figure 8-B. Progression of 3T3-L1 preadipocyte differentiation.
FBS: fetal bovine serum, MIX: methylisobutylxanthine, DEX: dexamethasone, Ins: insulin, C/EBP: CCAAT/enhancer binding protein, PPAR: peroxisome proliferator-activated receptor, ADD1/SREBP1c: sterol-regulatory element-binding protein-1c. LPL: lipoprotein lipase.
Adapted from Ntambi (2000),*J Nutr* 130: 3122S-3126S

The complex cascade of events that control adipogenesis includes regulation through signals that repress adipocyte development. Pref-1 is an inhibitor of differentiation; it is highly expressed in preadipocytes, and its mRNA levels decrease during differentiation (106). Therefore, it is an ideal preadipose marker. Finally, several cytokines are inhibitors of adipose differentiation *in vitro*, probably acting through their repective receptors (**65, 107, 108**). TNFα inhibits adipogenesis in part through the activation of the ERK pathway, and blocking the induction of PPARγ and C/EBPα. IL-1, IL-6, IL-11 and IFN-γ activate members of the signal transducers and activators of the transcription (STAT) family of transcription factors, that might have a role in regulating adipogenesis (107). Being adipocyte-secreted factors, these cytokines may have a role as local signals reflecting the state of the adipocyte and its triglyceryde store.

1.4.2.1 PPARγ

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily. To date, three isoforms have been identified, encoded by separate genes: PPARα, PPARδ (also known as PPARβ) and PPARγ. The different isoforms have different tissue distribution and biological activities (109, 110). PPARγ is expressed as three isoforms; PPARγ1 and 3 encode the same protein product, whereas PPARγ2 differs due to the addition of 28 amino acids at the PPARγ2 N-terminus (100). Both PPARγ isoforms are most highly expressed in adipose tissue (PPARγ2 is specific of this tissue). The PPARs are ligand-dependent transcription factors that regulate target gene expression by binding to DNA at specific

peroxisome proliferator response elements (PPREs) in enhancer sites of regulated genes. Each receptor binds to its PPRE as a heterodimer with a retinoid X receptor (RXR) (109, 111). The activity of the heterodimer is negatively regulated by co-repressors. Upon binding a ligand, the co-repressors are released and following conformational changes, the heterodimer recruits co-activators (112). The result is an increase in gene transcription. Genes regulated by PPARγ are fatty acid-binding-protein 4 (FABP4/ aP2), phosphoenol pyruvate carboxykinase (PEPCK), acyl-CoA synthase, lipoprotein lipase, and fatty acid transporters such as FATP-1 and CD36 (113-115). Natural PPARγ ligands include several prostanoids such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and 15-hydroxy-eicosatetrenoic acid, metabolites of arachidonic acid (116). Certain natural fatty acids, such as linoleic acids, can also bind PPARγ at low affinity. The pharmacological ligands are the thiazolidinediones, a class of oral antidiabetic agents. The regulation of PPARγ by its ligands remains controversial. On the one hand, the low affinity of the natural ligands described so far suggests that there is no high affinity natural ligand, so instead of responding to one individual metabolite, PPARγ acts as a response to the total sum of lipid status (**7**). On the other hand, it has been suggested that the synthesis of ligands for PPARγ is regulated by the action of transcriptional factors. Transcription factor SREBP1c, one of the main controllers of adipose differentiation, has been postulated to have a role regulating adipogenesis through mediating the production of an endogenous PPARγ ligand, as well as directly modulating PPARγ expression by binding to its promoter (117). Moreover, PPAR transcriptional activities are also regulated through post-translational

modifications, including phosphorilation (via ERK and JNK MAPK) and ubiquitination (**34, 111**).

After many studies, the role of PPARγ as a key controller of differentiation has been established. However, its role in fully differentiated adipocytes remains unclear. As PPARγ has been identified as the receptor for thiazolidinediones, and thiazolidinediones are antidiabetic, PPARγ has been claimed to have a role in maintaining insulin sensitivity, most likely by regulating the expression of genes that encode components of the insulin signalling pathway (114). Also PPARγ regulation of adipocyte-secreted factors, such as adiponectin, has suggested its role in regulating insulin sensitivity (110, 118, 119). Finally, PPARγ might function in the maintenance of the expression of genes whose products play a role in lipid metabolism, in particular those that encode proteins that function in fatty acid uptake (lipoprotein lipase) and intracellular storage of triglycerides (FABP4/aP2, acyl-Coa synthase, PEPCK), thus stimulating the storage of fatty acids in mature adipocytes (110). This reduction in circulating fatty acids could contribute to an improvement in overall insulin sensitivity in mature adipocytes (118, 120).

2 Procyanidins

2.1 Brief introduction

Procyanidins (also called proanthocyanidins) are a class of flavonoids. Flavonoids are natural phenolic compounds ubiquitous to green plant cells, where they function as plant pigments. A variety of classes of flavonoids are distributed into fruit, vegetables, and manufactured beverages. The type and quantity of flavonoids among plants vary enormously, and some food and beverages are specially richer in a particular flavonoid (121). Procyanidins are the main flavonoids in grapes (where they are located in the seeds and skins) and red wine (that contains the same structures as grape seeds (122)) (123-125). The importance of procyanidins for humans derives from the fact that they are consumed in considerable amounts, because they are found in fruits and vegetables, and a major source of such polyphenol intake is beverages, such as red wine, green tea or chocolate (123, 126). In Mediterranean countries, red wine, which is part of the usual diet, contributes much of the daily intake of flavonoids (127, 128).

The average intake of flavonoids by humans has not yet been well defined given the enormous variety of these compounds, and the difficulties for their reliable characterization and quantification (128); values estimated vary from 2,6 mg/day on a Nordic diet (121) up to 1-2 g per day (129). But those studies were mainly focused on a limited number of flavonoids, mainly monomers, and only a relatively small number of foods were used to make an estimation (128, 130).

Table 1. Polyphenol content of food (mg)
Adapted from Scalbert (2000). *J Nutr* 130: 2073S-2085S

		Flavonoids					Total phenols	
			Flavanols					
Foodstuff (quantity)	**Phenolic acids**	**Flavonols**	**Catechin monomers**	**Procyanidins**	**Flavanones**	**Anthocyanidins**	**Chromatography**	**Folin assay[3]**
Vegetables								
Potato (200g)	28						28	57
Tomato (100g)	8	0.5					8	37
Lettuce (100g)	8	1					9	23
Onion (20g)		7					7	18
Fruits								
Apple (200g)	11	7	21	200[1]			239	440
Cherry (50g)	37	1	3	35		200	276	
Other foods								
Wheat bran (10g)	50						50	
Dark chocolate (20g)			16	86[1]			102	168
Beverages					22		22	75
Orange juice (100ml)								
Coffee (200ml)	150						150	179
Black tea (200ml)		8	130				138	200
Red wine (125ml)	12	2	34	45[2]		4	97	225

[1]Oligomers up to decamers
[2]Oligomers up to trimers
[3]Estimated by the Folin-Ciocalteu colorimetric assay as catechin or gallic acid equivalents. Values are in excess for samples containing ascorbic acid

Another major problem to estimate the effective doses of flavonoids on the human organism, is the scarce knowledge of their bioavailability and metabolism (121, 130-132). Studies are currently under way to identify the forms of flavonoids effectively absorbed by humans: whether they are glucosides or not, if polymeric forms are also absorbed, the mechanism(s) involved in their absorption, and the metabolism in humans suffered by those factors (121, 128, 133). Nevertheless, research into the properties of procyanidins is of great importance since their daily consumption has been related to many beneficial properties that make them worth studying.

Flavonoids include molecules that share a common structure of three phenolic rings; however, they can vary in the ring strucure of the aglycone (basic structure), state of oxidation/reduction, degree of hydroxylation, and derivatization of the hydroxyl groups (metilation, glycosilation…); therefore among flavonoids, thousands of different structures are included and are further categorized by subgroups according to their chemical structure (121, 134). The main classes of flavonoids are anthocyanidins, flavonols, flavones, flavanones, isoflavonoids and flavanols. Procyanidins are flavanols, also named flavan-3-ols or catechins. The structure of procyanidins derives from the 3 phenolic ring-basic structure of flavonoids, thus having a benzene ring (A), condensed with a heterocyclic pyran (C) that carries a phenyl benzene ring (B). Procyanidins are highly hydroxylated polymers, comprised of the monomeric structure flavanol (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate. Procyanidins include from dimers to oligomers up to 10 units; further polymerisated structures are classified as condensed tannins (121, 135).

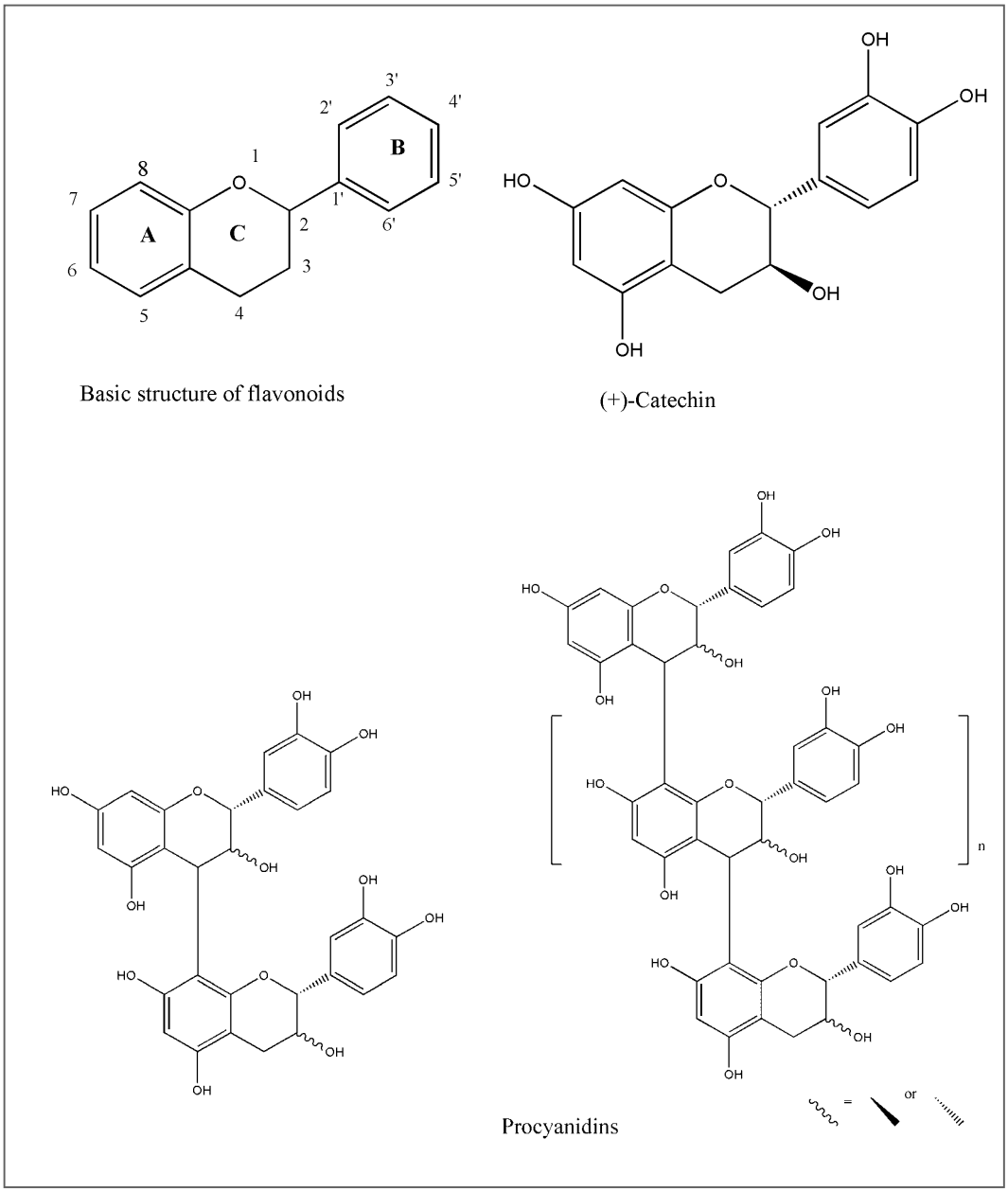

Figure 9. Chemical structure of flavonoids

The basic structure of procyanidins makes them highly susceptible to oxidation (136, 137). Its oxidation is catalysed by heavy metals and light. Actually, the main research concerning flavonoids and, concretely, procyanidins, has been focused on their properties as antioxidants, and many

of their beneficial effects have been attributed to this capacity (130). They are great free radical scavengers (138), and this makes them protective against oxidative stress and free radical-induced damage in membranes and nucleic acids (139, 140). But flavonoids are extensively metabolised *in vivo*, thus suffering alterations in their redox potentials. Besides, flavonoids are found in the organism in relatively small concentrations compared with those of other antioxidants (e.g. ascorbic acid, α-tocopherol). The structure of flavonoids favours their potential to bind to the ATP-binding site of a large number of proteins, including several kinases. Together this evidences suggest that flavonoids in the organism may act not only as antioxidants but by their interactions with specific proteins central to intracellular signalling cascades, specially modulation of protein and lipid kinase signalling cascades (141). Flavonoids can inhibit or stimulate these pathways and produce great changes in cellular function, by altering the phoshorilation state of target molecules and /or by modulating gene expression (141).

Red-wine procyanidins have a high affinity for proteins (142, 143). They also modulate gene expression and interfere with important signal transduction factors (144-146). Therefore, the beneficial effects of procyanidins may be as a result of their radical scavenging properties, their direct interaction with enzymes that are members of signalling pathways, their indirect modification of gene expression or a combination of all these factors.

Among the beneficial effects of procyanidins, they act as cardioprotective (147), antioxidants (136, 139), antigenotoxics (148), anti-inflamatories (149) and anticarcenogenics (150).

2.2 Effects of red wine procyanidins on lipid or glucose related anomalies.

One of the most studied effects of red wine regarding health is its role as a protector against coronary heart disease. Red wine was claimed to explain the French Paradox that emerged from the observation that despite the general correlation of mortality by coronary heart disease and saturated fat intake, France, a red wine consuming country, had a low death rate due to heart disease in relation to the high fat consumption (151, 152). Epidemiological studies that demonstrate the beneficial effects of moderate wine consumption are supported by many *in vivo* and *in vitro* studies (151, 153, 154). At least part of this protective effect against coronary heart disease was attributed to the procyanidins that red wine contains (139, 155).

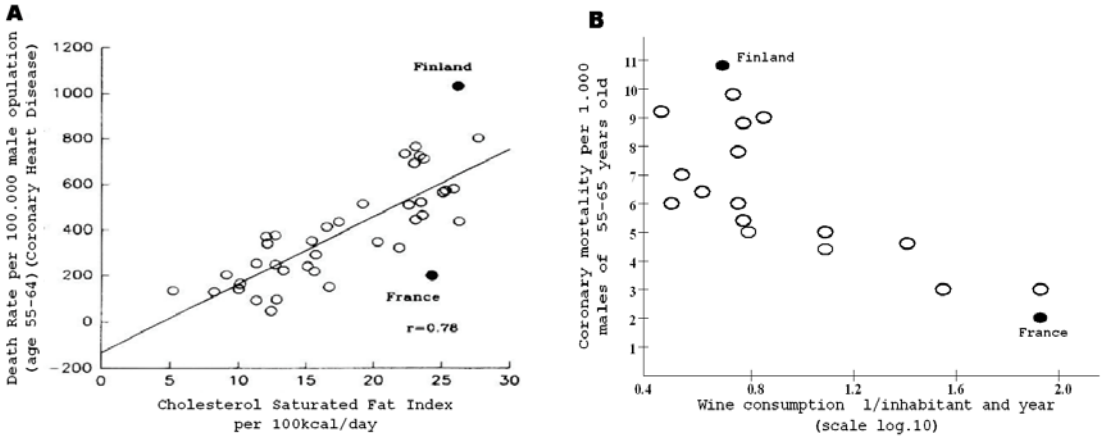

Figure 10. The French Paradox

A. From Artaud-Wild (1993). *Circulation* 88 (6): 2771-2779

B. Adapted from Bourzeix, M. and Caro G. (1990)De l'alcoholisme au bien boire. París: *L'Harmattan*

The search of an explanation for the protection by procyanidins against coronary heart disease has lead to the discovery of several metabolic processes that are targets for procyanidin effects. These include the modulation of cholesterol metabolism, changes in lipid plasmatic profile and involvement in glucose metabolism. Red wine may protect against coronary heart disease through the prevention of atherosclerosis. *In vivo* studies show antiatherosclerotic effects of red wine due to the procyanidins that it contains (156). Red wine procyanidins may protect from atherosclerosis by inhibiting LDL oxidation, and therefore preventing injures in the endothelial wall due to the formation of foam cells activated by oxidized LDL (157). This hypothesis, supported by epidemiological studies (158), is based on the antioxidant properties of red wine procyanidins (159) that *in vitro* inhibit human LDL oxidation (160). *In vivo* experiments with cholesterol-fed rabbits reinforce this hypothesis that the antiatherosclerotic role of procyanidins is related to the prevention of LDL oxidation (161) . In addition to its antioxidant activity, cardioprotective effects of red wine procyanidins involve other biological properties including the inhibition of platelet aggregation (145, 162-164), anti-inflamatory mechanisms (151), vasorelaxing activity (145), and the modulation of lipid metabolism and plasma lipid profile (146). Those properties of red wine procyanidins are achieved through modulation of intracellular signalling pathways, such as interaction with the β-platelet-derived growth factor receptor (βPDGFR) signalling pathway, involving PI3K and p38 MAPK (145, 165); through the modulation of enzymes, such as nitric oxide synthase (endothelium-derived nitric oxide being an important regulator of vascular tone) (145, 151) or Angiotensin I converting enzyme (important for the control of blood pressure) (166); and finally through the modulation of gene

expression, such as modulatory effects on apoptotic regulatory genes (147), changes in expression of transcription factors or genes involved in lipid metabolism (146).

The beneficial effects of procyanidins are also mediated by changes in lipidic plasmatic profile, as suggested by several *in vivo* experiments. Moderate and chronic consumption of red wine, but not of alcohol, reduce LDL cholesterol in normocholesterolemic rats (167). Grape seed procyanidins showed hypocholesterolemic effects in cholesterol-fed hamsters, due to an antioxidant-independent mechanism (168). In healthy rats, oral administration of a high acute dose of grape seed–derived procyanidins improved the plasma lipid profile, reducing TAG and fatty acids, decreasing LDL cholesterol and slightly increasing HDL-cholesterol (146). The reduction of plasma lipids might also be associated with a reduction of fat intake, as grape-seed procyanidins *in vitro* inhibit lipases such as pancreatic lipase (the most important enzyme for dietary triacylglycerols digestion), as well as lipoprotein lipase (169). Given that the pharmacologic inhibition of the digestion and fat absorption of dietary fat has been used as a strategy to treat obesity, a new role for procyanidins is emerging as being potential obesity preventive agents (169). Moderate red wine consumption has been inversely related to the development of obesity (170). In animal models the polyphenols found in grape seed have been shown to reduce food intake and body weight (171). The monomer epigallocatechin-gallate reduced or prevented an increase in body weight in lean and obese Zucker rats (172), and reduced food intake through a leptin receptor-independent appetite control pathway (173). In humans, grape-seed extract has also been shown to reduce energy intake (174). In fact, green tea and other plant extracts are postulated to modulate obesity (172,

175-177). The tea catechins reduced TAG and fatty acids in plasma in normally fed rats (178) and high-sucrose fed rats (179) and mice (176). They also maintained a lower body fat content in normal weight men (180, 181) and lowered body weight in a model of rats with high-fat diet-induced obesity (177). The mechanisms for tea catechins to exert those effects are through the stimulation of energy expenditure and fat oxidation (180, 181), the activation of lipid metabolism (177), and finally, like for red wine proyanidins, tea catechins inhibit digestive lipases (182).

Moderate red wine consumption might not only have beneficial effects on developing obesity but also on related disease factors, such as type 2 diabetes and insulin resistance (183, 184). Oxidative stress is believed to be a pathogenic factor in the development of diabetic complications, thus the antioxidant properties of procyanidins confers them a role in the prevention of these diseases (185-188). But since other classes of flavonoids show insulinomimetic and antidiabetic activities (189-192), procyanidins are likely to have a role in those pathologies through their action on intracellular signalling cascades. Thus far, there is scarce information on wine procyanidins interfering with the enzymes involved in insulin signalling. *In vitro* experiments showed that several flavonoids interact with such enzymes, such as PI3K and protein kinase C (PKC), but while they were shown to inhibit those enzymes, catechin had no effect on their activities (193, 194). Few studies have been carried out *in vivo* to support the hypothesis of the beneficial effects of red wine against diabetes or insulin resistance. These pathologies are characterized by hyperglycemia, and a role of procyanidins improving hyperglycemia has been shown in streptozotocin-treated rats (195).

Some monomers are also hypoglycemic *in vivo*: epicatechin, through β cell regeneration (196); catechin, through inhibition of glucose absorption (197); and also epigallocatechin-gallate (173). I*n vitro,* the monomers epicatechin gallate and specially epigallocatechin-gallate enhanced insulin activity (198). Epigallocatechin-gallate in hepatocytes shared insulin signalling pathways, including the repression of glucose production and PEPCK and glucose-6-phosphatase gene expression, possibly by modulation of the redox state of the cell (194). And also (-) epicatechin had insulin-like effects (199). But the exact relationship between wine or grape seed-procyanidins and insulin resistance or diabetes remains to be elucidated.

Despite results pointing to red wine procyanidins as protective against obesity and insulin resistant associated states, Cordain et al. failed to find the modulation of either body composition or plasma lipids in moderately obese women under moderate chronic wine consumption. Their results also showed no changes in insulin sensitivity of the subjects. They suggest the possibility that metabolic responses to alcohol differ between obese and lean subjects, as their results were opposite to the epidemiologic studies showing beneficial effects of red wine against insulin resistance (170).

The search for an explanation for the effects of red wine against coronary heart disease, highlights another biological property of procyanidins. Vascular smooth muscle cells contribute to the pathogenesis of the atherosclerotic lesions, since their proliferation and migration are critical events for the development of atherosclerosis. Red wine procyanidins have been demonstrated to inhibit vascular smooth muscle cells proliferation (145, 165). The mechanisms to do so are not clear, but might involve either the down-regulation of cyclin A expression or the up-regulation of p27 CKI (145, 200).

Thus, procyanidins have antiproliferative effects that have been studied in greater depth as this property signals procyanidins as cancer preventive agents. Grapes and wine procyanidins may induce the inhibition of the progression of the cell cycle in Caco-2 cells (201, 202). Grape seed extract also induces G1-arrest through the inhibition of ERK1/2 MAPK and p38 MAPK, the induction of $p21^{CIP}$ and a decrease in CDK4 in breast carcinoma and prostate carcinoma cell lines (203, 204). And an epicatechin pentamer arrested human breast cancer cells at G0/G1, while less polymeryzated forms did not (205). Instead, the monomer catechin was unable to inhibit proliferation and modify CKI levels on human melanoma cells (206) and human colon cancer cells (207). More details have been shown with the monomer epigallocatechin-gallate which has chemopreventive effects involving the modulation of intracellular cascades controlled by AP-1, NFκB or MAPK; changes in cell cycle regulatory proteins, such as p21 expression or pRb phosphorilation; and the induction of apoptosis in malignant cells (208-211).

Interestingly, dietary feeding of a grape seed extract also prevented photocarconogenesis, and that might be closely related to reduction in tissue fat levels (without changes in body weight) as well as inhibition of photo-oxidative damage of lipids (212). The causes for this tissue fat reduction were not assessed in this experiment, and were attributed to increased lipolysis, reduced fat synthesis or reduced fat absorption, effects already described for grape seed extract as antiobesity agent. It worth reflecting that there is a lack of studies relating the inhibition of adipose cell proliferation by grape-seed procyanidins and obesity.

2.3 Effects of procyanidins and other flavonoids on the adipocyte.

Our group initiated the study of the effects of grape seed procyanidins on adipocytes. In that paper it was shown that a grape-seed procyanidin extract induced long-term lipolysis in 3T3-L1 adipocytes, while the monomers (catechin and epicatechin) did not. Furthermore, this procyanidin extract caused a time-dependent reduction in HSL mRNA (213). On the other hand, Moreno et al. postulated an inhibition of HSL activity in 3T3-L1 adipocytes justified by a reduction of isoproterenol-stimulated glycerol release after a long term GSPE pre-treatment (169).

Similar effects were observed by other flavonoids, such as genisten, which in 3T3-L1 stimulated long term lipolysis (214). Genistein and other phytoestrogens were also tested in rat adipocytes (215, 216). All of them activated basal lipolysis, but this was mediated by different mechanisms: genistein did so by the activation of phosphodiesterase resulting in a cAMP increase, while daidzein acted on PKA. The effects of genistein, daidzein and zearelenone on lipolysis stimulated by epinephrine were dependent on concentrations, since high concentrations inhibited epinephrine-stimulated lipolysis by restriction of HSL action. Quercetin and fisetin also stimulated long-term lipolysis in rat adipocytes (217). Pycnogenol, a mixture of flavonoids containing procyanidins, also induced lipolysis (218) and decreased glycerol-3-phosphate dehydrogenase activity (219) in 3T3-L1. Lipolysis was also stimulated by bioflavones in 3T3-L1 correlating to their ability to inhibit cAMP phosphodiesterase (220). Tannic acid, a hydrolysable tannin found in red wine, increased stimulated cAMP accumulation in rat adipocytes, but failed to exert a lipolitic effect (221).

In addition to tryglyceride degradation, lipid synthesis was also studied under the action of certain flavonoids. Again the phytoestrogens genistein, daidzein, coumesterol and zearalenone had similar effects through different mechanisms. They attenuated lipogenesis in rat adipocytes, daidzein and coumesterol, via the inhibition of acetylCoA formation from glucose; zearalenone effects were more complex depending on its concentration and the presence or absence of insulin; and genistein, via restriction of glucose uptake plus other lipogenic pathways (215, 216). Genistein inhibition of glucose uptake was demonstrated to involve insulin signalling pathways downstream of the insulin receptor (222). Similarly, quercetin inhibited insulin-induced lipogenesis (190), as well as glucose uptake, probably due to their direct interaction with GLUT4 suggested by *in silico* experiments (223). Since glucose uptake into the insulin-sensitive cells is a key step in the process of glucose metabolism, several experimental approaches have focused on the potential of flavonoids to modulate glucose transport. Results are variable among different flavonoids. In addition to genistein and quercetin, other non-procyanidin flavonoids showed an inhibitory effect on glucose uptake. Naringenin inhibited glucose uptake through the inhibition of PI3K (224). Myricetin results are controversial; it inhibited glucose uptake in rat adipocytes, and it was also supported by *in silico* experiments that suggested direct interaction with GLUT4 (223). On the other hand, previous studies using myricetin demonstrated that it increased glucose transport in rat adipocytes, and it mimicked insulin stimulating lipogenesis without affecting insulin receptor function or GLUT4 translocation (189). Curiously, in Strobel work (223), despite catechin-gallate also showing an inhibitory effect on glucose transport, catechin and gallic acid neither block

glucose transport nor showed *in silico* evidences of direct interaction with GLUT4. Finally, shikonin stimulates glucose uptake in 3T3-L1 via a PI3K independent-pathway but involving PKB/Akt phophorilation (225). What these studies share is that they all establish a relationship between flavonoids and insulin-stimulated intracellular signalling pathways, despite the exact effects possibly varying within flavonoid structures and experimental conditions.

Other effects of procyanidins in adipocytes refer to differentiation; studies using the 3T3-L1 cell line demonstrated that catechin can inhibit or stimulate lipid accumulation depending on the isomer studied (226). ECGC inhibited 3T3-L1 preadipocyte proliferation and reduced triacylglycerol content (172). Concerning other flavonoids, genistein also inhibited differentiation at the onset of the differentiation program (214), probably through the inhibition of C/EBPβ activity (227). And daidzein showed biphasic effects depending on the concentration either activating or inhibiting adipogenesis in osteoprogenitor KS483 cells, through the activation of PPARs (α, β and γ) and estrogen receptor (228).

Anthocyanins, another class of non-procyanidin flavonoids, have been demonstrated to enhance adipocytokine (adiponectin and leptin) secretion in rat adipocytes; to increase the expression of PPARγ; and to enhance the expression of adipocyte-specific genes through an independent PPARγ ligand activity (229). Thus far, there are no studies investigating the role of grape-seed procyanidins as enhancers of adipocytokine secretion in adipocytes.

In conclusion, procyanidins interfere with a large number of regulatory pathways, such as energy metabolism, growth, cell division, inflammation and

stress response. In addition to their effects as antioxidants and free-radical scavengers, they also act as enzyme inhibitors, hormones, antihormones and regulators of gene expression, thus interacting with intracellular signalling pathways. Procyanidins are suggested to be protective against some pathologies. But, in spite of the adipocyte being a key organ in the development of some of those pathologies such as obesity and diabetes, which are associated to cardiovascular heart disease, there is a lack of information regarding the effects and pathways used by red wine or grape seed extract procyanidins in this cell type.

3 References

1. **H. Green and M. Meuth** (1974) *An established pre-adipose cell line and its differentiation in culture*. Cell 3, 127-133.

2. **P. Cornelius, et al.** (1994) *Regulation of adipocyte development*. Annu Rev Nutr 14, 99-129

3. **O. A. MacDougald and D. Lane** (1995) *Transcriptional regulation of gene expression during adipocyte differentiation*. Annu Rev Biochem 64, 345-373

4. **J. M. Ntambi and K. Young-Cheul** (2000) *Adipocyte differentiation and gene expression*. J Nutr 130, 3122S-3126S.

5. **H. Green and O. Kehinde** (1979) *Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line*. J Cell Physiol 101, 169-171.

6. **C. S. Hwang, et al.** (1997) *Adipocyte differentiation and leptin expression*. Annu Rev Cell Dev Biol 13, 231-259.

7. **Q. Tong and G. S. Hotamisligil** (2001) *Molecular mechanisms of adipocyte differentiation*. Rev Endocr Metab Disord 2, 349-355.

8. **A. Soukas, et al.** (2001) *Distinct Transcriptional Profiles of Adipogenesis in Vivo and in Vitro*. J Biol Chem 276, 34167

9. **H. Sell, et al.** (2004) *The brown adipocyte: update on its metabolic role*. Int J Biochem Cell Biol 36, 2098-2104

10. **D. M. Gibson and R. A. Harris** (2002) *Metabolic regulation in mammals*, Taylor & Francis, London

11. **K. N. Frayn, et al.** (2003) *Integrative physiology of human adipose tissue*. Int J Obes 27, 875-888

12. **A. Strawford, et al.** (2004) *Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with 2H2O*. Am J Physiol Endocrinol Metab 286, E577-588

13. **C. Forest, et al.** (2003) *Fatty acid recycling in adipocytes: a role for glyceroneogenesis and phosphoenolpyruvate carboxykinase*. Biochem Soc Trans 31, 1125-1129.

14. **G. Jiang and B. B. Zhang** (2003) *Glucagon and regulation of glucose metabolism*. Am J Physiol Endocrinol Metab 284, E671-678

15. **E. E. Kershaw and J. S. Flier** (2004) *Adipose tissue as an endocrine organ*. J Clin Endocrinol Metab 89, 2548-2556.

16. **M. Fasshauer and R. Paschke** (2003) *Regulation of adipocytokines and insulin resistance*. Diabetologia 46, 1594-1603.

17. **P. J. Havel** (2004) *Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism*. Diabetes 53, S143-151.

18. **J. Kopecky, et al.** (2004) *Energy metabolism of adipose tissue-physiological aspects and target in obesity treatment*. Physiol Res 53, S225-232.

19. **J. L. Miner** (2004) *The adipocyte as an endocrine cell*. J Anim Sci 82, 935-941.

20. **C. Londos, et al.** (1999) *On the control of lipolysis in adipocytes*. Ann N Y Acad Sci 892, 155-168.

21. **T. Nagatomo, et al.** (2001) *Beta-adrenoceptors: three-dimensional structures and binding sites for ligands*. Jpn J Pharmacol 87, 7-13.

22. **N. M. Nathanson** (2003) *An array of details on G-protein coupled receptor signaling: differential effects of alpha 1-adrenergic receptor subtypes on gene expression and cytokine receptor signaling*. Mol Pharmacol 63, 959-960

23. **C. Holm** (2000) *Molecular mechanisms regulating hormone-sensitive lipase and lipolysis*. Annu Rev Nutr 20, 365-393

24. **G. B. Carey** (1998) *Mechanisms regulating adipocyte lipolysis*. Adv Exp Med Biol 441, 157-170.

25. **S. J. Yeaman** (2004) *Hormone-sensitive lipase - new roles for an old enzyme*. Biochem J 379, 11-22

26. **C. Holm** (2003) *Molecular mechanisms regulating hormone-sensitive lipase and lipolysis*. Biochem Soc Trans 31, 1120-1124.

27. **F. B. Kraemer and W. J. Shen** (2002) *Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis*. J Lipid Res 43, 1585-1594.

28. **S. Mottagui-Tabar, et al.** (2003) *Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis*. Diabetologia 46, 789-797

29. **D. L. Brasaemle, et al.** (2000) *The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets*. Biochim Biophys Acta 1483, 251-262

30. **C. Sztalryd, et al.** (2003) *Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation*. J Cell Biol 161, 1093-1103

31. **V. W. Dolinsky, et al.** (2004) *Triacylglycerol hydrolase: role in intracellular lipid metabolism*. Cell Mol Life Sci 61, 1633-1651.

32. **V. W. Dolinsky, et al.** (2003) *Regulation of triacylglycerol hydrolase expression by dietary fatty acids and peroxisomal proliferator-activated receptors*. Biochim Biophys Acta 1635, 20-28

33. **S. Collins and R. S. Surwit** (2001) *The beta-adrenergic receptors and the control of adipose tissue metabolism and thermogenesis*. Recent Prog Horm Res 56, 309-328.

34. **E. Hu, et al.** (1996) *Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR gamma*. Science 274, 2100

35. **J. P. Warne** (2003) *Tumour necrosis factor alpha: a key regulator of adipose tissue mass*. J Endocrinol 177, 351-355.

36. **A. Green, et al.** (2004) *Stimulation of lipolysis by tumor necrosis factor-alpha in 3T3-L1 adipocytes is glucose dependent: implications for long-term regulation of lipolysis*. Diabetes 53, 74-81.

37. **S. Gasic, et al.** (1999) *Tumor necrosis factor-alpha stimulates lipolysis in adipocytes by decreasing Gi protein concentrations*. J Biol Chem 274, 6770-6775

38. **M. Ryden, et al.** (2004) *Targets for TNF-alpha-induced lipolysis in human adipocytes*. Biochem Biophys Res Commun 318, 168-175.

39. **M. Rosenstock, et al.** (2001) *Distinct long-term regulation of glycerol and non-esterified fatty acid release by insulin and TNF-alpha in 3T3-L1 adipocytes*. Diabetologia 44, 55-62

40. **S. C. Souza, et al.** (2003) *TNF-alpha induction of lipolysis is mediated through activation of the extracellular signal related kinase pathway in 3T3-L1 adipocytes*. J Cell Biochem 89, 1077-1086.

41. **T. Rahn-Landström, et al.** (2000) *Donwn-regulation of cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumor necrosis factor-alpha and cAMP*. Biochem J 346, 337-343

42. **L. M. Botion and A. Green** (1999) *Long.term regulation of lipolyis and hormone-sensitive lipase by insulin and glucose*. Diabetes 48, 1691-1697

43. **P. G. McTernan, et al.** (2002) *Insulin and rosiglitazone regulation of lipolysis and lipogenesis in human adipose tissue in vitro*. Diabetes 51, 1493-1498

44. **A. R. Saltiel and R. Kahn** (2001) *Insulin signalling and th regulation of glucose and lipid metabolism*. Nature 414, 799-806

45. **P. H. Ducluzeau, et al.** (2002) *Molecular mechanisms of insulin-stimulated glucose uptake in adipocytes*. Diabetes Metab 28, 85-92

46. **R. Somwar, et al.** (2002) *A dominant-negative p38 MAPK mutant and novel selective inhibitors of p38 MAPK reduce insulin-stimulated glucose uptake in 3T3-L1 adipocytes without affecting GLUT4 translocation*. J Biol Chem 277, 50386-50395

47. **L. M. Furtado, et al.** (2002) *Activation of the glucose transporter GLUT4 by insulin*. Biochem Cell Biol 80, 569-578

48. **K. V. Kandror** (2003) *A long search for Glut4 activation*. Sci STKE 2003, PE5.

49. **J. A. Chavez and S. A. Summers** (2003) *Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes*. Archives of Biochemistry and Biophysics 419, 101-109

50. **D. A. E. Cross, et al.** (1997) *Insulin activates protein kinase B, inhibits glycogen synthase kinase-3 and activates glycogen synthase by rapamycin-insensitive pathways in skeletal muscle and adipose tissue*. FEBS Lett 406, 211-21

51. **S. J. Orena, et al.** (2000) *Inhibition of glycogen-synthase kinase 3 stimulates glycogen synthase and glucose transport by distinct mechanisms in 3T3-L1 adipocytes*. J Biol Chem 275, 15765-15772

52. **S. A. Summers, et al.** (1999) *The role of glycogen synthase kinase 3beta in insulin-stimulated glucose metabolism*. J Biol Chem 274, 17934-17940

53. **T. Yamada, et al.** (2002) *Role of PDK1 in insulin-signaling pathway for glucose metabolism in 3T3-L1 adipocytes*. Am J Physiol Endocrinol Metab 282, E1385-1394

54. **M. J. Brady, et al.** (1998) *The activation of glycogen synthase by insulin switches from kinase inhibition to phosphatase activation during adipogenesis in 3T3-L1 cells*. J Biol Chem 273, 14063-14066

55. **K. Ueki, et al.** (1998) *Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis*. J Biol Chem 273, 5315-5322

56. **W. B. Benjamin, et al.** (1994) *ATP citrate-lyase and glycogen synthase kinase-3 beta in 3T3-L1 cells during differentiation into adipocytes*. Biochem J 300, 477-482.

57. **T. C. Jensen, et al.** (2000) *Specific desensitization of glycogen synthase activation by insulin in 3T3-L1 adipocytes. Connection between enzymatic activation and subcellular localization*. J Biol Chem 275, 40148-40154

58. **H. Ou, et al.** (2004) *Spatial reorganization of glycogen synthase upon activation in 3T3-L1 adipocytes*. Endocrinology, en.2004-1022

59. **K. J. Nadeau, et al.** (2004) *Insulin regulation of sterol regulatory element-binding protein-1 expression in L-6 muscle cells and 3T3 L1 adipocytes*. J Biol Chem 279, 34380-34387

60. **J. B. Kim, et al.** (1998) *Nutritional and insulin regulation of fatty acid synthetase and eptin gene expression through ADD1/SREBP1*. J Clin Invest 101, 1-9

61. **K. H. Kim, et al.** (2004) *Regulatory role of GSK3 for transcriptional activity of ADD1/SREBP1c*. J Biol Chem, M405522200

62. **S. L. Lay, et al.** (2002) *Insulin and sterol-regulatory element-binding protein-1c (SREBP-1C) regulation of gene expression in 3T3-L1 adipocytes. Identiafication of CCAAT/enhacner-binding protein-beta as an SREBP-1C target*. J Biol Chem 277, 35625-35634

63. **S. Kersten** (2001) *Mechanisms of nutritional and hormonal regulation of lipogenesis*. EMBO Rep 2, 282-286.

64. **E. D. Rosen, et al.** (2000) *Transcriptional regulation of adipogenesis.* Genes Dev 14, 1293-1307

65. **F. M. Gregoire, et al.** (1998) *Understanding adipocyte differentiation*. Physiol Rev 78, 783-809.

66. **A. Student, et al.** (1980) *Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes*. J Biol Chem 255, 4745-4750

67. **C. S. Rubin, et al.** (1978) *Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells*. J Biol Chem 253, 7570-7578

68. **G. R. Burton, et al.** (2002) *Microarray analysis of gene expression during early adipocyte differentiation*. Gene 293, 21-31

69. **B. A. Jessen and G. J. Stevens** (2002) *Expression profiling during adipocyte differentiation of 3T3-L1 fibroblasts*. Gene 299, 95-100

70. **X. Guo and K. Liao** (2000) *Analysis of gene expression profile during 3T3-L1 preadipocyte differentiation*. Gene 251, 45-53

71. **C. S. Rubin, et al.** (1978) *Development of hormone receptors and hormone responsiveness in vitro.* J Biol Chem 253, 7570-7578

72. **W. Schmidt, et al.** (1990) *Adipose conversion of 3T3-L1 cells in a serum-free culture system depends on epidermal growth factor, insulin-like growth factor I, corticosterone, and cyclic AMP*. J Biol Chem 265, 15489-15495.

73. **P. J. Smith, et al.** (1988) *Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes*. J Biol Chem 263, 9402-9408.

74. **H. Miki, et al.** (2001) *Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation*. Mol Cell Biol 21, 2521-2532.

75. **K. Tomiyama, et al.** (1995) *Wortmannin, a Specific Phosphatidylinositol 3-Kinase Inhibitor, Inhibits Adipocytic Differentiation of 3T3-L1 Cells*. Biochem Biophys Res Commun 212, 263-269

76. **R. Magun, et al.** (1996) *Expression of a constitutively activated form of protein kinase B (c- Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation*. Endocrinology 137, 3590-3593

77. **D. J. Klemm, et al.** (2001) *Insulin-induced adipocyte differentiation. Activation of CREB rescues adipogenesis from the arrest caused by inhibition of prenylation*. J Biol Chem 276, 28430-28435

78. **G. Wolf** (1999) *The molecular mechanism of the stimulation of adipocyte differentiation by a glucocorticoid*. Nutr Rev 57, 324-326

79. **T. Takamura, et al.** (2001) *Stage-specific effects of a thiazolidinedione on proliferation, differentiation and PPAR[gamma] mRNA expression in 3T3-L1 adipocytes*. Eur J Pharmacol 422, 23-29

80. **H. Staiger, et al.** (2002) *Serum-free differentiation of 3T3-L1 preadipocytes is characterized by only transient expression of peroxisome proliferator-activated receptor-[gamma]*. Biochem Biophys Res Commun 296, 125-128

81. **Z. Qiu, et al.** (2001) *DNA Synthesis and Mitotic Clonal Expansion Is Not a Required Step for 3T3-L1 Preadipocyte Differentiation into Adipocytes*. J Biol Chem 276, 11988-11995

82. **F. M. Gregoire** (2001) *Adipocyte differentiation: from fibroblast to endocrine cell*. Exp Biol Med (Maywood) 226, 997-1002.

83. **G. Entenmann and H. Hauner** (1996) *Relationship between replication and differentiation in cultured human adipocyte precursor cells*. Am J Physiol 270, C1011-1016.

84. **Q.-Q. Tang, Otto, T.C., and Lane, D.M.** (2003) *Mitotic clonal expansion: A synchronous process required for adipogenesis*. Proc Natl Acad Sci USA 100, 44-49

85. **Q.-Q. Tang, et al.** (2004) *Sequential gene promoter interactions by C/EBP[beta], C/EBP[alpha], and PPAR[gamma] during adipogenesis*. Biochem Biophys Res Commun 318, 213-218

86. **M. Tessema, et al.** (2004) *Cell cycle and no end*. Virchows Arch 444, 313-323

87. **A. B. Pardee** (1989) *G1 events and regulation of cell proliferation*. Science 246, 603-608

88. **A. K. Pearce and T. C. Humphrey** (2001) *Integrating stress-response and cell cycle checkpoint pathways*. TRENDS in cell biology 11, 426-433

89. **R. Agami and R. Bernards** (2002) *Convergence of mitogenic and DNA damage signaling in the G1 phase of the cell cycle*. Cancer Lett 177, 111-118

90. **R. F. Morrison and S. R. Farmer** (1999) *Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis*. J Biol Chem 274, 17088-17097

91. **N. Dyson** (1998) *The regulation of E2F by pRB-family proteins*. Genes Dev 12, 2245-2262.

92. **L. Zheng and W.-H. Lee** (2001) *The retinoblastoma gene: a prototypic and multifunctional tumor suppressor*. Exp Cell Res 264, 2-18

93. **A. P. Bracken, et al.** (2004) *E2F target genes: unraveling the biology*. Trends Biochem Sci 29, 409-417

94. **H. Muller and K. Helin** (2000) *The E2F transcription factors: key regulators of cell proliferation*. Biochim Biophys Acta - Reviews on Cancer 1470, M1-M12

95. **C. J. Sherr and J. M. Roberts** (1999) *CDK inhibitors: positive and negative regulators of G1-phase progression.* Genes Dev 13, 1501-1512

96. **L. Fajas** (2003) *Adipogenesis: a cross-talk between cell proliferation and cell differentiation*. Ann Med 35, 79-85.

97. **C. Wang, et al.** (2003) *Cyclin D1 repression of peroxisome proliferator-activated receptor {gamma} expression and transactivation*. Mol Cell Biol 23, 6159-6173

98. **L. Fajas, et al.** (2002) *E2Fs regulate adipocyte differentiation*. Developmental Cell 3, 39-49

99. **K. A. Cole, et al.** (2004) *Rb regulates C/EBP{beta}-DNA-binding activity during 3T3-L1 adipogenesis*. Am J Physiol Cell Physiol 286, C349-354

100. **S. Theocharis, et al.** (2004) *Peroxisome proliferator-activated receptor-gamma ligands as cell-cycle modulators*. Cancer Treat Rev 30, 545-554.

101. **Z. Cao, et al.** (1991) *Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells*. Genes Dev 5, 1538-1552

102. **Q.-Q. Tang and M. D. Lane** (1999) *Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation*. Genes Dev 13, 2231-2241

103. **Rosen E.D., et al.** (2002) *C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway*. Genes Dev 16, 22-26

104. **R. M. Umek, et al.** (1991) *CCAAT-enhancer binding protein: a component of a differentiation switch*. Science 251, 288

105. **T. Pederson and C. M. Rondinone** (2000) *Regulation of proteins involved in insulin signaling pathways in differentiating human adipocytes*. Biochem Biophys Res Commun 276, 162-168.

106. **H. S. Sul, et al.** (2000) *Function of pref-1 as an inhibitor of adipocyte differentiation*. Int J Obes 24, S15-S19

107. **O. A. MacDougald and S. Mandrup** (2002) *Adipogenesis: forces that tip the scales*. Trends Endocrinol Metab 13, 5-11.

108. **H. Xu, et al.** (1999) *Transmembrane tumor necrosis factor (TNF)-alpha inhibits adipocyte differentiation by selectively activating TNF receptor 1*. J Biol Chem 274, 26287-26295.

109. **S. A. Kliewer, et al.** (2001) *Peroxisome proliferator-activated receptors:from genes to physiology*. Recent Prog Horm Res 56, 239-263

110. **P. Ferre** (2004) *The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity*. Diabetes 53, S43-50.

111. **C. Blanquart, et al.** (2003) *Peroxisome proliferator-activated receptors: regulation of transcriptional activities and roles in inflammation*. J Steroid Biochem Mol Biol 85, 267-273

112. **G. J. Murphy and J. C. Holder** (2000) *PPAR-[gamma] agonists: therapeutic role in diabetes, inflammation and cancer*. Trends Pharmacol Sci 21, 469-474

113. **J. Berger and D. E. Moller** (2002) *The mechanisms of action of PPARs*. Annu Rev Med 53, 409-435.

114. **D. E. Moller and J. P. Berger** (2003) *Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation*. Int J Obes Relat Metab Disord 27, S17-21.

115. **B. Gustafson, et al.** (2003) *Adiponectin gene activation by thiazolidinediones requires PPAR[gamma]2, but not C/EBP[alpha]-evidence for differential regulation of the aP2 and adiponectin genes*. Biochem Biophys Res Commun 308, 933-939

116. **S. A. Kliewer, et al.** (1997) *Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma*. PNAS 94, 4318-4323

117. **L. Fajas, et al.** (1999) *Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism*. Mol Cell Biol 19, 5495-5503

118. **S. M. Rangwala and M. A. Lazar** (2004) *Peroxisome proliferator-activated receptor [gamma] in diabetes and metabolism*. Trends Pharmacol Sci 25, 331-336

119. **R. Walczak and P. Tontonoz** (2002) *PPARadigms and PPAradoxes: expanding roles for PPARgamma in the control of lipid metabolism*. J Lipid Res 43, 177-186

120. **Y. Tamori, et al.** (2002) *Role of peroxisome proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3-L1 adipocytes*. Diabetes 51, 2045-2055.

121. **S. A. Aherne and N. M. O'Brien** (2002) *Dietary flavonols: chemistry, food content, and metabolism*. Nutrition 18, 75-81

122. **M. Monagas, et al.** (2003) *Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from Vitis vinifera L. Cv. Graciano, tempranillo, and Cabernet Sauvignon*. J Agric Food Chem 51, 6475-6481

123. **J. Peterson and J. Dwyer** (1998) *Taxonomic classification helps identify flavonoid-containing foods on a semiquantitative food frequency questionnaire.* J Am Diet Assoc 98, 677-685

124. **P. M. Kris-Etherton, et al.** (2002) *Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer*. Am J Med 113, 71-88

125. **A. J. Shrikhande** (2000) *Wine by-products with health benefits*. Food Research International 33, 469-474

126. **J. F. Hammerstone, et al.** (2000) *Procyanidin content and variation in some commonly consumed foods*. J Nutr 130, 2086S-2092

127. **J.-B. Ruidavets, et al.** (2000) *Catechin in the Mediterranean diet: vegetable, fruit or wine?* Atherosclerosis 153, 107-117

128. **A. Scalbert** (2000) *Dietary intake and bioavailability of polyphenols*. J Nutr 130, 2073S-2085S

129. **B. H. Havsteen** (2002) *The biochemistry and medical significance of the flavonoids*. Pharmacol Ther 96, 67-202

130. **R. J. Nijveldt, et al.** (2001) *Flavonoids: a review of probable mechanisms of action and potential applications*. Am J Clin Nutr 74, 418-425

131. **Y. Nakamura and Y. Tonogai** (2003) *Metabolism of grape seed polyphenol in the rat*. J Agric Food Chem 51, 7215-7225.

132. **N. C. Cook and S. Samman** (1996) *Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources*. Nutr Biochem 7, 66-76

133. **M.-P. Gonthier, et al.** (2003) *Metabolism of dietary procyanidins in rats*. Free Radic Biol Med 35, 837-844

134. **J.-J. Macheix, et al.** (1990) *Fruit phenolics*, CRC Press, Inc., Boca Raton, Florida

135. **K. E. Heim, et al.** (2002) *Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships*. J Nutr Biochem 13, 572-584

136. **Y. Yilmaz and R. T. Toledo** (2004) *Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid*. J Agric Food Chem 52, 255-260.

137. **A. E. Hagerman, et al.** (1998) *High molecular weight plant polyphenolics (tannins) as biological antioxidants*. J Agric Food Chem 46, 1887 -1892

138. **T. De Bruyne, et al.** (1999) *Biological evaluation of proanthocyanidin dimers and related polyphenols*. J Nat Prod 62, 954-958.

139. **P. Simonetti, et al.** (2002) *Procyanidins from Vitis vinifera seeds: in vivo effects on oxidative stress*. J Agric Food Chem 50, 6217-6221.

140. **N. Saint-Cricq De Gaulejac, et al.** (1999) *Comparative study of polyphenol scavenging activities assessed by different methods*. J Agric Food Chem 47, 425-431.

141. **R. J. Williams, et al.** (2004) *Flavonoids: antioxidants or signalling molecules?* Free Radic Biol Med 36, 838-849

142. **M. J. Brunet, et al.** (2002) *Human Apo A-1 and rat transferrin are the principal plasma proteins that bind wine catechins*. J Agric Food Chem 50, 2708-2712

143. **H. Moini, et al.** (2000) *Molecular aspects of procyanidin biological activity: disease preventative and therapeutic potentials*. Drug Metabol Drug Interact 17, 237-259.

144. **G. G. Mackenzie, et al.** (2004) *Epicatechin, catechin, and dimeric procyanidins inhibit PMA-induced NF-{kappa}B activation at multiple steps in Jurkat T cells*. FASEB J 18, 167-169

145. **M. Dell'Agli, et al.** (2004) *Vascular effects of wine polyphenols*. Cardiovasc Res 63, 593-602

146. **J. M. del Bas, et al.** *Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats*. FASEB J In Press

147. **D. Bagchi, et al.** (2003) *Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract*. Mutat Res 523-524, 87-97

148. **N. Llópiz, et al.** (2004) *Antigenotoxic Effect of Grape Seed Procyanidin Extract in Fao Cells Submitted to Oxidative Stress.* J Agric Food Chem 52, 1083-1087

149. **W. G. Li, et al.** (2001) *Anti-inflammatory effect and mechanism of proanthocyanidins from grape seeds*. Acta Pharmacol Sin 22, 1117-1120.

150. **Y. Lu, et al.** (2004) *Procyanidins from grape seeds protect against phorbol ester-induced oxidative cellular and genotoxic damage*. Acta Pharmacol Sin 25, 1083-1089.

151. **M. Bohm, et al.** (2004) *Alcohol and red wine: impact on cardiovascular risk*. Nephrol Dial Transplant 19, 11-16

152. **J. Ferrieres** (2004) *The French paradox: lessons for other countries*. Heart 90, 107-111.

153. **A. A. de Lorimier** (2000) *Alcohol, wine, and health*. Am J Surg 180, 357-361

154. **G. J. Soleas, et al.** (1997) *Wine as a biological fluid: history, production, and role in disease prevention*. J Clin Lab Anal 11, 287-313.

155. **J. F. Tomera** (1999) *Current knowledge of the health benefits and disadvantages of wine consumption*. Trends in Food Science & Technology 10, 129-138

156. **J. A. Vinson, et al.** (2001) *Red wine, dealcoholized red wine, and especially grape juice, inhibit atherosclerosis in a hamster model*. Atherosclerosis 156, 67-72

157. **F. Ursini and A. Sevanian** (2002) *Wine polyphenols and optimal nutrition*. Ann NY Acad Sci 957, 200-209

158. **S. Kiechl, et al.** (1998) *Alcohol consumption and atherosclerosis: what is the relation? : prospective results from the bruneck study*. Stroke 29, 900-907

159. **N. Landrault, et al.** (2001) *Antioxidant capacities and phenolics levels of french wines from different varieties and vintages*. J Agric Food Chem 49, 3341-3348

160. **A. S. Meyer, et al.** (1997) *Inhibition of human low-density lipoprotein oxidation in relation to composition of phenolic antioxidants in grapes (Vitis vinifera)*. J Agric Food Chem 45, 1638

161. **J. Yamakoshi, et al.** (1999) *Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits*. Atherosclerosis 142, 139-149

162. **D. Shanmuganayagam, et al.** (2002) *Grape seed and grape skin extracts elicit a greater antiplatelet effect when used in combination than when used individually in dogs and humans*. J Nutr 132, 3592-3598

163. **D. Rein, et al.** (2000) *Cocoa and wine polyphenols modulate platelet activation and function*. J Nutr 130, 2120S-2126

164. **P. Russo, et al.** (2001) *Effects of de-alcoholated red wine and its phenolic fractions on platelet aggregation*. Nutr Metab Cardiovasc Dis 11, 25-29.

165. **S. Rosenkranz, et al.** (2002) *Inhibition of the PDGF receptor by red wine flavonoids provides a molecular explanation for the "French paradox"*. FASEB J 16, 1958-1960

166. **L. Actis-Goretta, et al.** (2003) *Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins*. FEBS Lett 555, 597-600

167. **E. Cascon, et al.** (2001) *Nonalcoholic components in wine reduce low density lipoprotein cholesterol in normocholesterolemic rats*. Lipids 36, 383-388.

168. **C. Auger, et al.** (2004) *Phenolics from commercialized grape extracts prevent early atherosclerotic lesions in hamsters by mechanisms other than antioxidant effect*. J Agric Food Chem 52, 5297-5302.

169. **D. A. Moreno, et al.** (2003) *Inhibitory effects of grape seed extract on lipases*. Nutrition 19, 876-879

170. **L. Cordain, et al.** (2000) *Influence of moderate chronic wine consumption on insulin sensitivity and other correlates of syndrome X in moderately obese women*. Metabolism 49, 1473-1478

171. **K. Tebib, et al.** (1996) *effects of dietary grape seed tannins on rat cecal fermentation and colonic bacterial enzymes*. Nutr Res 16, 105-110

172. **Y. H. Kao, et al.** (2000) *Modulation of obesity by a green tea catechin*. Am J Clin Nutr 72, 1232-1234.

173. **Y.-H. Kao, et al.** (2000) *Modulation of Endocrine Systems and Food Intake by Green Tea Epigallocatechin Gallate*. Endocrinology 141, 980-987

174. **N. Vogels, et al.** (2004) *The effect of grape-seed extract on 24 h energy intake in humans*. Eur J Clin Nutr 58, 667-673.

175. **M. Yoshikawa, et al.** (2002) *Salacia reticulata and its polyphenolic constituents with lipase inhibitory and lipolytic activities have mild antiobesity effects in rats*. J Nutr 132, 1819-1824

176. **G. Zheng, et al.** (2004) *Anti-obesity effects of three major components of green tea, catechins, caffeine and theanine, in mice*. In Vivo 18, 55-62.

177. **T. Murase, et al.** (2002) *Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver*. Int J Obes Relat Metab Disord 26, 1459-1464.

178. **L.-Y. Wu, et al.** (2004) *Effect of green tea supplementation on insulin sensitivity in Sprague-Dawley rats*. J Agric Food Chem 52, 643-648

179. **M.-H. Yang, et al.** (2001) *Green, oolong and black tea extracts modulate lipid metabolism in hyperlipidemia rats fed high-sucrose diet*. J Nutr Biochem 12, 14-20

180. **W. Rumpler, et al.** (2001) *Oolong tea increases metabolic rate and fat oxidation in men*. J Nutr 131, 2848-2852

181. **A. G. Dulloo, et al.** (1999) *Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans*. Am J Clin Nutr 70, 1040-1045

182. **C. Juhel, et al.** (2000) *Green tea extract (AR25(R)) inhibits lipolysis of triglycerides in gastric and duodenal medium in vitro*. J Nutr Biochem 11, 45-51

183. **E. J. Mayer, et al.** (1993) *Progression of coronary artery disease: alcohol consumption and insulin concentrations: role of insulin in associations of alcohol intake with high-density lipoprotein cholesterol and triglycerides*. Circulation 88, 2190-2197

184. **R. R. Zilkens and I. B. Puddey** (2003) *Alcohol and type 2 diabetes - another paradox?* J Cardiovasc Risk 10, 25-30

185. **A. Maritim, et al.** (2003) *Effects of pycnogenol treatment on oxidative stress in streptozotocin-induced diabetic rats*. J Biochem Mol Toxicol 17, 193-199.

186. **N. Landrault, et al.** (2003) *Effect of a polyphenols-enriched chardonnay white wine in diabetic rats*. J Agric Food Chem 51, 311-318.

187. **G. Caimi, et al.** (2003) *Diabetes mellitus: oxidative stress and wine*. Curr Med Res Opin 19, 581-586.

188. **A. Ceriello, et al.** (2001) *Red wine protects diabetic patients from meal-induced oxidative stress and thrombosis activation: a pleasant approach to the prevention of cardiovascular disease in diabetes*. Eur J Clin Invest 31, 322-328.

189. **K. C. Ong and H.-E. Khoo** (1996) *Insulinomimetic effects of myricetin on lipogenesis and glucose transport in rat adipocytes but not glucose transporter translocation.* Biochem Pharmacol 51, 423-429

190. **A. Shisheva and Y. Shechter** (1992) *Quercetin selectively inhibits insulin receptor function in vitro and the bioresponses of insulin and insulinomimetic agents in rat adipocytes*. Biochemistry 31, 8059-8063.

191. **K. C. Ong and H.-E. Khoo** (2000) *Effects of myricetin on glycemia and glycogen metabolism in diabetic rats*. Life Sci 67, 1695-1705

192. **M. Vessal, et al.** (2003) *Antidiabetic effects of quercetin in streptozocin-induced diabetic rats*. Comp Biochem Physiol C Toxicol Pharmacol 135, 357-364

193. **G. Agullo, et al.** (1997) *Relationship between flavonoid structure and inhibition of phophatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition*. Biochem Pharmacol 53, 1649-1657

194. **M. E. Waltner-Law, et al.** (2002) *Epigallocatechin gallate, a constituent of green tea represses hepatic glucose production*. J Biol Chem 277, 34933-34940

195. **N. Al-Awwadi, et al.** (2004) *Antidiabetic activity of red wine polyphenolic extract, ethanol, or both in streptozotocin-treated Rats*. J Agric Food Chem 52, 1008-1016

196. **M. J. Kim, et al.** (2003) *Protective effects of epicatechin against the toxic effects of streptozotocin on rat pancreatic islets: in vivo and in vitro*. Pancreas 26, 292-299.

197. **M. Shimizu, et al.** (2000) *Regulation of intestinal glucose transport by tea catechins*. Biofactors 13, 61-65.

198. **R. A. Anderson and M. M. Polansky** (2002) *Tea enhances insulin activity*. J Agric Food Chem 50, 7182-7186

199. **F. Ahmad, et al.** (1989) *Insulin like activity in (-) epicatechin*. Acta Diabetol Lat 26, 291-300.

200. **K. Iijima, et al.** (2000) *Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and downregulate expression of cyclin A gene*. Circulation 101, 805-811

201. **S. Carnesecchi, et al.** (2002) *Flavanols and procyanidins of cocoa and chocolate inhibit growth and polyamine biosynthesis of human colonic cancer cells*. Cancer Lett 175, 147-155

202. **C. Laurent, et al.** (2004) *Grape seed extract affects proliferation and differentiation of human intestinal Caco-2 cells*. J Agric Food Chem 52, 3301-3308.

203. **C. Agarwal, et al.** (2000) *A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468 cells by inhibiting mitogen-activated protein kinases activation and inducing G1 arrest and differentiation*. Clin Cancer Res 6, 2921-2930.

204. **C. Agarwal, et al.** (2000) *Anticarcinogenic effect of a polyphenolic fraction isolated from grape seeds in human prostate carcinoma DU145 cells: modulation of mitogenic signaling and cell-cycle regulators and induction of G1 arrest and apoptosis*. Mol Carcinog 28, 129-138.

205. **A. P. Kozikowski, et al.** (2003) *Studies in polyphenol chemistry and bioactivity. 4.(1) Synthesis of trimeric, tetrameric, pentameric, and higher oligomeric epicatechin-derived procyanidins having all-4beta,8-interflavan connectivity and their inhibition of cancer cell growth through cell cycle arrest*. J Org Chem 68, 1641-1658.

206. **F. Casagrande and J.-M. Darbon** (2001) *Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1*. Biochem Pharmacol 61, 1205-1215

207. **S.-M. Kuo** (1996) *Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells*. Cancer Lett 110, 41-48

208. **O. J. Park and Y.-J. Surh** (2004) *Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies*. Toxicol Letters 150, 43-56

209. **M. Liberto and D. Cobrinik** (2000) *Growth factor-dependent induction of p21CIP1 by the green tea polyphenol, epigallocatechin gallate*. Cancer Lett 154, 151-161

210. **J. Y. Chung, et al.** (1999) *Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells*. Cancer Res 59, 4610-4617

211. **R. Agarwal** (2000) *Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents*. Biochem Pharmacol 60, 1051-1059

212. **A. Mittal, et al.** (2003) *Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation*. Carcinogenesis 24, 1379-1388

213. **A. Ardévol, et al.** (2000) *Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes*. Int J Obes 24, 319-324

214. **A. W. Harmon and B. H. Joyce** (2001) *Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis.* Am J Physiol 280, C807-C813

215. **K. Szkudelska, et al.** (2000) *Genistein affects lipogenesis and lipolysis in isolated rat adipocytes*. J Steroid Biochem Mol Biol 75, 265-271

216. **K. Szkudelska, et al.** (2002) *Daidzein, coumestrol and zearalenone affect lipogenesis and lipolysis in rat adipocytes*. Phytomedicine 9, 338-345.

217. **U. R. Kuppusamy and N. P. Das** (1994) *Potentiation of β-adrenoreceptor agonist-mediated lipolysis by quercetin and fisetin in isolated rat adipocytes*. Biochem Pharmacol 47, 521-529

218. **N. Hasegawa** (1999) *Stimulation of lipolysis by pycnogenol*. Phytother Res 13, 619-620.

219. **N. Hasegawa** (2000) *Inhibition of lipogenesis by pycnogenol*. Phytother Res 14, 472-473.

220. **M. Dell'Agli and E. Bosisio** (2002) *Biflavones of Ginkgo biloba stimulate lipolysis in 3T3-L1 adipocytes*. Planta Med 68, 76-79.

221. **F. Wrisez and B. Lambert** (1998) *Opposite effects of tannic acid, a hydrolysable tannin, on cyclic AMP accumulation and on lipolysis in rat adipocytes*. Med Sci Res 26, 409-411

222. **A. Abler, et al.** (1992) *Genistein differentially inhibits postreceptor effects of insulin in rat adipocytes without inhibiting the insulin receptor kinase*. J Biol Chem 267, 3946-3951

223. **P. Strobel, et al.** (2004) *Myricetin, quercetin and catechin-gallate inhibit glucose uptake in isolated rat adipocytes*. Biochem J 7, 7

224. **A. W. Harmon, Patel, Y.M.** (2003) *Naringenin inhibits phosphoinositide 3-kinase activity and glucose uptake in 3T3-L1 adipocytes.* Biochem Biophys Res Commun 305, 229-234

225. **R. Kamei, et al.** (2002) *Shikonin stimulates glucose uptake in 3T3-L1 adipocytes via an insulin-independent tyrosine kinase pathway*. Biochem Biophys Res Commun 292, 642-651

226. **M. Mochizuki and N. Hasegawa** (2004) *Stereospecific effects of catechin isomers on insulin induced lipogenesis in 3T3-L1 cells*. Phytother Res 18, 449-450.

227. **A. W. Harmon, et al.** (2002) *Genistein inhibits CCAAT/enhancer-binding protein beta (C/EBPbeta) activity and 3T3-L1 adipogenesis by increasing C/EBP homologous protein expression*. Biochem J 367, 203-208.

228. **Z. Dang and C. W. Lowik** (2004) *The balance between concurrent activation of ERs and PPARs determines daidzein-induced osteogenesis and adipogenesis*. J Bone Miner Res 19, 853-861

229. **T. Tsuda, et al.** (2004) *Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes*. Biochem Biophys Res Commun 316, 149-157.

III~ Results

Intracellular mediators of procyanidin-induced lipolysis in 3T3-L1 adipocytes

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Intracellular Mediators of Procyanidin-Induced Lipolysis in 3T3-L1 Adipocytes

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We have previously reported that grape seed procyanidins stimulate long-term lipolysis on 3T3-L1 fully differentiated adipocytes. To unravel the molecular mechanism by which procyanidins exert this effect, we checked the involvement of two main cellular targets in adipose cells: protein kinase A (PKA) and peroxisome proliferator-activated receptor-γ (PPAR-γ). Procyanidin treatment increased intracellular cAMP levels in 3T3-L1 adipocytes, and their lipolytic effect was inhibited by simultaneous treatment with H89, a PKA specific inhibitor. BRL49653, a very highly specific ligand of PPAR-γ, totally abolished the lipolytic effect of procyanidins. Simultaneous to this long-term lipolytic effect, the mRNA levels of some differentiation adipocyte markers decreased, although there were no changes in the triglyceride content of the cells. BRL49653 did not antagonize the decrements of differentiation markers. These results support a mediation of PPAR-γ and PKA on the lipolytic effects of procyanidins on 3T3-L1 adipocytes.

KEYWORDS: Procyanidin; lipolysis; protein kinase A; PPAR-γ2; 3T3-L1

INTRODUCTION

Procyanidins, a group of flavonoids, are oligomeric forms of catechins that are abundant in red wine, grapes, chocolate, and apples (*1*). Our group has previously shown that long-term treatment with grape seed procyanidins increases lipolytic rate in 3T3-L1 adipocytes (*2*). We also know that procyanidins decrease mRNA levels of hormone-sensitive lipase (HSL) and the activity of glycerol-3-phosphate dehydrogenase (G3PDH) (*2*), but we do not know the underlying molecular mechanisms to explain these effects. There is scarce information about the mechanism of action of procyanidins. Only Rosenkranz has shown that wine procyanidins inhibit the activation of platelet-derived growth factor β (β-PDGF) receptor on vascular smooth muscle cells (*3*). There is more information about the cellular targets of some monomeric flavonoids, which also exert a lipolytic effect. Quercetin and fisetin act synergistically with epinephrine on β-adrenergic receptor (*4*), while genistein inhibits tyrosine kinase activity (*5*) and inhibits CCAAT/enhancer binding protein-β (C/EBP) activity (*6*). To determine the mechanisms of action of procyanidins that explain their lipolytic effect on the adipose cell, we must bear in mind that this effect only works with a long-term treatment. Hormonal short-term modulation of lipolysis is mainly driven by cAMP-dependent protein kinase A (PKA), which controls both HSL lipolytic activity (*7*, *8*) and non-HSL lipolytic activity (*9*). Long-term lipolysis is modulated by several factors such as cAMP, phorbol esters, dexamethasone, and tumor necrosis factor-α ((TNFα)) (*7*, *10*). Of all of these, TNF-α is the one whose lipolytic effect very much resembles that of the procyanidins and is counteracted by peroxisome proliferator-activated receptor-γ (PPARγ) ligands such as thiazolidinediones (TZD) (*11*), both in the presence of HSL and in the absence of HSL (*9*).

The aim of this study was to find cellular targets of procyanidins that explain their long-term lipolytic effect on adipocytes. To do this, we evaluated the interaction of procyanidins with PKA-mediated signaling pathways and the procyanidin modifications of PPAR-γ mediated pathways.

MATERIALS AND METHODS

Chemicals. Grape seed procyanidins extract (PE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract had a mean molecular weight of 1399 and contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5−13 units) (31.7%) procyanidins. Thiazolidinedione BRL49653 was kindly provided by GlaxoSmithKline (UK) and Galderma R&D (France). (±)Epinephrine and DL-propanolol were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were obtained from BioWhitthaker (Verviers, Belgium). H89 was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany).

Cell Culture and Differentiation. 3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (*2*). Briefly, confluent preadipocytes were treated with 0.25 μmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-methylxanthine, and 5 μg/mL insulin for 2 days in 10% fetal bovine serum containing DMEM. Cells were then switched to 10% FBS/DMEM media containing only insulin for 2 more days, and then switched to 10% FBS/DMEM media without insulin. Ten days after differentiation was induced, cells were treated with the grape

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seed procyanidins and various ligands (BRL49653, H89, epinephrine and propanolol), as shown in detail in the figures. We determined the lipid content by staining the cells with Oil Red O.

Measurement of Lipolytic Activity. Fully differentiated adipocytes were treated for 15 h with procyanidins, with or without H89, epinephrine, propanolol, or BRL49653, depending on the experimental design (see figures). Lipolysis was monitored as the amount of glycerol released into the media or as the amount of nonesterified fatty acid (NEFA) released into the media. Glycerol was measured by the Garland and Randle method (*12*). NEFA was measured by the NEFA-C kit (WAKO Chemicals, Germany). Glycerol and NEFA values were corrected by their protein content, which was measured by the Bradford method (*13*) using the Bio-Rad protein reagent.

Measurement of Glycerol-3-phosphate Dehydrogenase Activity. After treatment, differentiated 3T3-L1 adipocytes were rinsed twice with PBS, scraped into 750 μL of 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L β-mercaptoethanol, and sonicated. The resulting extract was used to measure glycerol-3-phosphate dehydrogenase activity (G3PDH), in accordance with the Wise and Green method (*14*).

Cyclic AMP Determination. Differentiated 3T3-L1 adipocytes were exposed for 8 min to procyanidin extract or epinephrine. After the treatment, the cells were scraped in PBS. Part of the cell suspension was deproteinized with 3% perchloric acid and neutralized with 1 mmol/L NaOH. We used the supernatant of this extract to measure cyclic AMP content in the cells with a cyclic AMP assay kit (Amersham). We used the rest of the cell suspension to quantify protein content by the Bradford method (*13*).

Quantitative RT-PCR. PPAR-γ2 and HSL mRNA levels were measured by real-time RT-PCR analyses in a fluorescent thermal cycler (GeneAmp 5700 Sequence Detection System, Applied Biosystems). The level of mRNA for each gene was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA detected in each sample. 3T3-L1 adipocytes were grown in 6-well multiplates, and total RNA was isolated using the High Pure RNA Isolation Kit (Roche). 1 μg of RNA was reverse transcribed by the SuperScript II Rnase H_ Reverse Transcriptase according to the manufacturer's instructions (LifeTechnologies). 2/100 of each RT reaction was amplified according to the protocols provided by the manufacturer (Applied Biosystems, Warrington, UK). The following primers were used: GAPDH CATGGCCTTCCGTGTTCCT (forward), and CCTGCTTCACCACCTTCTTGA (reverse); PPAR-γ2 CTGTTGACCCAGAGCATGGT (forward), and AGAGGTCCACAGAGCTGATTCC (reverse); HSL GGAGCACTACAAACGCAACGA (forward), and AATCGGCCACCGGTAAAGAG (reverse).

Statistics. Results are expressed as mean ± SEM. Effects were assessed using One-way or T-test. We used Tukey's Test of honestly significant differences to make pairwise comparisons. All calculations were performed using SPSS software.

RESULTS

PKA Participates in the Lipolytic Action of Procyanidins. PKA participates in the control of both the HSL-mediated and the non-HSL-mediated lipolytic activity of adipocyte (*9*,*15*). As **Figure 1** shows, the lipolytic effect of procyanidins requires PKA involvement, because treatment of cells with the PKA inhibitor H-89 reduced procyanidin-induced lipolysis in a dose-dependent manner. In control cells, 20 μmol/L H-89 did not significantly modify the lipolysis rate (1.25 ± 0.04 vs 1.01 ± 0.01). Procyanidins also increased cAMP levels of the cells, by a similar amount as epinephrine (**Figure 2**). Because of the oligomeric structure of procyanidins, the most suitable way to activate this intracellular-signaling pathway should be procyanidin interaction with β-adrenergic receptors. To check this hypothesis, we applied a simultaneous treatment with procyanidins and a β-adrenergic agonist (epinephrine) or a β-adrenergic antagonist (propanolol). **Figure 3** shows that procyanidin-induced lipolysis remains even at high epinephrine concentrations. And propanolol, both at 10 and at 100 μM, was not able to antagonize the lipolytic effect of procyanidins (PE 140 mg/L, 2.495 ± 0.212; PE + propanolol 10 μM, 1.945 ± 0.033; PE + propanolol 100 μM, 2.536 ± 0.553).

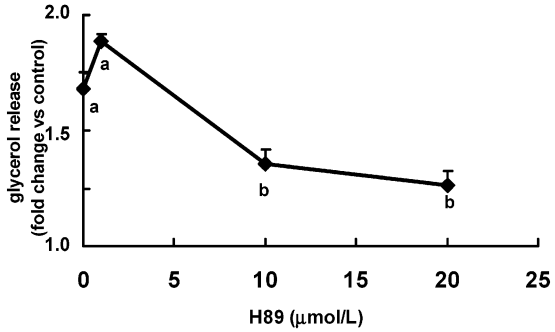

Figure 1. Effect of H89 on procyanidin-induced lipolysis. Fully differentiated 3T3-L1 adipocytes were treated with 140 mg/L grape seed procyanidin extract (PE) in the presence of various concentrations of H89 for 15 h. H89 treatment was done 10 min before PE addition. Data are expressed as glycerol release versus PE-induced lipolysis. Each treatment was previously corrected by its own vehicle control. Values represents mean ± SEM. The letters (a, b) indicate statistically significant differences between H89 concentrations.

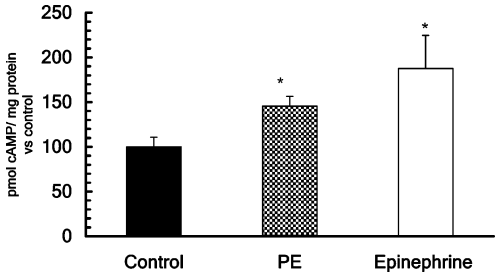

Figure 2. Effect of procyanidin and epinephrine on cyclic AMP levels. Differentiated 3T3-L1 adipocytes were exposed for 8 min to 210 mg/L grape seed procyanidin extract (PE) or 1 μmol/L epinephrine. cAMP levels (pmol/mg protein) are normalized to the control levels (100%). Each value represents mean ± SEM. *$p < 0.05$ as compared to control.

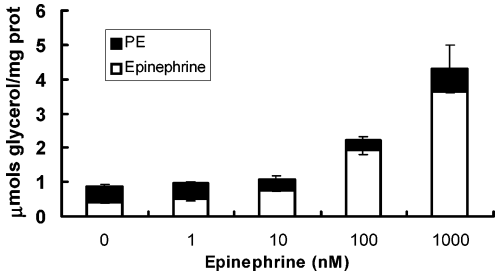

Figure 3. Effect of procyanidins on epinephrine-induced lipolysis. 3T3-L1 adipocytes were incubated for 15 h with 0–1000 nmol/L epinephrine in the absence or presence of 140 mg/L grape seed procyanidin extract (PE). Full column shows simultaneous treatment. ■ represents PE effect, and □ represents epinephrine effect. Values represent mean ± SEM.

PPARγ Participates in the Lipolytic Action of Procyanidins. Of all of the molecules so far described as having lipolytic action, the one that has effects most similar to those of the procyanidins is TNF-α. Thiazolidinedione BRL49653, a high affinity PPARγ agonist (*16*), blocks the lipolytic action of TNF-α (*17*). **Figure 4** shows that BRL49653 cancels out the lipolytic effect of procyanidins. This was a clear dose−response effect, with an IC50 ≈ 70 nmol/L. Total inhibition was achieved around 1 μmol/L BRL49653. However, this clear antagonism between BRL49653 and procyanidins was only observed in the lipolytic effect of procyanidins. Simultaneous treatment with

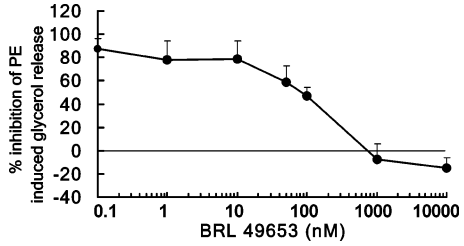

Figure 4. Effect of BRL49653 on procyanidin-induced lipolysis. Fully differentiated 3T3-L1 adipocytes were treated with 140 mg/L grape seed procyanidin extract (PE) in the presence of different concentrations of BRL49653 for 15 h. Data are expressed as % of PE-induced lipolysis. Values represents mean ± SEM.

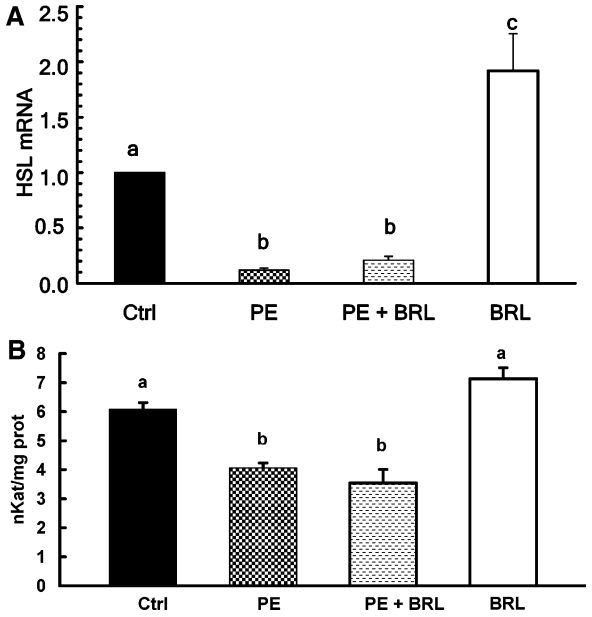

Figure 5. Effect of simultaneous treatment with BRL49653 and procyanidin on HSL mRNA levels (**A**) and G3PDH activity (**B**). Fully differentiated 3T3-L1 adipocytes were treated for 15 h with 140 mg/L grape seed procyanidin extract (PE), 0.1 μmol/L BRL49653 (BRL), and a combination of the two. (**A**) After treatment, total RNA was extracted and gene expression was quantified by real-time RT-PCR. HSL gene expression, normalized by GAPDH mRNA levels, is expressed relative to control cells. (**B**) After treatment, cells were scraped and sonicated. Glycerol-3-phosphate dehydrogenase activity (nKat/mg protein) was assayed in this homogenate. Values represent mean ± SEM. a, b, c indicate groups significantly different with $p < 0.05$.

BRL49653 and procyanidins did not antagonize the effects of procyanidins on adipocyte markers either in the previously published effects (*2*), that is, the decrease in HSL mRNA (**Figure 5A**) or the decrease in G3PDH activity (**Figure 5B**), or in the decrease in PPARγ mRNA levels shown in **Figure 6**.

Although we found no differences in lipid depots due to 24-h procyanidin treatment (**Figure 7**), there was a reduction of some adipocyte markers (mRNA HSL and PPARγ; G3PDH activity). To check whether cells dedifferentiate due to procyanidin treatment, we first exposed cells to 140 mg/L procyanidins for 24 h, and then changed the cells to a new cell culture medium without procyanidins for 3 days. The lipolytic rate, measured as glycerol or as nonesterified fatty acid (NEFA) released to the medium, decreased very strongly after 24 h of procyanidin treatment (**Figure 8A**). Three days after treatment, HSL and PPARγ mRNA levels tended to recover (**Figure 8B**).

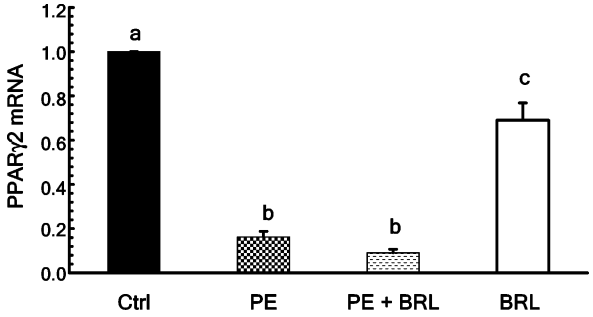

Figure 6. Procyanidin effects on PPARγ2 mRNA levels. 3T3-L1 adipocytes were incubated for 15 h with 140 mg/L grape seed procyanidin extract (PE), 0.1 μmol/L BRL49653 (BRL), and a combination of the two. After treatment, total RNA was extracted and gene expression was quantified by real-time RT-PCR. PPARγ2 gene expression, normalized by GAPDH mRNA levels, is expressed relative to control cells. Values represents mean ± SEM. a, b, c indicate groups significantly different with $p < 0.05$.

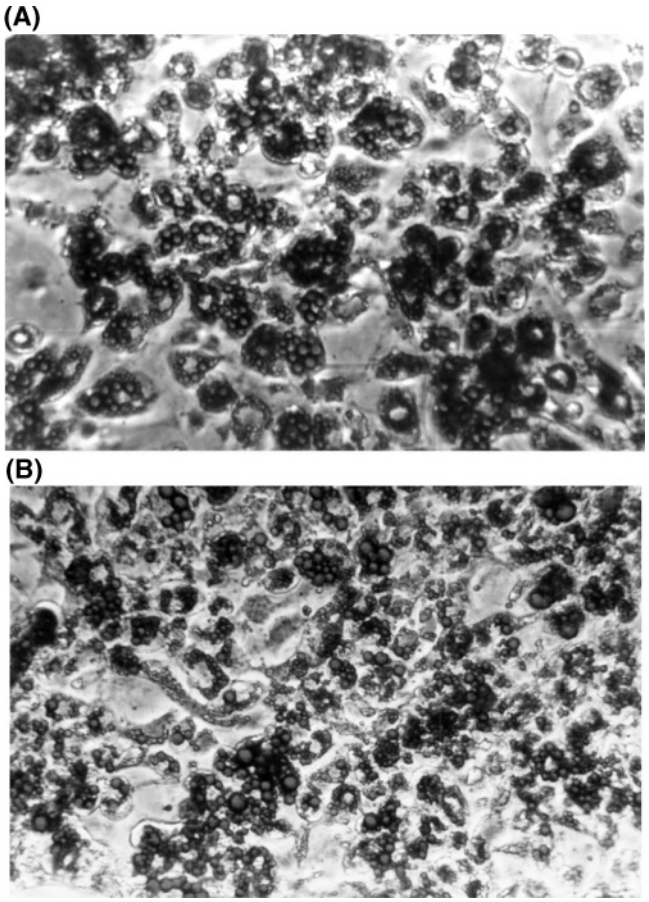

Figure 7. Oil Red O staining of differentiated cells. Fully differentiated (day 10) 3T3-L1 cells were treated for 15 h with (**B**) and without (**A**) 140 mg/L grape seed procyanidin extract (PE) and subsequently stained for lipid accumulation with Oil Red O.

DISCUSSION

We previously described the lipolytic and antilipogenic effects of grape seed-derived procyanidins on 3T3-L1 adipocytes (*2*). We now describe two intracellular mediators (PKA and PPAR-γ) that help to explain the long-term lipolytic effect of procyanidins. Two pieces of evidence support PKA participation: the inhibition of the lipolytic effect by H-89 and the increase in cAMP induced by procyanidins. PPAR-γ involvement was deduced from BRL49653 experiments. BRL49653, at concentrations very close to those at which it binds to PPARγ

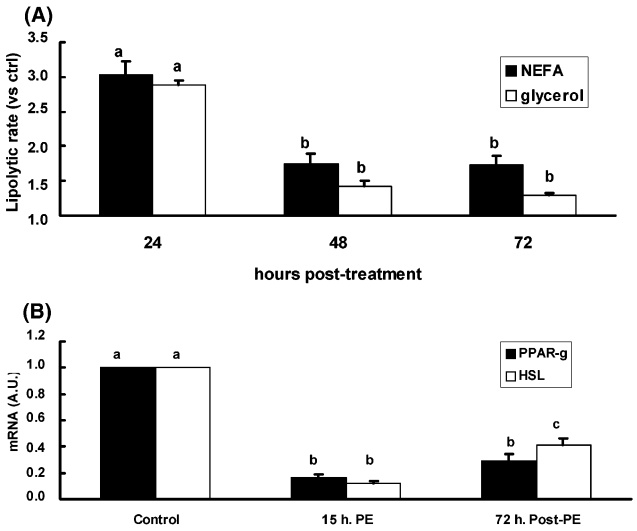

Figure 8. Lypolitic rate and mRNA levels post-procyanidin treatment. 3T3-L1 adipocytes were first incubated for 24 h with 140 mg/L grape seed procyanidin extract (PE). Cells were then changed to a new cell culture medium without PE for 3 days. (**A**) Lypolitic rate versus nontreated cells was measured as glycerol or as NEFA released to the medium. (**B**) PPARγ2 and HSL mRNA levels were quantified by real-time RT-PCR. PPARγ2 and HSL gene expression, normalized by GAPDH mRNA levels, is expressed relative to control cells. Values represents mean ± SEM. a, b, c indicate groups significantly different with $p < 0.05$.

(*16*), antagonized the procyanidin effect, and the inhibition obtained with the simultaneous treatment was total. From our experiments, we cannot say how procyanidins interact with these intracellular mediators. Liang and co-workers reported that some flavonoids (those with a monomeric structure) act as allosteric effectors, bind to PPARγ, and activate it (*18*).

Although Kupussamy (*4*) and Harmon (*19*) reported that flavonoids potentiate β-adrenergic agonist-mediated lipolysis, our results suggest that procyanidins do not interact with β-adrenergic receptors for two reasons: first, because a typical β-adrenergic stimulation of lipolysis works in a short time and the lipolytic effect of procyanidins was observed only after long-term exposure (*2*) and, second, because β-adrenergic antagonists do not remove the lipolytic effect of procyanidins and this lipolytic activity remains after simultaneous treatment with epinephrine. We believe it is much more likely that procyanidins act on some signal transduction pathway that modulates PPARγ activity through PKA and/or mitogen-activated protein kinases (MAPK) (*20*, *21*). PKA involvement has already been shown, and the previous results of our group support procyanidin modulation of MAP kinases (*22*). Williams and co-workers recently revised the role of flavonoids as signaling molecules or as antioxidants (*23*). Their revision reinforced the idea that flavonoids may exert modulatory actions in cells as signaling molecules.

Procyanidin effects show similarities to the TNF-α reported effects (*17*, *24*, *25*): long-term lipolytic activity, down-regulation of adipocyte markers, removal by PPAR-γ agonists of their lipolytic activity, and hypothetical modulation of MAPK signaling mechanism. However, PPAR-γ agonists also remove the down-regulation of adipocyte markers induced by TNF-α (*17*, *25*). In our results, this did not occur. Another difference were the effects on the triglyceride content of the cells. Doses of TNF-α that down-regulate mRNA HSL significantly decrease the amount of triglycerides in the cells (*26*). We did not find statistical differences between the triglyceride contents of procyanidin-treated cells and those of control cells. Despite all of these differences between TNF-α effects and procyanidins effects, we decided to check what post-treatment effect procyanidins had. Most effects of procyanidins after 15−24 h of treatment were reversed 24 or 72 h after treatment. At 72 h after treatment, only PPARγ showed mRNA levels close to those observed during procyanidins treatment. However, adipogenic compounds such as thiazolidinediones also led to decreases in PPARγ mRNA levels in mature adipocytes (*20*). There are many different opinions about the PPARγ mRNA levels that are required for PPARγ to play their important role in maintaining the characteristics of mature adipocyte (*20*, *26*).

In conclusion, from our present results, we define two intracellular mediators of grape-procyanidin-induced lipolysis. The main one is the peroxisome proliferator-activated receptor-γ (PPAR-γ), although protein kinase A (PKA) is also involved. More work is needed to determine the mechanisms that explain the complete sequence of signaling events and ascertain the role of procyanidins on the adipocyte differentiation program.

ABBREVIATIONS USED

HSL, hormone sensitive lipase; G3PDH, glycerol-3-phosphate dehydrogenase; β-PDGF, platelet-derived growth factor β; C/EBPβ, CCAAT/enhancer binding protein-β; PKA, cAMP-dependent protein kinase A; TNFα, tumor necrosis factor-α; PPARγ, peroxisome proliferator-activated receptor-γ; TZD, thiazolidinediones; PE, grape seed procyanidin extract; NEFA, nonesterified fatty acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinases.

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LITERATURE CITED

(1) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S−2085S.

(2) Ardévol, A.; Bladé, C.; Salvadó, M. J.; Arola, L. Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes. *Int. J. Obesity* **2000**, *24*, 319−324.

(3) Rosenkranz, S.; Knirel, D.; Dietrich, H.; Flesch, M.; Erdmann, E.; Bohm, M. Inhibition of the PDGF receptor by red wine flavonoids provides a molecular explanation for the "French paradox". *FASEB J.* **2002**, *16*, 1958−1960.

(4) Kuppusamy, U. R.; Das, N. P. Potentiation of β-adrenoreceptor agonist-mediated lipolysis by quercetin and fisetin in isolated rat adipocytes. *Biochem. Pharmacol.* **1994**, *47*, 521−529.

(5) Akiyama, T.; Ishida, J.; Nakagawa, S.; Ogawara, H.; Watanabe, S.; Itoh, N.; Shibuya, M.; Fukami, Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **1987**, *262*, 5592−5595.

(6) Harmon, A. W.; Patel, Y. M.; Harp, J. B. Genistein inhibits CCAAT/enhancer-binding protein β (C/EBPβ) activity and 3T3-L1 adipogenesis by increasing C/EBP homologous protein expression. *Biochem. J.* **2002**, *367*, 203−208.

(7) Holm, C.; Osterlund, T.; Laurell, H.; Contreras, J. A. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annu. Rev. Nutr.* **2000**, *20*, 365−393.

(8) Birnbaum, M. J. Lipolysis: more than just a lipase. *J. Cell Biol.* **2003**, *161*, 1011−1012.

(9) Okazaki, H.; Osuga, J.-i.; Tamura, Y.; Yahagi, N.; Tomita, S.; Shionoiri, F.; Iizuka, Y.; Ohashi, K.; Harada, K.; Kimura, S.; Gotoda, T.; Shimano, H.; Yamada, N.; Ishibashi, S. Lipolysis in the Absence of Hormone-Sensitive Lipase: Evidence for a Common Mechanism Regulating Distinct Lipases. *Diabetes* **2002**, *51*, 3368−3375.
(10) Carey, G. B. Mechanisms regulating adipocyte lipolysis. *Adv. Exp. Med. Biol.* **1998**, *441*, 157−170.
(11) Zhang, B.; Berger, J.; Hu, E.; Szalkowski, D.; White-Carrington, S.; Spiegelman, B. M.; Moller, D. E. Negative regulation of peroxisome proliferator-activated receptor-γ gene expression contributes to the antiadipogenic effects of tumor necrosis factor-α. *Mol. Endocrinol.* **1996**, *10*, 1457−1466.
(12) Garland, P. B.; Randle, R. J. A rapid enzymatic assay for glycerol. *Nature* **1962**, *196*, 987−988.
(13) Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(14) Wise, L. S.; Green, H. Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. *J. Biol. Chem.* **1979**, *254*, 273−275.
(15) Holm, C. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem. Soc. Trans.* **2003**, *31*, 1120−1124.
(16) Lehmann, J. M.; Moore, L. B.; Smith-Oliver, T. A.; Wilkinson, W. O.; Willson, T. M.; Kliewer, S. A. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J. Biol. Chem.* **1995**, *270*, 12953−12956.
(17) Souza, S. C.; Yamamoto, M. T.; Franciosa, M. D.; Lien, P.; Greenberg, A. S. BRL49653 blocks the lipolytic actions of tumor necrosis factor-α. *Diabetes* **1998**, *47*, 691−695.
(18) Liang, Y. C.; Tsai, S. H.; Tsai, D. C.; Lin-Shiau, S. Y.; Lin, J. K. Suppression of inducible cyclooxygenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor-γ by flavonoids in mouse macrophages. *FEBS Lett.* **2001**, *496*, 12−18.
(19) Harmon, A. W.; Joyce, B. H. Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. *Am. J. Physiol. Cell Physiol.* **2001**, *280*, C807−C813.
(20) Watanabe, M.; Inukai, K.; Katagiri, H.; Awata, T.; Oka, Y.; Katayama, S. Regulation of PPAR-γ transcriptional activity in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* **2003**, *300*, 429−436.
(21) Lazennec, G.; Canaple, L.; Saugy, D.; Wahli, W. Activation of Peroxisome Proliferator-Activated Receptors (PPARs) by Their Ligands and Protein Kinase A Activators. *Mol. Endocrinol.* **2000**, *14*, 1962−1975.
(22) Pinent, M.; Blay, M.; Blade, M. C.; Salvado, M. J.; Arola, L.; Ardevol, A. Grape Seed-Derived Procyanidins Have an Antihyperglycemic Effect in Streptozotocin-Induced Diabetic Rats and Insulinomimetic Activity in Insulin-Sensitive Cell Lines. *Endocrinology* **2004**, *145*, 4985−4990.
(23) Williams, R. J.; Spencer, J. P. E.; Rice-Evans, C. Flavonoids: antioxidants or signaling molecules? *Free Radical Biol. Med.* **2004**, *36*, 838−849.
(24) Perrey, S.; Ishibashi, S.; Yahagi, N.; Osuga, J.; Tozawa, R.; Yagyu, H.; Ohashi, K.; Gotoda, T.; Harada, K.; Chen, Z.; Iizuka, Y.; Shionoiri, F.; Yamada, N. Thiazolidinedione- and tumor necrosis factor alpha-induced downregulation of peroxisome proliferator-activated receptor gamma mRNA in differentiated 3T3-L1 adipocytes. *Metabolism* **2001**, *50*, 36−40.
(25) Rosenbaum, S. E.; Greenberg, A. S. The short- and long-term effects of tumor necrosis factor-α and BRL49653 on peroxisome proliferator-activated receptor (PPAR)γ2 gene expression and other adipocyte genes. *Mol. Endocrinol.* **1998**, *12*, 1150−1160.
(26) Tamori, Y.; Masugi, J.; Nishino, N.; Kasuga, M. Role of Peroxisome Proliferator-Activated Receptor-γ in Maintenance of the Characteristics of Mature 3T3-L1 Adipocytes. *Diabetes* **2002**, *51*, 2045−2055.

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Grape Seed-Derived Procyanidins Have an Antihyperglycemic Effect in Streptozotocin-Induced Diabetic Rats and Insulinomimetic Activity in Insulin-Sensitive Cell Lines

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Flavonoids are functional constituents of many fruits and vegetables. Some flavonoids have antidiabetic properties because they improve altered glucose and oxidative metabolisms of diabetic states. Procyanidins are flavonoids with an oligomeric structure, and it has been shown that they can improve the pathological oxidative state of a diabetic situation. To evaluate their effects on glucose metabolism, we administered an extract of grape seed procyanidins (PE) orally to streptozotocin-induced diabetic rats. This had an antihyperglycemic effect, which was significantly increased if PE administration was accompanied by a low insulin dose. The antihyperglycemic effect of PE may be partially due to the insulinomimetic activity of procyanidins on insulin-sensitive cell lines. PE stimulated glucose uptake in L6E9 myotubes and 3T3-L1 adipocytes in a dose-dependent manner. Like insulin action, the effect of PE on glucose uptake was sensitive to wortmannin, an inhibitor of phosphoinositol 3-kinase and to SB203580, an inhibitor of p38 MAPK. PE action also stimulated glucose transporter-4 translocation to the plasma membrane. In summary, procyanidins have insulin-like effects in insulin-sensitive cells that could help to explain their antihyperglycemic effect *in vivo*. These effects must be added to their antioxidant activity to explain why they can improve diabetic situations. (*Endocrinology* 145: 4985–4990, 2004)

FLAVONOIDS ARE PHENOLIC compounds that are widely found in fruits and vegetables (1). They have a broad range of biological activities (2–5). They function as powerful antioxidants, as phytoestrogens, and can alter the activities of important cell-signaling enzymes, such as tyrosine kinases, phosphodiesterases, and phosphoinositide kinases (6). Some may also have antidiabetic activity (7–10). Studies of the *in vivo* and *in vitro* effects of various flavonoids on glucose metabolism have shown opposite and often controversial results. This is probably because of the different structural characteristics of the molecules and the different experimental designs used (11–13). Procyanidins, a group of flavonoids, are oligomeric forms of catechins that are abundant in red wine, grapes, cocoa, and apples (1). It has been shown that they have a role in protecting against the altered oxidative state of diabetic situations (14–17). The only reported information about how they affect glucose metabolism derives from the recent work of Al-awwadi and colleagues (18) that describes an antidiabetic activity of a red wine polyphenolic extract in streptozotocin (STZ)-treated rats.

Hyperglycemia is characteristic of type I and type II diabetes mellitus, which is mainly caused by insulin deficiency and/or a failure of normal insulin levels to stimulate glucose uptake in tissues. Although defects in glucose homeostasis have been recognized for decades, the molecular mechanisms involved in impaired whole body glucose uptake are still not understood. It is now clear, however, that the aggressive control of hyperglycemia can attenuate the development of chronic complications. Significant progress has been made in identifying the molecular components and signaling pathways involved in the short-term regulation of glucose uptake (19, 20). Insulin binds to the cell surface insulin receptor and activates its intrinsic tyrosine kinase activity. The subsequent activation of phosphatidylinositol 3-kinase (PI3K) is necessary for the recruitment of glucose transporter-4 (GLUT-4) to the cell surface. In addition, emerging evidence suggests that a second signaling cascade, which functions independently of the PI3K pathway, is required for the insulin-dependent translocation of GLUT-4 (21, 22). However, GLUT-4 translocation to the plasma membrane does not account for all the increase in glucose uptake due to insulin stimulation. It has recently been shown that insulin also stimulates GLUT-4 activation through p38 MAPK signaling (23).

A suitable antidiabetic agent should have actions similar to those of insulin, or it should bypass the defects in insulin action characterized by insulin resistance. In this report we assess how effective a grape seed-derived procyanidin is at lowering hyperglycemia in an insulin-deficient rat model of diabetes: the STZ-induced diabetic rat. To analyze the biochemical mechanism of this postulated effect, we evaluate the insulinomimetic activity of this grape seed-derived procyanidin working with two cell culture lines of insulin-sensitive tissues: L6E9 myotubes and 3T3-L1 adipocytes.

Abbreviations: FBS, Fetal bovine serum; GLUT-4, glucose transporter-4; LDM, low density microsome; PE, grape seed procyanidin extract; PI3K, phosphatidylinositol 3-kinase; PM, plasma membrane; STZ, streptozotocin.

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Materials and Methods

Cells, reagents, and materials

Grape seed procyanidin extract (PE) was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this PE extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5–13 U; 31.7%) procyanidins.

Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium), and SB203580 was purchased from Calbiochem (Merck, Darmstadt, Germany). Insulin (Actrapid) was obtained from Novo Nordisk (Bagsvaerd, Denmark). Bradford protein reagent was obtained from Bio-Rad Laboratories (Hercules, CA). L6E9 cells and anti-GLUT-4 antibody were provided by Dr. Marta Camps and Prof. Antonio Zorzano (University of Barcelona, Barcelona, Spain), and antirabbit peroxidase-conjugated antibody, wortmannin, and STZ were obtained from Sigma-Aldrich Corp. (St. Louis, MO). 2-Deoxy-D-[^{3}H]glucose and enhanced chemiluminescence detection reagent were purchased from Amersham Biosciences (Little Chalfont, UK).

Induction of experimental diabetes

Male Wistar rats, weighing 250 g, were purchased from Charles River Laboratories (Barcelona, Spain). The animals were housed in animal quarters at 22 C with a 12-h light, 12-h dark cycle and were fed *ad libitum*. All procedures were approved by the animal ethics committee of our university. Type 1 diabetes mellitus was induced by ip injection of a freshly prepared solution of STZ (70 mg/kg) in 50 mM citrate buffer, pH 4.5. The only diabetic animals used were those with polyuria, glycosuria, and hyperglycemia (~20 mM) 2–3 d postinduction. All studies were carried out 1 wk after STZ had been injected.

Animal experimental procedures

STZ-diabetic rats were divided into five groups of six or seven rats each. In the control group rats were fed an oral gavage with vehicle (tap water). In the PE group rats were given an oral gavage of PE in aqueous solution (250 mg/kg body weight). In the insulin group rats were given 9 nmol/animal insulin, ip, and fed an oral gavage with tap water. In the low insulin group rats were given 1.26 nmol/animal insulin, ip, and fed an oral gavage with tap water. In the PE plus low insulin group rats were given 1.26 nmol/animal insulin, ip, and fed an oral gavage with PE (250 mg/kg body weight).

At 1100 h, blood glucose levels were measured, and then (time zero) the respective treatments were administered. Blood samples were collected by tail bleeding, and glucose was measured at 30, 60, 90, 120, 150, 210, and 270 min.

An additional experiment was performed with the same experimental model, but only with fasted animals from the control and PE groups. Food was withdrawn at 0700 h, and at 1400 h (time zero), a blood sample was collected by tail bleeding. The respective treatments were then administered. Next, blood was again extracted after 60 min. Blood glucose was determined with a glucometer (Glucocard, Menarini, Barcelona, Spain).

Cell culture and differentiation

L6E9 myoblasts were cultured in supplemented DMEM [10% (vol/vol) fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM glutamine, and 25 mM HEPES (pH 7.4)]. Preconfluent myoblasts (80–90%) were induced to differentiate by lowering FBS to a final concentration of 2% (vol/vol).

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (24). Briefly, confluent preadipocytes were treated with 0.25 μM dexamethasone, 0.5 mM 3-isobutylmethylxanthine, and 5 μg/ml insulin for 2 d in 10% FBS containing DMEM. Cells were switched to 10% FBS/DMEM containing only insulin for 2 d, then to 10% FBS/DMEM without insulin. Ten days after differentiation had been induced, cells were treated with PE and used for the experiments.

Cell treatment

Fully differentiated adipocytes and myotubes were washed twice with PBS and incubated at 37 C with serum-free, supplemented DMEM containing 0.2% BSA (depletion medium) for 2 and 5 h, respectively. Acute treatment with PE and/or insulin was carried out during the last 30 or 60 min of depletion in adipocytes and myotubes, respectively. All PE concentrations assayed were nontoxic for both 3T3-L1 adipocytes (24) and L6E9 myotubes (25).

Glucose uptake assay

Glucose transport was determined by measuring the uptake of 2-deoxy-D-[^{3}H]glucose. Cells were cultured on six- or 12-well plates. Transport assay was initiated by washing the cells twice in a transport solution (20 mM HEPES, 137 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, and 2 mM pyruvate, pH 7.4). Cells (myotubes and adipocytes) were then incubated for 7–10 min in the transport solution, which contained 0.1 mM 2-deoxy-D-glucose and 1 μCi 2-deoxy-D-[^{3}H]glucose (10 mCi/mmol). Glucose uptake was stopped by adding 2 vol ice-cold 50 mM glucose in PBS and washing twice in the same solution. Cells were disrupted by adding 0.1 M NaOH/0.1% PBS, and radioactivity was determined by scintillation counting (Packard 1500 Tri-Cab, Izasa SA, Madrid, Spain). Glucose transport values were corrected for protein content, which was determined by the Bradford method (26). Each condition was run in triplicate.

To determine whether PI3K and p38 MAPK were involved in the signaling pathways that were potentially used by PE, we performed assays using specific inhibitors of these kinases. Wortmannin (1 μM) and SB 203580 (100 μM) were added 15 and 20 min, respectively, before PE and insulin treatments. Glucose uptake was then assayed.

Cell fractionation method

To obtain plasma membranes (PM) and low density microsomes (LDM), cells were grown on 75-cm^2 flasks and treated as described above. Then a subcellular fractionation was carried out with slight variations to the method used by Simpson and colleagues (27). Briefly, cells were homogenized, and the homogenate was centrifuged for 20 min at 15,800 $\times$ *g* to pellet the PM fraction. The supernatant was centrifuged for 75 min at 180,000 $\times$ *g* to pellet the LDM fraction. The fractions were then resuspended in HES (20 mM HEPES, 1 mM EDTA, and 255 mM sucrose, pH 7.4) and subjected to Western blotting analysis.

Electrophoresis and immunoblot analysis

Aliquots of fractions (PM and LDM) were resolved on 7.5% SDS-PAGE and transferred to nitrocellulose membranes, and the membranes were blocked with PBS 5% low-fat dry milk. Incubation with the primary antibody (anti-GLUT-4; 1:1000 dilution) was performed overnight. Membranes were washed in PBS/0.1% Tween and incubated with the peroxidase-conjugated secondary antibody (1:1000 dilution) for 2 h. Then they were washed again in PBS/0.1% Tween. Bands were visualized with enhanced chemiluminescence detection reagents.

Calculations and statistical analysis

Results are expressed as the mean $\pm$ SEM. Effects were assessed using one-way ANOVA or *t* test. We used Tukey's test of honestly significant differences to make pairwise comparisons. All calculations were performed using SPSS software (SPSS, Inc., Chicago, IL).

Results

Acute administration of procyanidins reduced hyperglycemia in STZ-induced diabetic rats

To assess the potential role of procyanidins in improving hyperglycemia, we used an animal model in which there is no, or very little, insulin secretion: STZ-induced diabetic rats. Figure 1A shows that an acute gavage of PE (250 mg PE/kg body weight) significantly reduced blood glucose levels. Although we started our experiment 2 h after the light period had begun, the control group showed that there was some intestinal glucose absorption. To ascertain whether the inhibition of glucose absorption in the intestine was not the

only thing to mediate the glycemia-lowering effect of PE, we administered the same dose of PE to fasted animals. In this case, Table 1 shows that 1 h after PE treatment, blood glucose levels also significantly decreased ($P = 0.004$) *vs.* those in the control group. Figure 1B shows that simultaneous administration of a low dose of insulin (1.26 nmol) and PE caused an additive effect, but it was not as high as that of an effective insulin dose (9 nmol). Table 2 summarizes the ANOVA results of all treatments on nonfasted animals and shows that the PE effect was greater than that caused by a low dose of insulin.

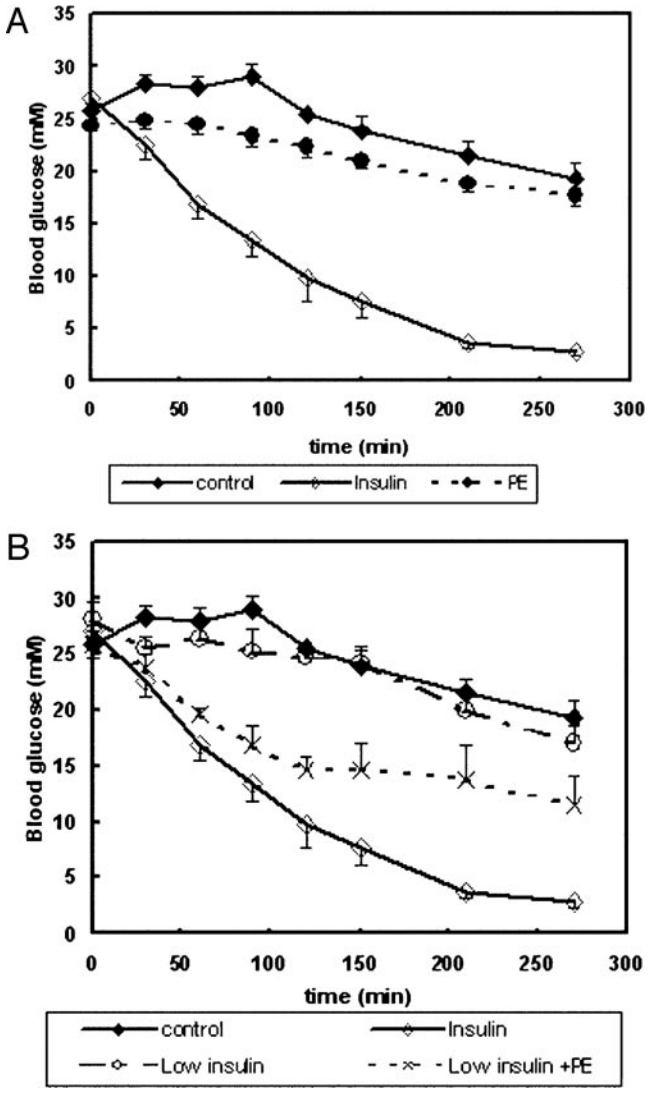

FIG. 1. Effect of PE treatments on glucose levels in STZ-induced diabetic rats. At 1100 h (time zero), a blood sample was collected by tail bleeding, and respective treatments were administered. Blood samples were extracted at the times indicated in the figure. Glucose levels were quantified by a glucometer. The data are the mean ± SEM of six animals. A, Effect of PE on glucose levels. B, Additive effect of a simultaneous treatment with PE plus a low dose of insulin. Table 2 summarizes the ANOVA results of the overall effect of the treatments shown in A and B. The *different letters* indicate statistically significant differences between treatments.

TABLE 1. Changes in blood glucose levels in fasted animals due to procyanidin treatment

	Blood glucose (mM)		
	0 h	1 h	Decrease
Control	19.31 ± 0.64	20.61 ± 1.40	1.31 ± 0.72
PE	19.65 ± 0.70	18.26 ± 1.12	−1.39 ± 0.52[a]

At 0700 h, food was removed from animal cages. At 1400 h (time zero), a blood sample was collected by tail bleeding, and respective treatments were administered. After 1 h, blood glucose was measured. The data are the mean ± SEM of six animals.

[a] Significant differences *vs.* the control group at $P < 0.05$.

TABLE 2. ANOVA results of all treatments on nonfasted animals

Treatment	Control	Low insulin	PE	Low insulin + PE	Insulin
ANOVA result	a	a, b	b	c	d

Different letters indicate statistically significant differences between treatments.

Procyanidins increase glucose uptake in insulin-sensitive cell lines (L6E9 myotubes and 3T3-L1 adipocytes)

To determine whether the effects of procyanidins *in vivo* were due to their action on insulin-sensitive tissues, we used the differentiated cell lines L6E9 myotubes and 3T3-L1 adipocytes. Figure 2 shows that PE treatment caused a dose-dependent increase in glucose uptake in both cell lines. These dose-response effects showed some differences between myotubes and adipocytes. L6E9 myotubes, at 0.14 mg PE/liter, increased their glucose uptake *vs.* untreated cells, and their maximal stimulation was 1.6-fold greater than basal rates. 3T3-L1 adipocytes, however, needed a higher concentration of PE to stimulate their glucose uptake (35 mg PE/liter), but at their maximal nontoxic PE concentration (140 mg PE/liter), its stimulation was stronger (7-fold above basal). Nevertheless, in both cell lines the effect of PE on glucose uptake at the maximal doses assayed was 50% that of insulin (55.3 ± 13.85% for L6E9; 45.32 ± 3.35% for 3T3-L1). To evaluate the effects of simultaneous administration of PE and insulin, we incubated adipocytes with a low dose of insulin (10 nM) and several doses of PE. Figure 3 shows that the presence of PE in the cell culture medium boosted the effect of a low insulin concentration. In 3T3-L1 adipocytes, 7 mg/liter of PE, a nonstimulating dose by itself, together with a low insulin dose (10 nM) almost doubled the capacity, induced by insulin in the cell, to capture glucose.

Procyanidin-induced increase in glucose uptake shares some mediators of insulin mechanism of action

Previous results show that procyanidins have an insulin-like effect. To elucidate the mechanism by which PE stimulates glucose uptake, we attempted to determine whether PE shared some of main cellular mediators of insulin signaling. Firstly, the combination of the highest stimulating concentration of PE and a supramaximal insulin concentration did not cause an additive stimulation of glucose transport in myotubes (Table 3). In adipocytes, there was some additive effect, although it did not reach the total sum of both the independent effects (Table 3). To determine whether the effects of PE on glucose transport required intact PI3K activity, wortmannin was added before PE to the cell culture

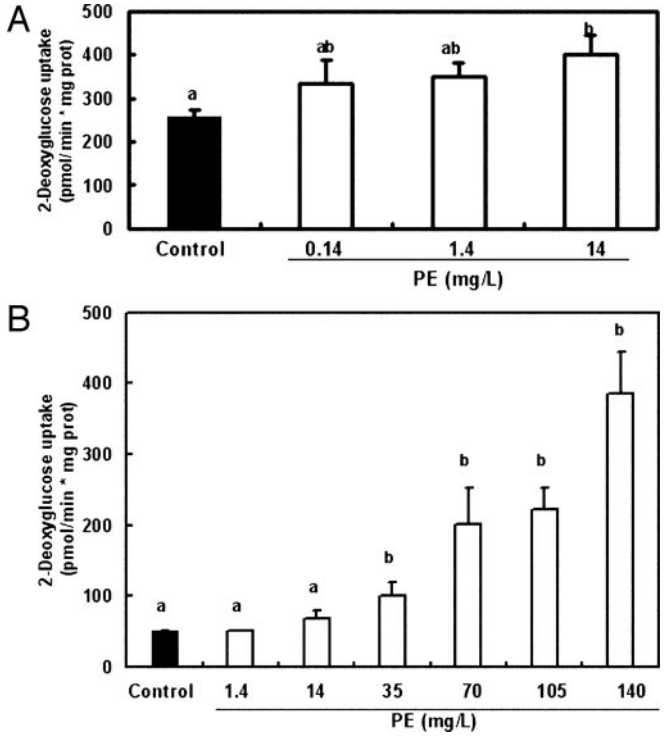

FIG. 2. Dose-response effects on glucose uptake due to PE treatment in L6E9 myotubes and 3T3-L1 adipocytes. Fully differentiated myotubes (A) and adipocytes (B) were incubated with serum-free medium supplemented DMEM containing 0.2% BSA for 5 and 2 h, respectively. Acute treatment with several doses of PE was carried out during the last 60 or 30 min of depletion in myotubes and adipocytes, respectively. At the end of the treatment, 2-deoxyglucose uptake was assayed. The data are the mean ± SEM of at least three different experiments. a and b, Statistically significant differences between PE concentrations.

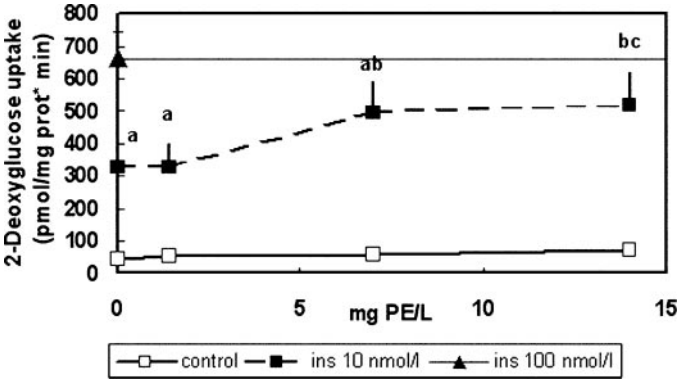

FIG. 3. Dose-response effect on glucose uptake due to simultaneous treatment with a low dose of insulin plus PE in 3T3-L1 adipocytes. Fully differentiated adipocytes were serum-depleted as indicated in Fig. 2. Simultaneous treatment with several doses of PE with or without 10 nM insulin was applied during the last 30 min of depletion. Cells treated with 100 nM insulin were used as a positive control. At the end of the treatment, the 2-deoxyglucose uptake was assayed. The data are the mean ± SEM of three different experiments. a, b, and c, Statistically significant differences between PE concentrations.

medium. Figure 4 shows that PE-induced glucose transport was inhibited by wortmannin in both cell lines. As positive control, we simultaneously treated 3T3-L1 adipocytes and

TABLE 3. Effects due to simultaneous treatment with high doses of PE and insulin in L6E9 myotubes and 3T3-L1 adipocytes

	L6E9	3T3-L1
Insulin	100	100
PE	43.18 ± 29.97	45.32 ± 3.35
Insulin + PE	113.7 ± 10.41	121.9 ± 6.23[a]

Fully differentiated myotubes and adipocytes were serum-depleted as described in Fig. 2. They were treated with their respectives high doses of PE (L6E9, 14 mg/liter; 3T3-L1, 140 mg/liter), without or with insulin 1 μmol/liter, during the last 60 or 30 min of depletion in myotubes and adipocytes, respectively. At the end of treatment, 2-deoxyglucose uptake was assayed. Results are expressed as the percentage of the insulin effect (100%). The data are the mean ± SEM of three different experiments.

[a] Significant differences *vs.* insulin treatment at $P < 0.05$.

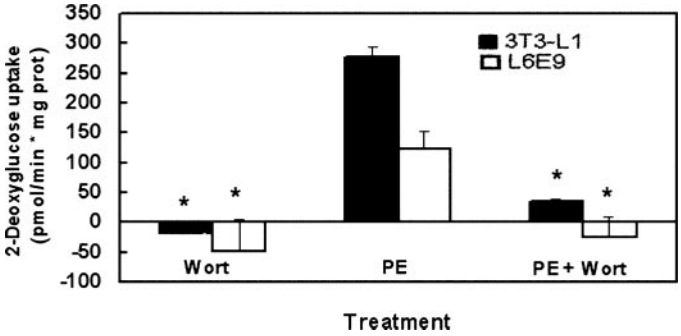

FIG. 4. Effects of wortmannin on PE-stimulated glucose uptake in L6E9 myotubes and 3T3-L1 adipocytes. Differentiated cells were serum-depleted as indicated in Fig. 2. L6E9 myotubes and 3T3-L1 adipocytes were treated with wortmannin (1 μM) before PE (14 mg/liter for 1 h for L6E9; 140 mg/liter for 30 min for 3T3-L1) was added to the cell culture medium. At the end of the treatment, 2-deoxyglucose uptake was assayed. The data are the mean ± SEM of three different experiments. *, Significant differences *vs.* PE-treated group at $P < 0.05$.

L6E9 myotubes with insulin or insulin plus wortmannin. In both cell lines, wortmannin inhibited insulin-induced glucose uptake (data not shown). These results prove that PE requires PI3-kinase activity to be intact if it is to have a stimulatory effect on cellular glucose uptake.

Insulin stimulation of glucose uptake activates two responses on GLUT-4: a very well established effect recruiting GLUT-4 transporters from intracellular reserves to the plasma membrane, and an increase in the intrinsic activity of the transporter, a p38 MAPK-mediated effect. We studied whether PE also activated both responses on GLUT-4. 3T3-L1 adipocytes treated with 140 mg/liter PE increased the amount of GLUT-4 in the plasma membrane (PE, 9.26 ± 4.55; control, 3.61 ± 1.33; Fig. 5). To determine whether the effects of PE on glucose transport required p38MAPK activity, SB203580 (100 μM) was added 20 min before PE treatment. Figure 6 shows that SB203580 inhibited PE-induced glucose transport to a similar extent as it inhibited insulin-induced glucose transport.

Discussion

In this study we show that PE mimics the role of insulin *in vivo* and *in vitro*. We show that an acute dose of PE has an antihyperglycemic effect on an insulin-deficient animal

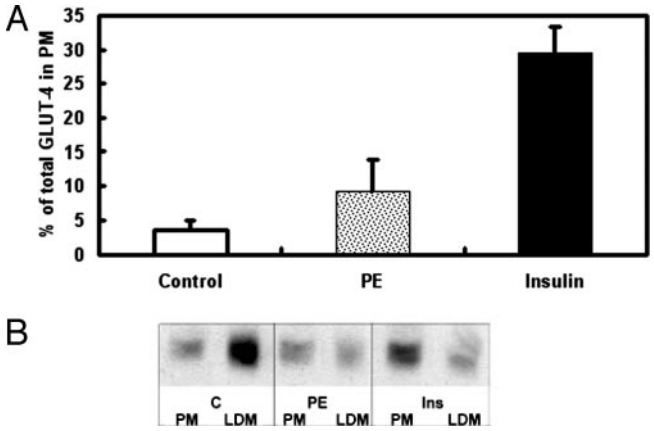

FIG. 5. Effect of PE on distribution of GLUT-4 between plasma membrane and intracellular membranes in 3T3-L1 adipocytes. Fully differentiated adipocytes were serum-depleted as indicated in Fig. 2. They were treated with 140 mg/liter PE or 100 nM insulin or were untreated during the last 30 min of depletion. PM and LDM fractions were obtained and assayed by Western blot to determine the abundance of the GLUT-4 glucose transporter. A, Percentage of total GLUT-4 that was located in the PM. B, Representative autoradiogram [30 μg (PM) and 5 μg (LDM) protein/lane]. The data are the mean ± SEM of at least three different experiments.

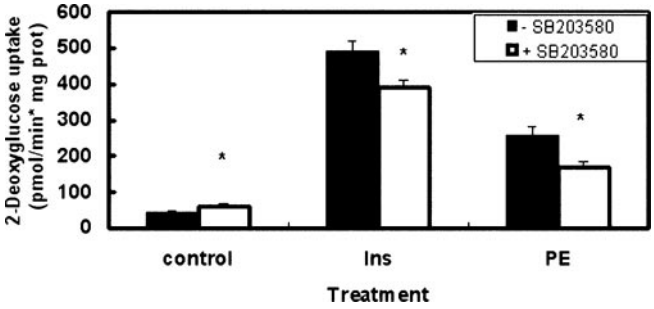

FIG. 6. Effects of SB203580 on PE-stimulated glucose uptake in 3T3-L1 adipocytes. Adipocytes were serum-depleted as indicated in Fig. 2. Cells were pretreated with SB203580 (100 μM) for 20 min before 140 mg/liter PE were added to the cell culture medium. After 30 min of simultaneous treatment, 2-deoxyglucose uptake was assayed. The data are the mean ± SEM of at least three different experiments. *, Significant differences *vs.* non-SB203580 group at $P < 0.05$.

model, the STZ-induced diabetic rat. The antihyperglycemic role of PE by itself was moderate, but simultaneous administration with a low dose (one seventh the effective dose) of insulin boosted the effect to such an extent that it was similar to that of other typical antihyperglycemic agents, such as metformin and troglitazone (28). To our knowledge, this is the first report of an antihyperglycemic effect mediated by PE *in vivo*. Previous studies have shown that wine flavonoids improve the altered oxidative status of the diabetic situation, but they have not found any effect on glycemia. Landrault and colleagues (14) reported that the daily administration of an ethanol-free white wine enriched in phenolics to STZ-induced diabetic rats improved the oxidative stress associated with diabetes. Also, Ceriello and colleagues (16) found that acute moderate red wine consumption during a meal (300 ml red wine, ~300 mg of flavonoids) improved oxidative status in the absorptive phase in human type 2 diabetic patients.

The differences between our results and those obtained by others may be due to the different composition and dose of procyanidins used and the diabetic situation tested, although little work has been done with procyanidins. The blood glucose-lowering activity of other flavonoids, however, has been defined for epicatechin (29), catechin (30), and epicatechin gallate (7, 31). These monomeric forms act through different mechanisms: epicatechin induces pancreatic β-cell regeneration (29), catechin inhibits intestinal glucose absorption (30), and epicatechin gallate increases hepatic glycogen synthesis (7, 31). Only Al-awwadi and colleagues (18) observed that a polyphenol extract from a red wine administered for 6 wk to STZ-induced diabetic rats has an antidiabetic activity, but they do not describe any possible mechanisms to explain this effect. Our *in vivo* experiments suggest that PE can lower glucose levels by at least two mechanisms: a delay in intestinal glucose absorption and an insulin-like effect on insulin-sensitive tissues. Both effects were present in nonfasted animals: 1 h after PE treatment, the glucose level of PE-treated animals was 24.51 ± 1.00 mM, approximately 4 mM lower than that of the control group (28.01 ± 1.08 mM). And when the animals had fasted for 7 h, thus eliminating the effect on glucose absorption, we found a smaller difference (~2 mM) between the glycemia in the PE-treated group (18.26 ± 1.12 mM) and that in the control group (20.61 ± 1.4 mM). To explain the biochemical basis of this last effect, we hypothesized that procyanidins act on insulin-sensitive tissues. It has been reported that some flavonoids stimulate glucose uptake in cultured cells (8, 12, 13). Others, however, are *in vitro* inhibitors of glucose transport in rat adipocytes (11, 32–34).

To date, however, the effects of procyanidins on glucose uptake in insulin-sensitive cells have not been studied. In this respect we show that PE has an insulin-like effect on glucose uptake in two insulin-sensitive cell lines: 3T3-L1 adipocytes and L6E9 myotubes. In both cell lines, PE alone did not cause maximal insulin stimulation, but there was a synergic effect between PE and insulin in 3T3-L1. With this simultaneous treatment, a low dose of insulin and PE, we managed to stimulate glucose uptake to a similar extent as that caused by a maximal insulin concentration. There are various molecular mechanisms by which this stimulation of glucose uptake could take place. Waltner-Law and colleagues (31) showed that epigallocatechin gallate has an insulin-like effect on hepatocytes mediated by changes in the redox state that affect the functional states of some intracellular mediators of insulin signaling. We do not discard this possibility, because procyanidins are also very well described antioxidants (35). However, the high affinity of procyanidins for binding to proteins is also very well described (36–38). Our present results show that the PE stimulation of glucose uptake does not use a different or complementary mechanism to that of insulin in myotubes, because simultaneous treatment with saturating doses of insulin and PE did not cause an additive effect. We do not discard a complementary mechanism in adipocytes, because we observed that simultaneous treatment leads to some addition, but it did not reach the sum of both independent effects. Therefore, procyanidins in adipocytes must also use insulin mechanisms.

Our results also support the idea that the PE stimulation of glucose uptake in adipocytes and myotubes acts through

some of the main intracellular mediators described for the insulin signaling pathway (PI3K and p38 MAPK). In both cell lines, the inhibition of PE-stimulated glucose uptake caused by wortmannin was similar to that caused by insulin stimulation. The p38 MAPK inhibitor, SB203580, led to a similar situation. PE also increased the amount of insulin-sensitive glucose transporter, GLUT-4, in the plasma membrane. Like the effect of PE on the stimulation of glucose uptake, the effect of PE on GLUT-4 translocation was less than that of insulin.

In conclusion, our results indicate that PE is an antihyperglycemic agent with insulinomimetic properties. We have shown that procyanidins mimic and/or influence insulin effects by directly acting on specific components of the insulin-signaling transduction pathway. This insulin-like role of procyanidins must be added to their very well described effect of improving altered oxidative states.

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References

1. **Scalbert A, Williamson G** 2000 Dietary intake and bioavailability of polyphenols. J Nutr 130(8S Suppl): 2073S–2085S
2. **Ross JA, Kasum CM** 2002 Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr 22:19–34
3. **Knekt P, Kumpulaineu J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T, Aromaa A** 2002 Flavonoid intake and risk of chronic diseases. Am J Clin Nutr 76:560–568
4. **Middleton EJR, Kandaswami C, Theoharides TC** 2000 The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52:673–751
5. **Havsteen BH** 2002 The biochemistry and medical significance of the flavonoids. Pharmacol Ther 96:67–202
6. **Gamet-Payrastre L, Manenti S, Gratacap M-P, Tulliez J, Chap H, Payrastre B** 1999 Flavonoids and the inhibition of PKC and PI 3-kinase. Gen Pharmacol 32:279–286
7. **Kao Y-H, Hiipakka RA, Liao S** 2000 Modulation of endocrine systems and food intake by green tea epigallocatechin gallate. Endocrinology 141:980–987
8. **Ong KC, Khoo H** 1996 Insulinomimetic effects of myricetin on lipogenesis and glucose transport in rat adipocytes but not glucose transporter translocation. Biochem Pharmacol 51:423–429
9. **Ahmad FKP, Khan MM, Rastogi AK, Kidwai JR** 1989 Insulin like activity in (−)epicatechin. Acta Diabetol Lat 26:291–300
10. **Vessal M, Hemmati M, Vasei M** 2003 Antidiabetic effects of quercetin in streptozotocin-induced diabetic rats. Comp Biochem Physiol C 135:357–364
11. **Harmon A, Patel YM** 2003 Naningerin inhibits phosphoinositide 3-kinase activity and glucose uptake in 3T3–L1 adipocytes. Biochem Biophys Res Commun 305:229–234
12. **Kamei RKM, Kitagawa Y, Hazeki O, Oikawa S** 2003 2′-Benzyloxychalcone derivatives stimulate glucose uptake in 3T3–L1 adipocytes. Life Sci 73:2091–2099
13. **Jarvill-Taylor JK, Anderson RA, Graves DJJ** 2001 A hydroxychalcone derived from cinnamon functions as a mimetic for insulin in 3T3–L1 adipocytes. Am Coll Nutr 4:327–336
14. **Landrault N, Poucheret P, Azay J, Krosniak M, Gasc F, Jenin C, Cros G, Teissedre PL** 2003 Effect of a polyphenols-enriched chardonnay white wine in diabetic rat. J Agric Food Chem 51:311–318
15. **Maritim ADB, Sanders RA, Watkins III JB** 2003 Effects of pycnogenol treatment on oxidative stress in streptozotocin-induced diabetic rats. J Biochem Mol Toxicol 17:193–199
16. **Ceriello A, Bortolotti N, Motz E, Lizzio S, Catone B, Assaloni R, Tonutti L, Taboga C** 2001 Red wine protects diabetic patients from meal-induced oxidative stress and thrombosis activation: a pleasant approach to the prevention of cardiovascular disease in diabetes. Eur J Clin Invest 31:322–328
17. **Caimi GCC, Lo Presti R** 2003 Diabetes mellitus: oxidative stress and wine. Curr Med Res Opin 19:581–6
18. **Al-Awwadi NAJ, Poucheret P, Cassanas G, Krosniak M, Auger C, Gasc F, Rouanet JM, Cros G, Teissedre PL** 2004 Antidiabetic activity of red wine polyphenolic extract, ethanol, or both in streptozotocin-treated rats. J Agric Food Chem 52:1008–1016
19. **Lange K** 2001 Regulation of glucose uptake in differentiated cells. Front Biosci 6:D630–D359
20. **Zierath JR, Wallberg-Henriksson H** 2002 From receptor to effector: insulin signal transduction in skeletal muscle from type II diabetic patients. Ann NY Acad Sci 967:120–134
21. **Khan AHPJ** 2002 Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. Diabetologia 45:1475–1483
22. **Simpson F, Whitehead J, James D** 2001 GLUT4: at the cross roads between membrane trafficking and signal transduction. Traffic 2:2–11
23. **Michelle Furtado L, Poon V, Klip A** 2003 GLUT4 activation: thoughts on possible mechanisms. Acta Physiol Scand 178:287–296
24. **Ardévol A, Bladé C, Salvadó MJ, Arola L** 2000 Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3–L1 adipocytes. Int J Obesity 24:319–324
25. **Blay M, Pinent M, Baiges I, Ardévol I, Salvadó M, Bladé C, Arola L** 2003 Grape seed proanthocyanidins increase glucose uptake in muscular cell line L6E9. In: Lonvaud-Funel A, deRevel G, Darriet P, eds. Oenologie '03. 7th International Symposium of Oenology. Bordeaux: Editions Tec&Doc; 669–673
26. **Bradford MM** 1976 A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254
27. **Simpson IA, Yver DR, Hissin PJ, Wardzala LJ, Karnieli E, Salans LB, Cushman SW** 1983 Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. Biochim Biophys Acta 763:393–407
28. **Reed M, Meszaros K, Entes L, Claypool M, Pinkett J, Gadbois T, Reaven G** 2000 A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. Metabolism 49:1390–1394
29. **Kim M-J RG, Chung J-S, Sim SS, Min DS, Rhie D-J, Yoon SH, Hahn SJ, Kim M-S, Jo Y-H** 2003 Protective effects of epicatechin against the toxic effects of streptozotocin on rat pancreatic islets: in vivo and in vitro. Pancreas 26:292–299
30. **Shimizu M, Kobayashi Y, Suzuki M, Satsu H, Miyamoto Y** 2000 Regulation of intestinal glucose transport by tea catechins. Biofactors 13:61–65
31. **Waltner-Law ME, Wang XL, Law BK, Hall RK, Nawano M, Granner DK** 2002 Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. J Biol Chem 277:34933–34940
32. **Smith RM, Tiesinga JJ, Shah N, Smith JA, Jarett L** 1993 Genistein inhibits insulin-stimulated glucose transport and decreases immunocytochemical labeling of GLUT4 carboxyl-terminus without affecting translocation of GLUT4 in isolated rat adipocytes: additional evidence of GLUT4 activation by insulin. Arch Biochem Biophys 300:238–246
33. **Shisheva A, Shechter Y** 1992 Quercetin selectively inhibits insulin receptor function in vitro and the bioresponses of insulin and insulinomimetic agents in rat adipocytes. Biochemistry 31:8059–8063
34. **Kotyk A, Kolinska J, Veres K, Szammer J** 1965 Inhibition by phloretin and phlorizin derivatives of sugar transport in different cells. Biochem Z 342:129–138
35. **Counet CCS** 2003 Effect of the number of flavanol units on the antioxidant activity of procyanidin fractions isolated from chocolate. J Agric Food Chem 51:6816–6822
36. **Hagiwara M, Inoue S, Tanaka T, Nunoki K, Ito M, Hidaka H** 1988 Differential effects of flavonoids as inhibitors of tyrosine protein kinases and serine/threonine protein kinases. Biochem Pharmacol 37:2987–2992
37. **Brunet MJ, Bladé C, Salvado MJ, Arola L** 2002 Human apo A-I and rat transferrin are the principal plasma proteins that bind wine catechins. J Agric Food Chem 50:2708–2712
38. **Moini HGQ, Packer L** 2000 Enzyme inhibition and protein-binding action of the procyanidin-rich French maritime pine bark extract, pycnogenol: effect on xanthine oxidase. J Agric Food Chem 48:5630–5639

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Metabolic fate of glucose on 3T3-L1 adipocytes treated with grape seed-derived procyanidin extract (GSPE). Comparison with the effects of insulin.

M Pinent, MC Bladé, MJ Salvadó, L Arola, A Ardévol

Nutrition (Submitted)

Abstract

Objective: To investigate the effects of a grape seed procyanidin extract (GSPE) on the metabolic fate of glucose in 3T3-L1 adipocytes.

Research methods: Differentiated 3T3-L1 cells were treated with GSPE or insulin for a short period (1 hour, acute treatment) or a long period (15 hours, chronic treatment). 2-[^{14}C]deoxyglucose uptake and 1-[^{14}C]-glucose incorporation into cells, glycogen and lipid were measured.

Results: GSPE mimicked the anabolic effects of insulin but there were several important differences. GSPE stimulated glycogen synthesis less than insulin, after both an acute and a chronic treatment. After chronic exposure, GSPE induced a higher incorporation of glucose into lipid. This was mainly due to the increase in glucose directed to glycerol synthesis. Also, after chronic exposure to GSPE, the insulin stimulation of glucose uptake was similar to when there was no pre-treatment.

Conclusion: GSPE has insulinomimetic properties, increases glucose uptake, and activates glycogen and lipid synthesis. However, the differences between the effects of GSPE and the effects of insulin indicate that GSPE uses complementary mechanisms to insulin signalling pathways to bring about these effects.

Keywords: Procyanidin, Triacylglycerol synthesis, glycogen synthesis, 3T3-L1 adipocytes

Introduction

Procyanidins, a group of flavonoids, are oligomeric forms of catechins that are abundant in red wine, grapes, cocoa and apples (1). They show beneficial effects on human health, being cardioprotective, antioxidant, antigenotoxic, anti-inflamatory and anticarcenogenic (2). However, few studies have been carried out to determine their effects on diabetic situation and glucose metabolism. Their antioxidant properties make them candidates for improving insulin resistant states. In fact, most of their beneficial effects have so far been attributed to their antioxidant properties (2), (3), (4). It has recently been suggested, however, that they also act on cells by modifying or interacting with specific proteins of important intracellular signalling pathways ((5),(6)) and in fact their role in improving hyperglycemia in streptozotocin-induced diabetes in rats has been reported (7). Our group previously showed that at least part of this antihyperglycemic effect is explained by the stimulation of glucose uptake by grape-seed derived procyanidins (GSPE) in insulin-sensitive cell lines (3T3-L1 adipocytes and L6E9 myotubes) (8). We reported that these GSPE effects showed insulinomimetic properties (e.g. they increased the amount of GLUT-4 in the plasma membranes and needed an active PI3K and a p38 MAPK to be observed). However, there is a lack of information about how procyanidins modify glucose metabolism in the cells and which mechanism they use to do it. Some studies have been done with monomeric catechins. Valsa et al. (9) showed that catechin has a hypoglycemic action due to an increase in glycogenesis and a decrease in glycogenolysis in the liver. Ahmad et al. (10) showed that (-)epicatechin has an insulin-like activity, increasing the glycogen content of the diaphragm in a dose-dependent manner and

increasing glucose uptake. They also showed that epicatechin does not share any binding site with insulin.

In this study we further analyse the effects of GSPE on glucose metabolism in the adipose cells. We evaluate the metabolic fate of the glucose taken up by 3T3-L1 cells in both acute and chronic GSPE treatments. We compare these effects with those of insulin and show that GSPE must use different mechanisms from those described for insulin to exert its insulinomimetic effects on 3T3-L1 adipocytes.

Materials and Methods

Cells, Reagents and Materials

Grape seed procyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5-13 units) (31.7%) procyanidins.

Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium). Insulin (Actrapid) was from Novo Nordisk (Bagsvaerd, Denmark). Bradford protein reagent was from Bio-Rad Laboratories (Life Sciences Group, Hercules, California, USA). 2-deoxy-[H3]-glucose was from Amersham Biosciences (Buckinghamshire, England). ARC-120-Glucose, D[1-14C] was from American Radiolabelled Chemicals Inc. (St Louis, M.O.)

Cell culture and differentiation

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (11). Briefly, confluent preadipocytes were treated with 0.25 μmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-methylxanthine, and 5 μg/mL insulin for 2 days in 10% FBS containing DMEM. The cells were switched to 10% FBS/DMEM media containing only insulin for 2 more days, and then switched to 10% FBS/DMEM media without insulin. Ten days after differentiation had been induced, the cells were treated as indicated below and used for experiments.

Cell treatment

For experiments on glucose incorporation into glycogen and lipid, there were two treatments, one acute and one chronic. For the acute treatment, fully differentiated adipocytes were washed twice with PBS and incubated at 37º C with serum-free supplemented DMEM containing 0.2% BSA (depletion medium) for 2 hours. During the last 60 minutes of this depletion treatment, the cells were treated with GSPE or insulin. For the chronic treatment, fully differentiated adipocytes were treated with GSPE or insulin for 15 hours without previous depletion.

For experiments on glucose uptake there was only the chronic GSPE treatment. The cells were treated with GSPE for 15 hours and then incubated with depletion medium for 2 hours. Finally, during the last 60 minutes of this depletion, the cells were treated with insulin.

2-Deoxyglucose uptake

Glucose transport was determined by measuring the uptake of 2-deoxy-D-[3H]

glucose. The cells were cultured on 6-well or 12-well plates. The transport assay was initiated by washing the cells twice in a transport solution (20mmol/L Hepes, 137 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 2.5 mmol/L $CaCl_2$, 2mmol/L pyruvate, pH 7.4). The cells were then incubated for 7 minutes in the transport solution, which contained 0.1 mmol/L 2-deoxy-D-glucose and 1μCi 2-deoxy-D-[3H]-glucose (10 mCi/mmol). Glucose uptake was stopped by adding 2 volumes of ice-cold 50mmol/L glucose in PBS and washing twice in the same solution. Cells were disrupted by adding 0.1 mol/L NaOH, 0.1% PBS, and radioactivity was determined by scintillation counting (Packard 1500 Tri-Cab) (Izasa SA, Madrid, Spain). Glucose transport values were corrected for protein content, which was determined by the Bradford method (12). Each condition was run in triplicate.

Glucose incorporation into glycogen and lipids

The cells were cultured on 25 cm^2 flasks. 2 μCi of D[1-^{14}C] glucose was added during the last 45 minutes of GSPE or insulin treatment in both the acute and the chronic treatments. The cells were then washed twice with PBS, scrapped with 2.5 ml PBS and homogenized. Aliquots of this homogenate were used to count total D[1-^{14}C] glucose incorporation into cells (50μL), quantify protein (25 μL), and determine incorporation into lipids (800μL) and glycogen (1200μL).

To determine the incorporation into glycogen, homogenate was hydrolized by incubating it at 100 ºC with 50 % KOH for 15 min. 5 mg of carrier glycogen was also added to the sample during this boiling time. Glycogen was then precipitated with 95% cold ethanol and incubated for 30 min at –20ºC. After

centrifugation (30 min, 2,000g), the pellet was resuspended in 1 ml hot water and washed again with ethanol. The final glycogen precipitated was resuspended in 1ml hot water and radioactivity was measured.

To quantify the lipid synthesis, the total lipids fraction was extracted with chloroform:methanol (2:1) overnight. It was then washed with 0.45 % NaCl and the organic phase was washed twice with 0.9 % NaCl. The sample was dried with a stream of N_2 and the pellet was resuspended in 2 ml chloroform:methanol (2:1). Half the sample was used directly to measure the radioactivity incorporated into the lipids. The other half was dried again with N_2, dissolved in heptane, and incubated with 1 ml of 6M KOH in 75% (v/v) ethanol at 50ºC for 4 hours to saponify the lipids. The solution was then acidified and fatty acids were extracted into 2 ml heptane. Both the heptane and the aqueous phases were used to determine the incorporation of D[1-^{14}C] glucose into fatty acid and glycerol portions of the triacylglicerol.

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.

Results

Metabolic fate of glucose in GSPE-treated adipocytes

We previously showed that an acute GSPE treatment stimulated 2-deoxyglucose uptake by up to seven times (8). In figure 1 we now show that a chronic GSPE treatment also stimulated 2-deoxyglucose uptake 2.12 ± 0.1 fold

over the base situation. Moreover, acute insulin stimulation after this chronic treatment induced a total increase in 2-deoxyglucose uptake that was similar to an acute insulin stimulation without pretreatment.

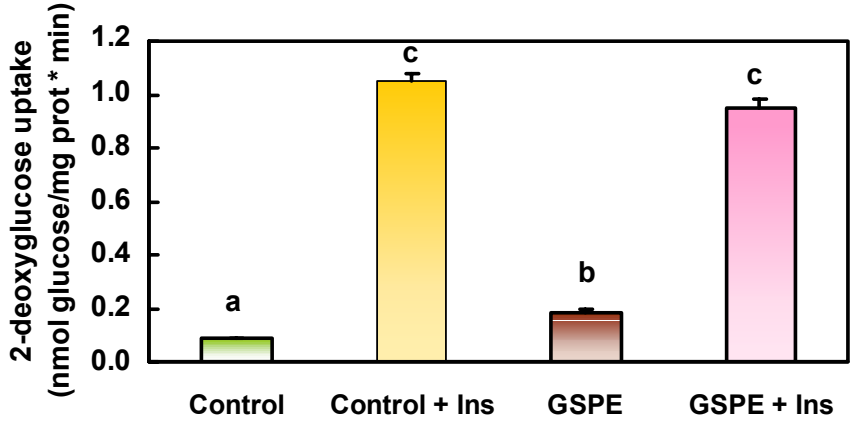

Figure 1. Effects of GSPE pre-treatment on insulin-stimulated 2-deoxyglucose uptake. 3T3-L1 adipocytes were treated for 15 hours with GSPE. This was followed by 2-hour depletion (cells without pre-treatment are called controls). For the last 30 minutes acute insulin stimulation (100nM) was performed (bars indicated as +Ins) and at the end of the treatments 2-deoxyglucose uptake was assayed. The data are the means ± SEM of three independent experiments. (a, b, c) indicate statistically significant differences between treatments.

However, 2-deoxyglucose only allows measurements of coupled transport and phosphorylation, since 2-deoxyglucose is phosphorylated but not further metabolized by cells (13). To assay the metabolic fate of this uptaken glucose, we measured the radiolabelled glucose incorporated into the cells and into the glycogen and lipids. Figure 2A shows that after an acute treatment of 140 mg/L of GSPE for 60 minutes, total glucose incorporated into the cell increased ~6 fold over the base situation. This effect was similar to that induced on 2-deoxyglucose uptake. Figures 2B and 2C show two metabolic fates of this glucose: glycogen synthesis (figure 2B) and lipid synthesis (figure 2C).

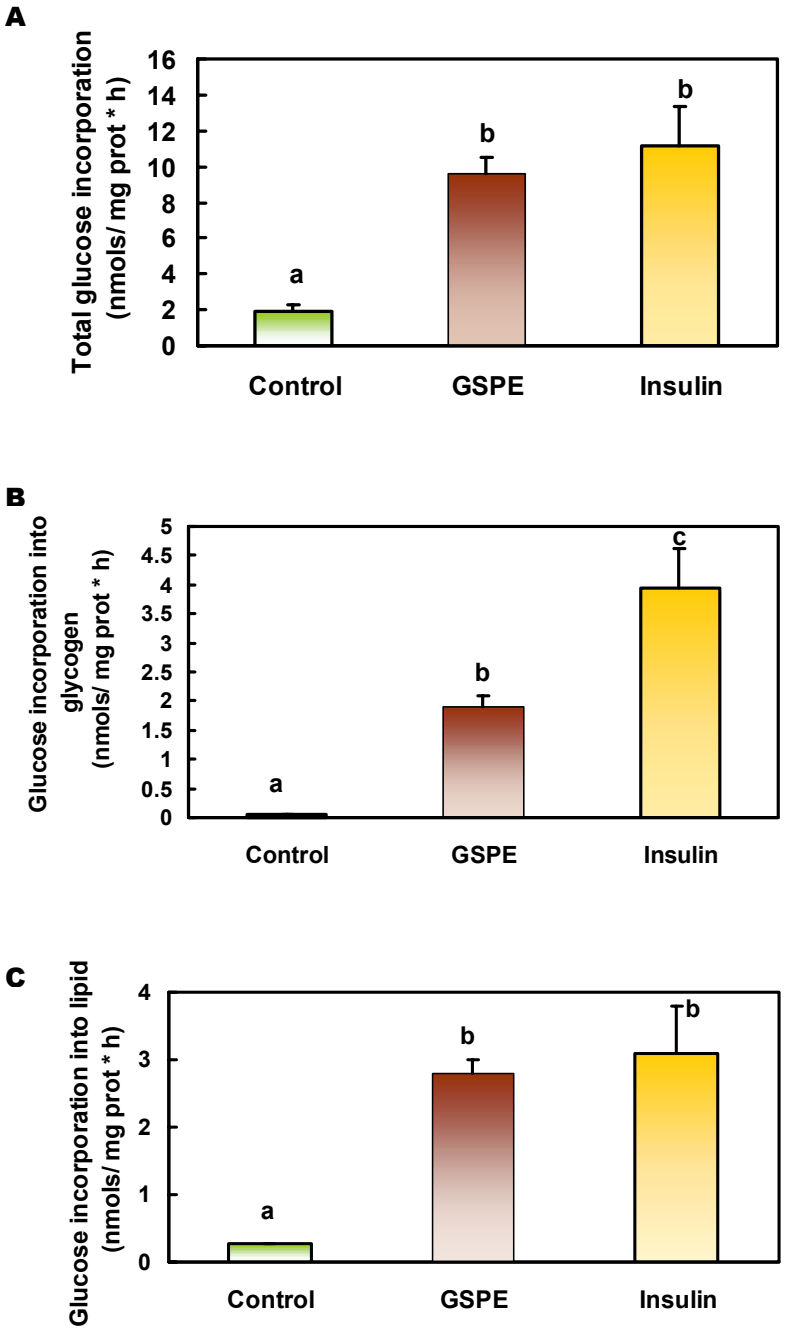

Figure 2 Radiolabelled glucose incorporation after an acute GSPE or insulin treatment in 3T3-L1 adipocytes. Fully differentiated adipocytes were treated with 140 mg/L GSPE or 100 nM insulin for 1 hour and D[1-^{14}C] glucose incorporation was assayed as described in materials and methods. **(A)** total glucose incorporation into the cells, **(B)** glucose incorporated into glycogen and **(C)** glucose incorporated into lipid. The data are the means ± SEM of three independent experiments. (a, b, c) indicate statistically significant differences between treatments.

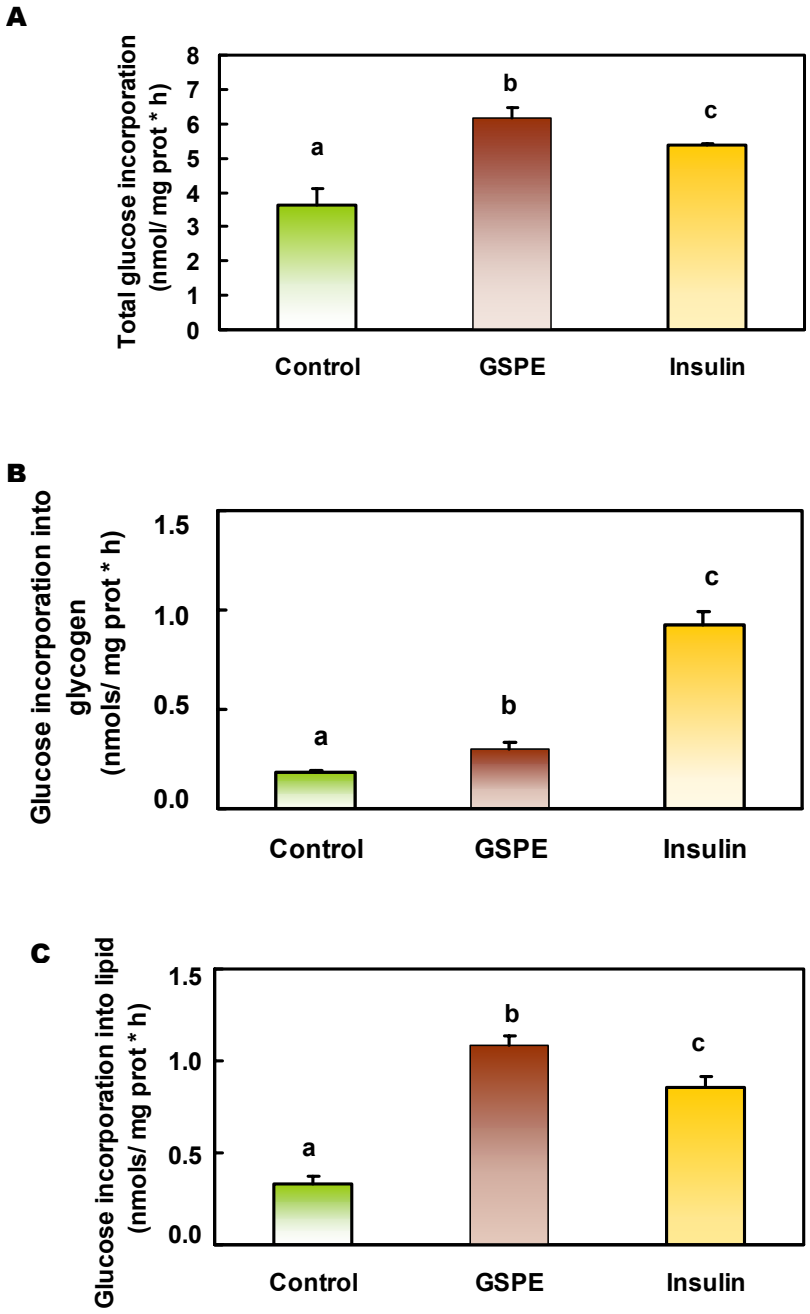

Figure 3 Radiolabelled glucose incorporation after a chronic GSPE or insulin treatment in 3T3-L1 adipocytes. Fully differentiated adipocytes were treated with 140 mg/L GSPE or 100 nM insulin for 15 hours and D[1-^{14}C] glucose incorporation was assayed as described in materials and methods. **(A)** total glucose incorporation into the cells, **(B)** glucose incorporated into glycogen and **(C)** glucose incorporated into lipid. The data are the means ± SEM of three independent experiments. (a, b, c) indicate statistically significant differences between treatments.

The same methodological approach was carried out after a chronic treatment of 140 mg/L of GSPE for 15 hours. Total glucose incorporated into the cell increased 1.83 ± 0.24 fold (figure 3A), which was similar to the stimulation of 2-deoxyglucose uptake. In the chronic treatment, glycogen synthesis (figure 3B) and lipid synthesis (figure 3C) were again metabolic fates of this glucose. The main differences between the metabolic fates of glucose after acute GSPE treatment and after chronic GSPE treatment were that with the acute treatment the stimulation of glycogen synthesis was higher than the stimulation of lipid synthesis, and with the chronic treatment the stimulation of lipid synthesis was higher than glycogen synthesis.

GSPE effects versus insulin effects on metabolic fate of glucose in 3T3-L1 adipocytes

The role of insulin as an anabolic hormone is clearly established. We now show that GSPE also stimulates glycogen synthesis and lipid synthesis in 3T3-L1 adipocytes. However, the anabolic effects of GSPE were somewhat different from those of insulin. Acute treatments of GSPE and insulin increased the total glucose incorporated into the cell by similar amounts (figure 2A), but GSPE activated glycogen synthesis less than insulin (figure 2B). There were no differences between the effects of GSPE treatment and the effects of insulin treatment on glucose incorporated into lipids, either as total lipids (figure 2C) or fractionated lipid components (figure 4A).

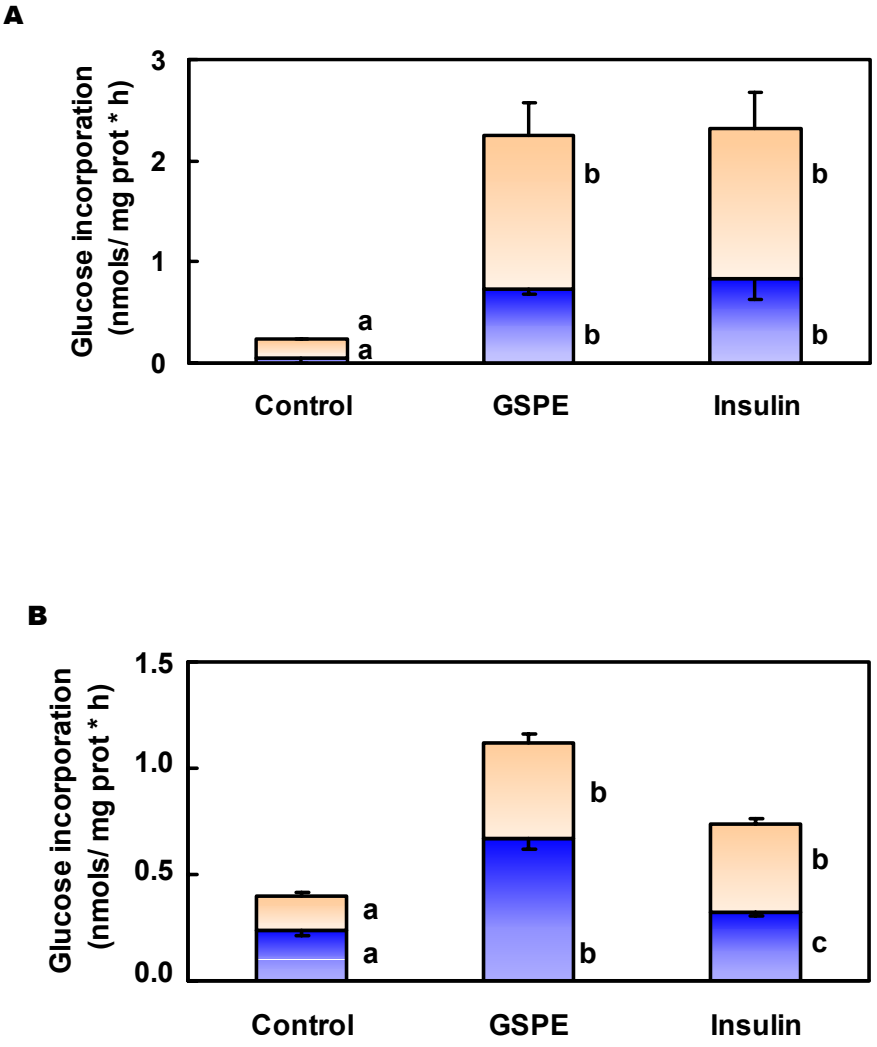

Figure 4. Distribution of D[1-^{14}C] glucose incorporated into lipids after GSPE or insulin treatment in 3T3-L1 adipocytes. Fully differentiated adipocytes were treated with 140 mg/L GSPE or 100 nM insulin for 1 hour (A) or 15 hours (B), and D[1-^{14}C] glucose incorporation into the cells was performed. Lipid fraction was obtained from the homogenates; fatty acids (upper bars) and glycerol (lower bars) were extracted from total lipids, and radioactivity of each phase was quantified. The data are the means ± SEM of three independent experiments. (a, b, c) indicate statistically significant differences between treatments.

The differences between the treatments were greater after the chronic treatment. Figure 3A shows that GSPE treatment stimulated total glucose incorporation into the cells more than insulin did. This difference is partially explained by the relative difference in the metabolic fate of glucose due to GSPE and the metabolic fate of glucose due to insulin. GSPE activated the synthesis of glycogen to a lesser extent than insulin (figure 3B) but directed a higher amount of glucose towards lipid synthesis (figure 3C). Specifically, most of this glucose was directed towards the synthesis of glycerol (figure 4B).

Table 1

Differences between the effects of GSPE and the effects of insulin on 2-deoxyglucose uptake in 3T3-L1 adipocytes

	Insulin	**GSPE**	P<0.05 vs insulin
Maximal stimulation vs control	16.23 ± 0.59	8.49 ± 0.81	yes
% uptake after simultaneous treatment with high doses	100	121.9 ± 6.23	yes
% uptake after inhibition by wortmannin	0.93 ± 0.62	18.85 ± 1.86	yes
% uptake after inhibition by SB 203580	73 ± 5.3	54.9 ± 4.2	yes

This table summarizes our previous results on 2-deoxyglucose uptake in 3T3-L1 after short-term treatment with 140mg/L GSPE or 100 nM insulin. Assays were carried out as already described (**8**). The concentrations of the inhibitors used were 1 µM wortmanin and 100 µM SB203580.

Discussion

We previously showed that an acute treatment with GSPE had an antihyperglycemic effect in streptozotocin-diabetic animals(8). Similarly, Al-awwadi and col. showed that a polyphenol extract from a red wine, chronically administered to streptozotocin-diabetic animals, also induced a reduction in glycaemia (7). To understand the mechanism behind this effect we found that at least part of the acute effect could be explained by the action of GSPE on insulin-sensitive cells, where GSPE shares some of the main intracellular mediators of insulin-signalling that control glucose uptake in insulin-sensitive cells (8). However, the mechanisms behind these effects are not fully understood and the metabolic fate of this glucose has not yet been described. In this paper we partially address these issues. The results of this study show that GSPE has similar anabolic properties to insulin, though there are some differences. Both acute and chronic treatments increased the synthesis of glycogen and lipids. Specifically, lipid synthesis was more enhanced than glycogen synthesis after chronic treatment. This was mainly due to the glucose that is directed towards glycerol synthesis. This effect helps to explain the previously described effects of GSPE on adipose cells i.e. GSPE increases glycerol release to the medium after a chronic treatment (11) without changing the triglyceride contents in the adipocytes (6). Since glycerol kinase (14) activity in white adipose tissue is scarce, the main source of glycerol-3-phosphate for triglyceride synthesis derives from glucose. Therefore, chronic GSPE treatment increased glucose uptake and directed it towards triglyceride synthesis. This increase in the synthesis of the glycerol moiety helps to explain how the triglyceride content of the cells could be

maintained simultaneously with a greater glycerol release to the cell culture medium.

This is the first paper to describe this anabolic role of procyanidins. An anabolic role has previously been described for monomeric catechins: catechin (9) and epicatechin (10). These studies showed that these monomeric catechins increase glycogen synthesis in the liver (9) and in the diaphragm (10) but they did not describe the mechanisms behind these effects. Some clues to understanding the mechanisms by which procyanidins exert these effects can be found by comparing the effects of GSPE with the effects of insulin. We previously showed that procyanidins use some intracellular mediators of insulin signalling pathways to exert their effects on glucose uptake (8). Table 1 shows the main differences between the effects of GSPE and insulin on 2-deoxyglucose uptake. The effect was 20% greater under a simultaneous treatment and there was a 20% difference in the effects of two different specific inhibitors (a PI3K inhibitor and a p38 MAPK inhibitor). Also, the responsiveness to an acute dose of insulin after a chronic pre-treatment with GSPE was higher than the responsiveness after a chronic insulin pre-treatment. Several reports have shown that prolonged exposure to insulin induces insulin resistance (15), (16). The degree of insulin resistance depends on the insulin doses for pre-treatment and acute treatment but, unlike after chronic GSPE treatment, it never reaches the stimulation obtained by the acute treatment. We also find this lower resistance to the chronic effect of procyanidins if we compare the effects on glucose incorporation after chronic treatment with those after acute treatment, since GSPE usually obtains a greater relative effect than insulin. With regard to glucose incorporation, the main difference between GSPE and insulin lies in their stimulation of

glycogen synthesis. GSPE stimulation of glycogen synthesis was always lower than insulin stimulation. Kohn et al. obtained a similar metabolic effect to that induced by GSPE (an enhanced glucose transport with a lower glycogen synthesis and unaltered lipid synthesis) with a constitutively active Akt kinase (17). This approach also leads to high levels of lipogenesis in quiescent 3T3-L1 adipocytes (18). Akt is mainly activated by PI3K (19) but its activation has also been described by other PI3K independent pathways such as the p38 MAP kinase pathway (20), (21), b3-adrenoreceptors (22), heat shock and osmotic stress (23). Therefore, GSPE could activate Akt by PI3K-driven pathways and/or p38 MAP kinase pathways or by other still undescribed pathways. In fact, Kamei et al. (24), who showed that shikonin (another flavonoid) stimulates glucose uptake via insulin-independent tyrosine kinase pathways, also suggest that Akt could be a potential point of convergence between shikonin and insulin. Nevertheless, this possible role for Akt should be analysed because its involvement in glycogen synthesis remains controversial (19). Another approach, specific desensitization by pretreatment with insulin, also showed improved glucose transport with a lower glycogen synthesis, unaltered lipid synthesis and no effects on Akt (25).

In conclusion, we have shown that GSPE has similar anabolic properties to insulin but that it also has several important differences. GSPE is less efficient at activating glycogen synthesis and, after chronic exposure, it activates triglyceride turnover by simultaneously activating synthetic and degrading pathways and maintaining the total triglyceride content of the adipocytes. These differences, and the differences in the control of glucose uptake by insulin signalling pathway mediators, suggest that GSPE uses complementary

mechanisms to that of insulin to induce its insulinomimetic properties in 3T3-L1 adipocytes.

Summary

We show that grape seed procyanidins extract (GSPE) stimulated glycogen and lipid synthesis in 3T3-L1 adipocytes, though glycogen synthesis was lower than with insulin. Also, after chronic exposure, GSPE directed more glucose to glycerol synthesis. The GSPE insulinomimetic properties are therefore obtained via a complementary mechanism to insulin signalling pathways.

Abbreviations: GSPE, grape seed procyanidin extract; PI3K, phosphatidylinositol 3-kinase; p38 MAPK, p38 mitogen-activated protein kinase

Acknowledgments

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References

1. **A. Scalbert and G. Williamson** (2000) *Dietary intake and bioavailability of polyphenols*. J. Nutr. 130, 2073S-2085S

2. **P. Cos, et al.** (2004) *Proanthocyanidins in health care: current and new trends*. Cur Med Chem 11, 1345-1359

3. **Shi J, et al.** (2003) *Polyphenolics in grape seeds-biochemistry and functionality.* J Med Food 6, 291-299

4. **Caimi G, et al.** (2003) *Diabetes mellitus: oxidative stress and wine.* Curr Med Res Opin 19, 581-586

5. **S. Rosenkranz, et al.** (2002) *Inhibition of the PDGF receptor by red wine flavonoids provides a molecular explanation for the "French paradox"*. FASEB J 16, 1958-1960

6. **M. Pinent, et al.** (2004) *Intracellular mediators of procyanidin-induced lipolysis in 3T3-L1 adipocytes*. J Agric Food Chem in press

7. **Al-Awwadi N, et al.** (2004) *Antidiabetic activity of red wine polyphenolic extract, ethanol, or both in streptozotocin-treated rats.* J Agric Food Chem 52, 1008-1016

8. **M. Pinent, et al.** (2004) *Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines*. Endocrinology 145, 4985-4990

9. **Valsa AK, et al.** (1997) *Effect of catechin on carbohydrate metabolism*. Indian J Biochem Biophys 34, 406-408

10. **Ahmad F, et al.** (1989) *Insulin like activity in (-) epicatechin.* Acta Diabetol Lat 26, 291-300

11. **A. Ardévol, et al.** (2000) *Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes*. Int. J. Obesity 24, 319-324

12. **Bradford and MM.** (1976) *A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding.* Anal Biochem 72, 248-254

13. **R. F. Kletzien and J. F. Perdue** (1973) *The inhibition of sugar transport in chick embryo fibroblasts by cytochalasin B. Evidence for a membrane-specific effect*. J Biol Chem 248, 711-719

14. **E. Jequier and L. Tappy** (1999) *Regulation of body weight in humans*. Physiol Rev 79, 451-480

15. **V. P. Knutson, et al.** (1995) *Insulin resistance is mediated by a proteolytic fragment of the insulin receptor*. J Biol Chem 270, 24972-24981

16. **G. A. Rosenstock M, Rudich A.** (2001) *Distinct long-term regulation of glycerol and non-esterified fatty acid release by insulin and TNF-alpha in 3T3-L1 adipocytes.* Diabetologia 44, 55-62

17. **A. D. Kohn, et al.** (1996) *Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter4 translocation*. J Biol Chem 271, 31372-31378

18. **A. D. Kohn, et al.** (1996) *Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation*. J Biol Chem 271, 21920-21926

19. **E. L. Whiteman, et al.** (2002) *Role of Akt/protein kinase B in metabolism*. Trends Endocrinol Metab 13, 444-451

20. **Y. Taniyama, et al.** (2004) *Role of p38 MAPK and MAPKAPK-2 in angiotensin II-induced Akt activation in vascular smooth muscle cells*. Am J Physiol Cell Physiol 287, C494-499

21. **E. Anter, et al.** (2004) *Activation of endothelial nitric-oxide synthase by the p38 MAPK in response to black tea polyphenols*. J Biol Chem 279, 46637-46643

22. **S. K. Moule, et al.** (1997) *Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and beta -adrenergic agonists in rat epididymal fat cells. Activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms*. J Biol Chem 272, 7713-7719

23. **H. Konishi, et al.** (1996) *Activation of RAC-protein kinase by heat shock and hyperosmolarity stress through a pathway independent of phosphatidylinositol 3-kinase*. PNAS 93, 7639-7643

24. **R. Kamei, et al.** (2003) *2'-benzyloxychalcone derivatives stimulate glucose uptake in 3T3-L1 adipocytes*. Life Sci 73, 2091-2099

25. **T. C. Jensen, et al.** (2000) *Specific desensitization of glycogen synthase activation by insulin in 3T3-L1 adipocytes. Connection between enzymatic activation and subcellular localization*. J Biol Chem 275, 40148-40154

Grape-seed derived procyanidins interfere with adipogenesis of 3T3-L1 cells at the onset of differentiation

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Abstract

Objective: Our group's previous results on the effects of a grape seed procyanidin extract (GSPE) on adipose metabolism showed that peroxisome proliferator-activated receptor-γ (PPARγ) plays a central role in the lipolytic effects of GSPE on adipocytes. Since PPARγ2 is a main regulator of the differentiation process of adipocytes, we investigated whether GSPE affects the adipogenesis of 3T3-L1 cells.

Design: We performed a time point screening by treating 3T3-L1 cells with GSPE during the differentiation process for 24 hours.

Measurements: Differentiation markers and differential gene expression due to GSPE treatment (using the microarray technique).

Results: 24 hour-GSPE treatment at the onset of differentiation reduces adipose specific markers and maintains the expression of preadipocyte marker preadipocyte factor-1 (Pref-1) significantly elevated. These effects were not found in other time points. Microarray analysis of gene expression after GSPE treatment at the early stage of differentiation showed a modified gene expression profile in which cell cycle and growth related genes were down-regulated by GSPE.

Conclusion: These results suggest that GSPE affects adipogenesis, mainly at the induction of differentiation, and that procyanidins may have a new role in which they impede the formation of adipose cells.

Keywords: Procyanidin, differentiation, 3T3-L1 adipocytes, PPARγ, microarray

Introduction

Flavonoids are phenolic compounds that are ubiquitously found in plants, fruits and beverages. They have been widely studied and shown to have beneficial effects on human health (1). Flavonoids have traditionally been studied due to their properties as antioxidants, though more recently it has been suggested that they act through the modulation of intracellular signalling cascades (2). Among other properties, flavonoids are antigenotoxic (3), antiatherogenic (1, 4) and ameliorators of diabetes (5). Their effects on proliferation and differentiation have been studied both in cancerous and non-cancerous cell lines (1, 6, 7). However, there is little information about their effects on adipocytes (6, 8, 9). Few studies have focused on the action of procyanidins, an oligomeric class of flavonoids, in the adipose cell (10) or on the relationship between procyanidins and the adipose differentiation process.

The study of the adipocyte metabolism is important because obesity is a growing problem in industrialized societies. A key step to determining the possible mechanisms of potential ameliorators of obesity is to define their role in the process of differentiation from preadipocytes to adipose cells. A good model to study differentiation is the 3T3-L1 cell line, provided it can differentiate from preadipocytes to adipocytes under certain stimulating conditions (11). Adipose differentiation is a complex process that is highly regulated by hormones, cytokines and growth factors. Insulin, insulin-like growth factor-1 (IGF-1), glucocorticoids (as dexamethasone), cAMP generating agents as isobutyl-methylxanthine (IBMX) (12), as well as other molecules such as thiazolidinediones (13), have been shown to trigger the

differentiation of preadipocytes to adipocytes. Before starting the differentiation program, 3T3-L1 may re-enter the cell cycle. Intracellular signals promote the phosphorilation of the retinoblastoma (Rb) protein, which results in the release of the E2F family of transcription factors. This class of transcription factors initiates subsequent events needed for transition through the cell cycle (14). A highly regulated expression of cyclins, CDKs (cyclin-dependent kinases) and CIKs (cyclin-dependent kinase inhibitors) drives the cell through cell cycle progression. This event, called mitotic clonal expansion, ceases coincident with the expression of the key transcription factors PPARγ and CCAAT/enhancer binding protein-α (C/EBPα) (15). PPARγ is the regulator of many genes involved in adipose phenotype and lipid metabolism, such as fatty acid synthase and adipocyte-specific fatty acid binding protein (aP2) (15, 16). After 6 days post induction of differentiation, cells acquire adipose morphology and accumulate triglyceride droplets. Although the transcriptional regulation of adipose differentiation has been widely studied, the intracellular signalling cascades that control this process are not fully understood. MAPK (17) and STAT (18) among others are intracellular mediators shown to be involved in the process of adipogenesis.

In addition to the induction of differentiation factors, inhibitors of adipogenesis, such as myostatin (19), tumor necrosis factor-α (TNFα) (20), retinoic acid (21) and rapamycin (22), have also been described. Genistein, a class of flavonoid, has also been shown to inhibit 3T3-L1 proliferation and differentiation (6).

Previous results from our group showed that a grape-seed procyanidin extract (GSPE) modified the adipose metabolism (10). Long term GSPE treatment increased the lipolytic rate in 3T3-L1 adipocytes and down-

regulated some mature adipocyte markers, such as glycerol-3-phosphate dehydrogenase (G3PDH) activity, PPARγ and hormone sensitive lipase (HSL) mRNA (23) Due to the central role of PPARγ2 in the control of the differentiation process of adipose cells, we hypothesized that GSPE affects the adipogenesis of 3T3-L1 cells.

This study was designed to determine whether GSPE affects the differentiation of 3T3-L1 into adipocytes. We studied differentiation cell markers at various time points and performed a more in depth screening of GSPE effects on gene expression profile in order to clarify its involvement in adipose cell differentiation.

Methods

Cells, Reagents and Materials

Grape seed procyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5-13 units) (31.7%) procyanidins.

Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium). Bradford protein reagent was obtained from Bio-Rad Laboratories (Life Sciences Group, Hercules, California, USA). Reagents for G3PDH activity assay were obtained from SIGMA (St Louis, M.O.), as were amino allyl dUTP for microarrays. RNA isolation was carried out with the High Pure RNA Isolation Kit (Roche). The reagents for reverse transcription were purchased

from Invitrogen. The probes for PCR and the reagents for microarray verification were obtained from Applied Biosystems. N-Hydroxysuccinimide esters of Cy3 and Cy5 were from Amersham.

Cell culture and treatment

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (10). Briefly, post-confluent cells were induced to adipocyte differentiation (day 0) with 0.25 µmol/l dexamethasone, 0.5 mmol/l 3-isobutyl-methylxanthine, and 5 µg/ml insulin in 10% FBS/supplemented DMEM; after 48 hours (day 2) cells were switched to 10% FBS/supplemented DMEM containing only insulin, and at day 4 insulin was removed.

3T3-L1 post-confluent preadipocytes were treated at various time points with 140 mg/l GSPE (dissolved in water) for 24 hours at day 0, day 2, and day 4 (figure 1). After treatment, the medium was replaced for the corresponding following differentiation protocol. At day 10, the triglyceride content, G3PDH activity and gene expression were measured. For the microarray assays, RNA was extracted at each time point, immediately after the GSPE treatment (Figure 1). Water treatments were used as controls.

Measurement of glycerol-3-phoshate dehydrogenase (G3PDH) activity

After treatment, differentiated 3T3-L1 adipocytes were rinsed twice with PBS, scraped into 750 µl of 50 mmol/l Tris-HCl, 1 mmol/l EDTA, 1 mmol/l β-mercaptoethanol and sonicated. The resulting extract was used to measure G3PDH, in accordance with the Wise and Green method (24).

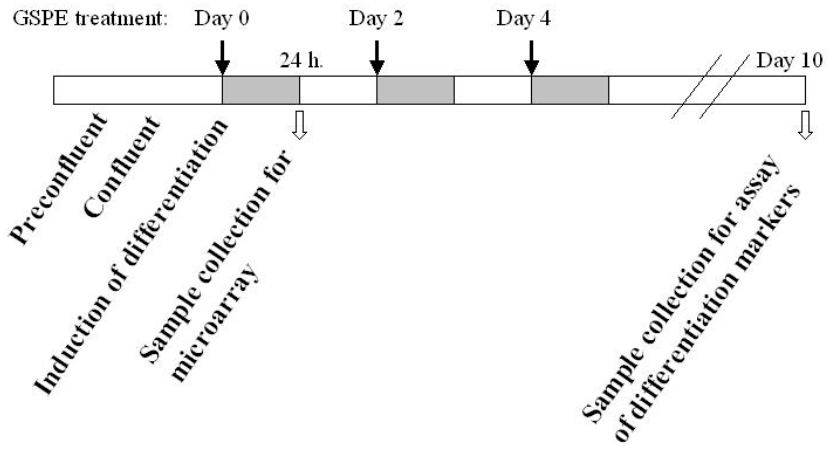


Figure 1. Experimental design. Post-confluent 3T3-L1 cells were induced to differentiate and treated with 140mg/l GSPE for 24 hours at several time points, as described in Methods. For microarray experiments RNA was extracted just after the 24-hour GSPE treatment at the onset of differentiation. The other analyses were performed with cells grown until 10 days post-induction of differentiation.

Triglyceride assay and lipid content

Remaining cell lisates, as described above, were used to determine the total triacylglyceride (TAG) content, measured using the enzymatic test: glycerol-phosphate oxidase method (QCA) following the manufacturer's instructions. Values were corrected by their protein content, which was measured by the Bradford method (25).We determined the lipid content by staining the cells with Oil Red O (26).

Microarray assay and data analysis

Total RNA was isolated and its quality was checked by Agilent 2100 Bioanalyzer RNA assays. The labelling and hybridation procedures used were based on those developed at The Institute for Genomic Research (TIGR) (27). The mouse slides, also obtained from TIGR, consisted of 27,648 elements that can be found on the web page:

http://www.genome.tugraz.at/adipocyte/Microarray.html.

Briefly, cDNA was prepared from 25 μg total RNA with Random Hexamers and Superscript Reverse Transcriptase II, in the presence of amino allyl dUTP. cDNA sample was purified (QIAquick kit, QIAGEN), according to the manufacturer's instructions, but using potassium phosphate wash and elution buffer instead of supplied buffers. N-Hydroxysuccinimide esters of Cy3 and Cy5 were coupled to the aadUTP incorporated in the cDNA. Coupling reactions were quenched by 0.1 M Sodium acetate (pH=5,2) and unincorporated dyes were removed using QIAquick columns (QIAGEN). Fluorescent samples were dried, resuspended in hybridization buffer (50% formamide, 5X SSC, 0.1% SDS) and combined. 20 μg mouse Cot1 DNA and 20 μg poly(A) DNA were added and denatured at 95ºC for 3 min. Sample was applied to prehybridized slide (incubation at 42ºC for 45 min in 5X SSC, 0.1 % SDS, 1% BSA), and hybridized in a humidified chamber overnight at 42ºC in the dark. The slides were washed at room temperature twice for 2 min. in a1X SSC, 0.2% SDS solution; for 4 min. in 0.1X SSC, 0.2% SDS; for 4 min in 0.1X SSC; for two minutes more in 0.1X SSC; and dipped twice in MQ water. The slides were dried and scanned with a GenePix 4000B microarray scanner (Axon Instruments), and the resulting TIFF images were analyzed with GenePix Pro 4.1 (Axon instruments).

Microarray assay was performed by triplicate with dye-swap, corresponding to three independent experiments (biological replicates). Features were filtered for low quality spots, and arrays were global mean normalized using ArrayNorm (28). We chose a 2-fold change cut-off to the mean of the ratios to find the most variable genes. Differentially expressed genes were classified according to the Gene Ontology™ (GO) Consortium (29), considering the biological process description for each gene.

Quantitative RT-PCR

Microarray results were verified by quantitative real-time PCR. cDNA corresponding to each RNA experiment was generated using TaqManR Reverse Transcription Reagents (Applied Biosystems) and quantitative PCR amplification and detection were performed using specific TaqMan Assay-on-Demand probes (Mm00438064_m1, for Cyclin A2; Mm00772471_m1 for Cell division control protein 2 homolog; Mm00495703_m1 for DNA topoisomerase II alpha isozyme), and the TaqMan PCR Core Reagent Kit as recommended by the manufacturers. Quantifications were performed in triplicate. mRNA 18S was used as the reference gene (HS99999901_sl, human mRNA 18S).

PPAR-γ2, HSL and Pref-1 were analysed as previously described (23). Briefly, 1 μg of total RNA was reverse transcribed and mRNA levels were measured by real-time RT-PCR analyses in a fluorescent thermal cycler (GeneAmp 5700 Sequence Detection System, Applied Biosystems) according to the manufacturer's instructions. The level of mRNA for each gene was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA detected in each sample. Amplification was performed with the probes already

described plus Pref-1, TGC GCC AAC AAT GGA ACT T (forward), TGG CAG TCC TTT CCA GAG AAC (reverse).

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.

Results

Effects of GSPE on differentiation markers

To determine the effect of GSPE on the differentiation program of 3T3-L1 cells, we checked some differentiation markers after a 24-hour GSPE treatment at different time points, and subsequent differentiation of cells (see figure 1). Figures 2 A and 2 B show that after 10 days post-induction of differentiation, the triglyceride content on cells treated at day 0 (day of induction of differentiation) was significantly reduced (32%), whereas when treatment was performed at day 2 or day 4, triglyceride content remained unchanged (Figure 2A).

G3PDH activity was also reduced at day 0 and day 2 of GSPE treatments (~26% reduction), but there were no changes at day 4 (figure 3).

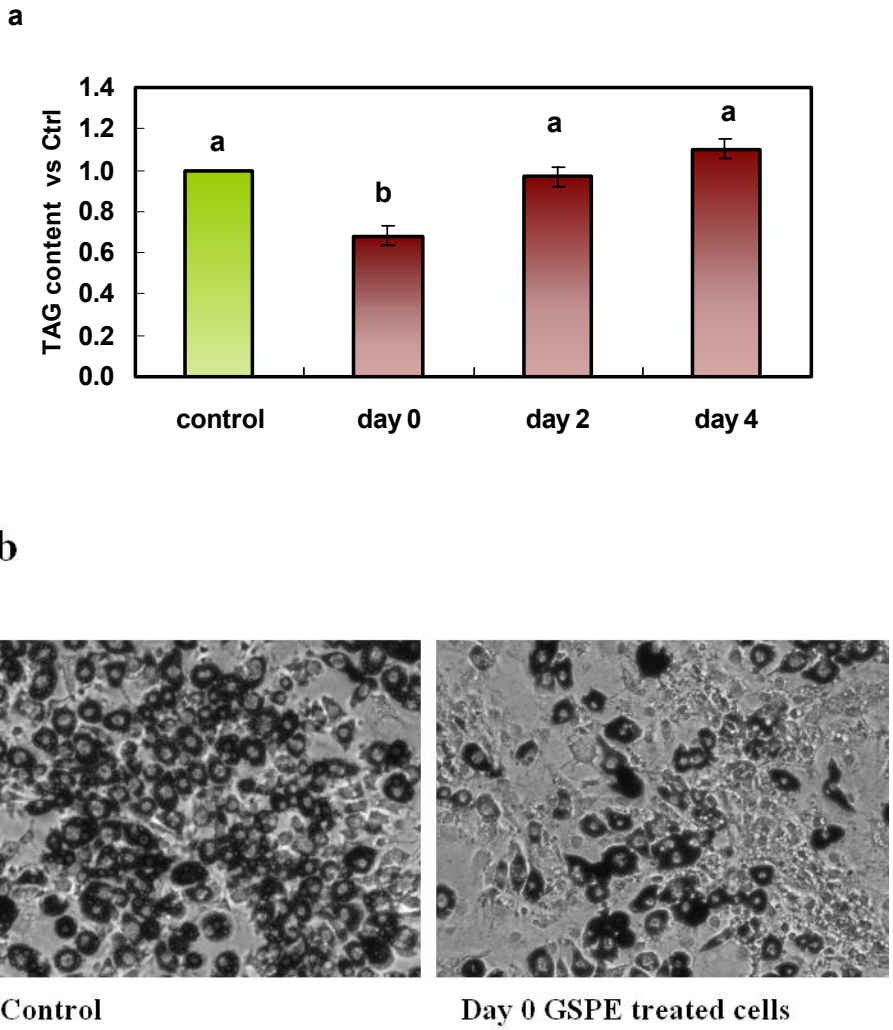


Figure 2. Effect of GSPE on triglyceride cell content. 3T3-L1 preadipocytes were induced to differentiate as described in Methods. Cells were incubated 24 hours with 140 mg/l of GSPE at different time points: day 0 (induction of differentiation), day 2 and day 4. (A) TAG content was assayed after 10 days of differentiation for all treatments. (B) Oil red staining for lipid accumulation was assayed after 10 days of differentiation for cells treated at day 0. Data reflect the means ± SEM of at least three independent experiments; values were corrected to controls in each experiment. (a, b) indicate statistically significant differences between means with $p<0.05$

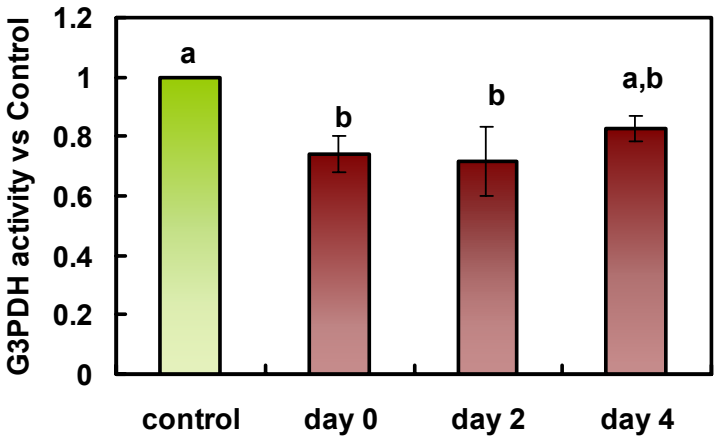
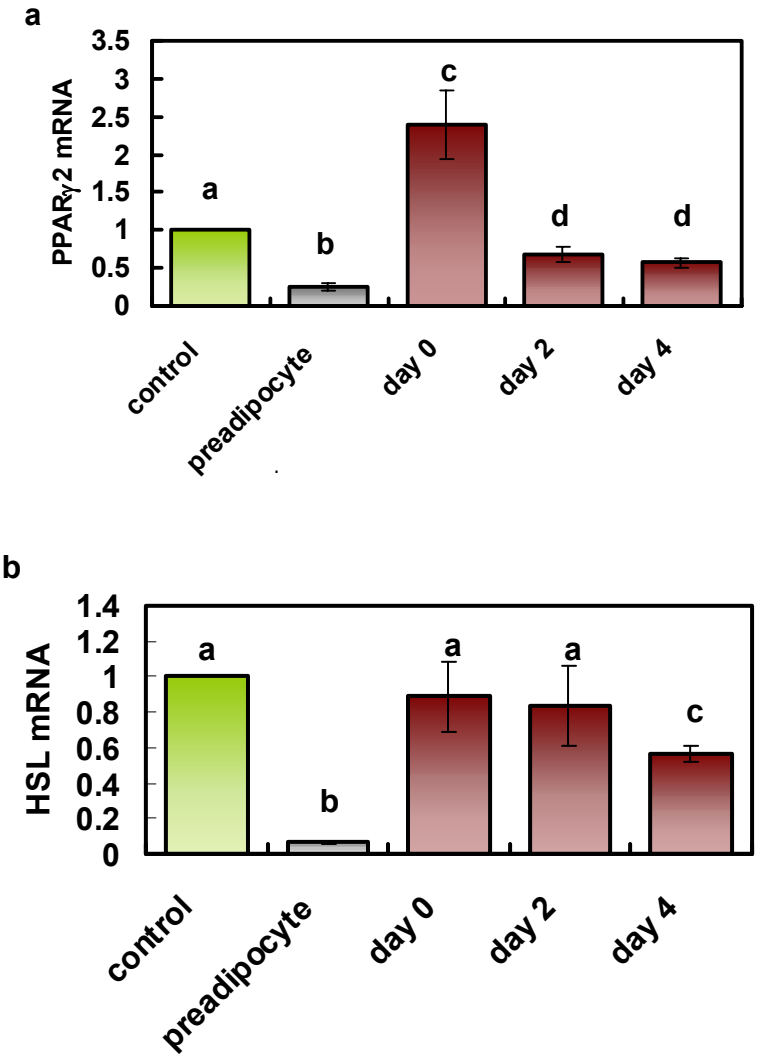


Figure 3. Effect of GSPE on glycerol-3-phophate dehydrogenase (G3PDH) activity of 3T3-L1 adipocytes. Post-confluent 3T3-L1 preadipocytes were induced to differentiate. At several time points (day 0, day 2, day 4), cells were incubated with 140 mg/l GSPE for 24 hours. After 10 days post induction of differentiation, cells were collected and G3PDH activity was assayed. Data reflect the means ± SEM of at least three independent experiments; values were corrected to controls in each experiment. (a, b) indicate statistically significant differences between means with $p<0.05$.

Gene expression markers were also analysed at the end of differentiation (10 days) after the three time point treatments. mRNA of preadipocytes corresponding to each biological replicate was isolated and analysed in order to check the behaviour of each marker during differentiation. Figure 4A shows that PPARγ2 mRNA levels increased after GSPE treatment at day 0. On the other hand, GSPE treatments at day 2 and day 4 reduced PPARγ2 gene expression (31% and 43% respectively). A late differentiation marker of adiposity is HSL, a key enzyme in the lipid metabolism. The mRNA levels of

this enzyme did not change when treatment was performed at day 0 or at day 2, but at day 4 mRNA levels showed a 44% reduction (figure 4B). Pref-1 was chosen as a preadipocyte marker, as its levels have been shown to be down-regulated during adipogenesis (30). Pref-1 mRNA levels due to GSPE treatment after induction of differentiation remained 5-fold up-regulated (figure 4C) but did not reach preadipocyte levels (10-fold increase). When treatments were performed at day 2 and day 4 Pref-1 showed the normal down-regulation in the process of differentiation.



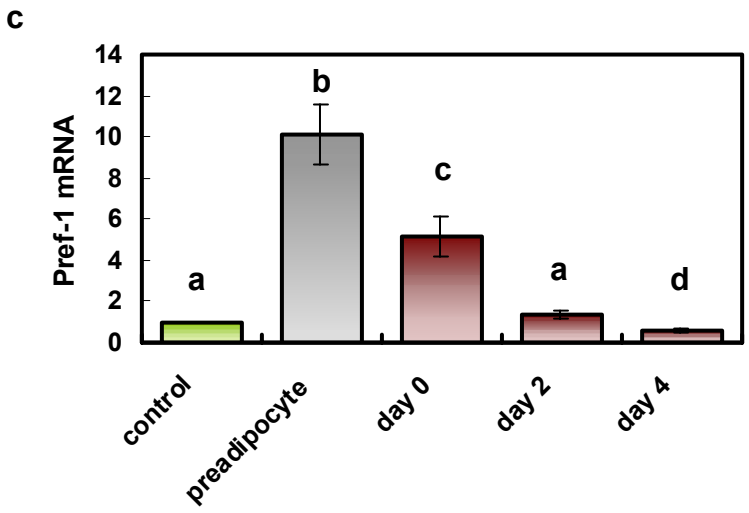


Figure 4. Procyanidin effects on mRNA levels (a, PPARγ-2; b, HSL; c, Pref-1). 3T3-L1 post confluent preadipocytes were induced to differentiate and 24 hours GSPE treatment (140 mg/l) was performed at day 0 (induction of differentiation), day 2 and day 4. At day 10, total RNA was extracted and gene expression was quantified by real-time RT-PCR. Gene expression, normalized by GAPDH mRNA levels, is expressed relative to control cells. Control: untreated fully differentiated cells; preadipocyte: untreated post-confluent fibroblasts. Values represent mean ± SEM. a, b, c indicate significantly different groups with $p<0.05$.

Gene profile is highly modified due to GSPE treatment during induction of differentiation

The time point screening showed that GSPE had stronger effects when added, together with the hormonal cocktail, at the onset of differentiation. We therefore decided to analyse the gene profile after 24 hour GSPE treatment of post-confluent preadipocytes (day 0).

We found 398 differentially expressed transcripts (more than 2-fold change). Of these, 38.3 % were up-regulated and 61.7 % were down-regulated. 206

genes could be categorized based on the biological processes in which they are involved. We did this classification according to the GO consortium, obtaining a view of the biological processes in which the differentially expressed genes are involved. The products of the genes are usually involved in several biological processes, so the results of the classification were redundant because some EST were classified into several categories. This redundancy helped us to get a more complete idea of the biological processes affected by GSPE.

Table 1 summarizes the number of differentially expressed EST classified according to their biological function (complete list of the data files are available on the web site: http://genome.tugraz.at/procyanidins). Most differentially expressed transcripts fell into the categories related to cell growth and maintenance, including cell cycle, citokynesis, cell organization and biogenesis. The protein metabolism category also contained a large number of differentially expressed genes, despite the fact that many of them were also contained in the cell growth group. Table 2 lists the genebank accession numbers of these growth and cell-cycle-related differentially expressed genes. These include cyclins (A2, B2, F, etc.), cell division control protein 2, histone H2A.X. and other cell cycle regulators. Almost all these genes were down-regulated, with mRNA expression differences that achieved more than 4-fold change. We also checked the expression of p21, a cell cycle-related gene involved in the effects of flavonoids on cell proliferation (31-35). We found that p21 (GeneBank ID BE282021), despite not being classified in the first screening of 2-fold change cut off, was 1.41-fold up-regulated.

Table 1. Distribution of differentially expressed genes (at least 2-fold change) by 24-hour GSPE treatment in function of the biological process in which they are involved. Classification was obtained with GO database. "Others" include 15 categories that contained only one or two EST.

Biological Process	total EST differentially expressed	nº EST down-regulated	nº EST up-regulated
protein metabolism	40	24	16
cell cycle	34	32	2
response external stimulus	21	14	7
cell organization and biogenesis	21	20	1
cell communication	19	11	8
cytokinesis	17	17	0
transcription	16	15	1
ion transport	16	8	8
phosphorus metabolism	15	8	7
intracellular transport	14	14	0
morphogenesis	14	10	4
carbohydrate metabolism	14	6	8
response to stress	14	11	3
alcohol metabolism	10	6	4
DNA metabolism	9	9	0
protein transport	9	9	0
nucleotide metabolism	7	0	7
hydrogen transport	7	0	7
energy pathways	7	6	1
acid organic metabolism	6	3	3
secretory pathway	5	5	0
amine metabolism	5	0	5
lipid metabolism	5	3	2
cell death	4	2	2
vitamin metabolism	3	0	3
response to endogenous stimulus	3	3	0
regulation of metabolism	3	3	0
amine transport	3	3	0
organic acid transport	3	3	0
vesicle-mediated transport	3	0	3
embrionic development	3	2	1
circulation	3	0	3
others	22	4	18

Table 2. Differentially expressed genes (24 h. GSPE treated cells vs control ratio at least 2-fold change) with functions related with cell growth and maintenance. Ratio represents the mean of three biological replicates. The genes in bold were validated with quantitative PCR, and the PCR value is shown in brackets.

	genes > 2-fold change down-regulated	
GeneBank ID	**Gene name**	**Ratio**
AW536751	Serine/threonine-protein kinase PLK (PLK-1) (Serine- threonine protein kinase 13)	4.41
AW536176	Homolog to UBIQUITIN-CONJUGATING ENZYME E2 H10	4.32
AU046168	Centromere protein A (CENP-A) (Centromere autoantigen A)	4.26
AW539821	Serine/threonine kinase 6	4.17
AW536849	G2/mitotic-specific cyclin B1	3.94
AU045643	Cyclin B1	3.78
AU022747	Centromeric protein E (CENP-E protein)	3.58
AW555095	Similar to pituitary tumor-transforming 1	3.48
AI836990	Pituitary tumor-transforming 1~putative	3.32
AW552385	Talin	3.20
C77497	Similar to kinesin-like 6 (mitotic centromere-associated kinesin)	3.19
AW536234	**Cell division control protein 2 homolog (P34 protein kinase)**	3.10 **(3.26)**
AU014747	**Cyclin A2 (Cyclin A)**	3.05 **(3.53)**
AU045362	Similar to cyclin B2	3.03
AU023746	Similar to transforming acidic coiled-coil containing protein 3	2.93
AW552691	Similar to kinesin-like 4	2.90
AW538770	Similar to cyclin F	2.85
AW558188	Similar to protein regulator of cytokinesis 1	2.85
AW544881	G2/mitotic-specific cyclin F	2.71
AW537338	Cell cycle protein P55CDC	2.71
AI844648	Histone H2A.X	2.58
AI849291	Ribosomal protein L28	2.42
AI838282	Tubulin beta polypeptide	2.41
AI837422	Tubulin beta-5 chain	2.41
BE380760	Epithelial membrane protein-1 (EMP-1) (Tumor-associated membrane protein)	2.41
AI839587	P19-Ink4d From Mouse Nmr 20 Structures	2.39
AW552266	Probable mitotic checkpoint control protein kinase BUB1	2.39
AW537237	TBX2 protein	2.38
C87726	Mitotic spindle assembly checkpoint protein MAD2A (MAD2-like 1)	2.36
AI851968	Chromosome condensation-related SMC-associated protein 1	2.36
AA407134	Inner centromere protein INCENP	2.33
AU045741	Fizzy-related protein	2.25
AI836347	Tubulin alpha-1 chain (Alpha-tubulin 1)	2.25
AI837019	Tubulin, beta 3	2.23
AW554229	**DNA topoisomerase II alpha isozyme**	2.23 **(2.49)**
AI840757	Tubulin alpha-1 chain	2.13
C77054	NIMA (never in mitosis gene a)-related expressed kinase 2	2.10
AU023348	Putative SMC4 protein	2.03
	genes > 2-fold change up-regulated	
AI844810	MAPK 6	2.11
AW546186	p96 protein	5.21

Discussion

In this paper we studied the effect of a GSPE on the differentiation process of 3T3-L1 adipocytes. First we performed a time point screening of 24 hour 140 mg/l GSPE, analysing several adipose differentiation markers after 10 days post-induction of differentiation. Simultaneous GSPE treatment with the hormonal cocktail at the onset of differentiation reduced triglyceride accumulation and decreased glycerol-3-phosphate dehydrogenase (G3PDH) activity. This shows that GSPE interferes negatively with the differentiation process, prohibiting cells from acquiring complete adipose phenotype. Moreover, at the molecular level Pref-1, a preadipose marker and inhibitor of adipogenesis (30), remained up-regulated in day 0 treated cells. Surprisingly, at this time point of treatment, PPARγ2 mRNA increased. It has been reported that this nuclear transcription factor (PPARγ2) is up-regulated during differentiation, with mRNA levels starting to rise at 24 hours and remaining stable until the end of the differentiation regimens (36). Therefore, a higher expression than the controls is a rare feature that suggests that GSPE not only partially inhibits differentiation but also produces other gene expression changes that are not characteristic of the differentiation process. When GSPE treatment was performed 2 or 4 days after induction, cell triglyceride content remained unchanged and morphology and G3PDH activity remained unaffected after day 4 of treatment. On the other hand, PPARγ2 levels were significantly down-regulated. The induction of PPARγ mRNA at the early stages of differentiation and its action as a stimulator of the transcription of adipogenic genes has been well described (reviewed in (15, 37, 38)). Despite the well-known role of PPARγ in the induction of adipose maturation,

however, its role in the maintenance of adipocyte phenotype is still unclear. Both the up-regulation of PPARγ when treatment was carried out at the beginning of differentiation and its down-regulation in later time-point treatments point to PPARγ as a target for GSPE irrespective of its actions on differentiation. This hypothesis is reinforced by previous results from our group that pointed to PPARγ as a target for GSPE in fully differentiated adipocytes, since BRL 49653, a high affinity PPARγ ligand, removed the GSPE lipolytic effects on 3T3-L1 (23).

As GSPE had inhibitory effects at the early stage of differentiation, we took our study further. We studied the gene expression profile of day 0-GSPE treated cells after 24 hours. We used microarray technology to obtain a wide view of the intracellular dynamics after GSPE treatment. This paper shows that GSPE modifies the gene expression profile of differentiating cells, since 398 transcripts were found to be 2-fold up/down regulated. Their functional classification showed that many of the differentially expressed genes were cell cycle related genes. This was not unexpected because within 24-36 hours of induction, cells re-enter the cell cycle and undergo mitotic clonal expansion. Also, microarray-based studies of adipose differentiation have shown that mRNAs maximally expressed at 16-24 hours correspond to many genes associated with the cell cycle (36, 39-41). Most of the cell cycle related genes in our microarray were down-regulated, which suggests that GSPE-treated cells were not in the mitotic clonal expansion phase. Opinions on whether mitotic clonal expansion is necessary to allow adipose differentiation are controversial (42), but recent studies support the idea that mitotic clonal expansion is required for adipogenesis (43). In fact, Tang et al. (44) demonstrated that PD98059 treatment, an inhibitor of MEK (MAPK/

extracellular signal-regulated kinase kinase), delayed mitotic clonal expansion and that this delay was proportional to decreases in differentiation markers and triacylglyceride accumulation. As 24-hour GSPE treatment is followed by a 24-hour normal induction cocktail, it is likely that GSPE acts delaying mitotic clonal expansion and therefore partially inhibiting differentiation. Although in this study we did not assay the potential inhibition of proliferation by GSPE, in breast carcinoma cells 24 hour-procyanidin treatment up to 75 mg/l—a time and dose quite similar to the one we used—has been shown to have anti-proliferative properties that correlated to an increase in p21 (45) (a cyclin-dependent kinase inhibitor (CIK)). CIKs are also key regulators of cell cycle re-entry because they inhibit cyclin-dependent kinase actions.

As far as we know, there are no studies on effects of procyanidins on adipose differentiation. Although most studies of the antiproliferative effects of flavonoids have been carried out in cancerous cells (46-49), there is also evidence that proliferation is inhibited by flavonoids in 3T3-L1. Genistein, a soy flavonoid, was shown to inhibit both proliferation and differentiation in 3T3-L1 when added at the induction of differentiation (6), and this induced growth arrest was accompanied by increase in p21 expression and subsequent cyclin E/CDK 2 supression (31). In fact, several flavonoids have been shown to exert growth-inhibitory effects through up-regulation of P21 in different cell lines (32-35).

We also found an up-regulation of p21 by GSPE treatment at day 0. P21 up-regulation favours its binding to cyclin E/cdk2 and the inhibition of its kinase activity, so cyclin E/cdk2 cannot contribute to pRB phosphorilation. Unphosphorilated pRb maintain its ability to repress E2F transcriptional

activity (50). The E2F family are transcription factors that regulate several genes necessary for progression through the cell cycle (recently reviewed in (51)). Concomitantly to the GSPE up-regulation of p21 mRNA, in our microarrays we also observed some of the E2F target genes (and therefore downstream of p21) down-regulated due to GSPE treatment, such as cyclin A, cell division control protein 2, DNA topoisomerase II, polo-like kinase (PLK) and other cell cycle related genes. This agrees with the hypothesis that, in 3T3-L1, GSPE induce growth-arrest by an up-regulation of p21. However, more studies are needed to confirm this hypothesis.

In conclusion, this paper shows for the first time that a grape-seed procyanidin extract (GSPE) interferes with the differentiation process of 3T3-L1 adipocytes, mainly at the onset of differentiation. At this time point the significantly modified gene expression profile of 3T3-L1 due to GSPE treatment suggests that GSPE can interfere with the progression of adipocytes through the cell cycle necessary to fully differentiate. These effects support the hypothesis that grape-seed procyanidins help to prevent the development of obesity and obesity-related pathologies by reducing the formation of new fat cells.

Acknowledgments

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References

1. **B. H. Havsteen** (2002) *The biochemistry and medical significance of the flavonoids*. Pharmacol Ther 96, 67-202

2. **R. J. Williams, et al.** (2004) *Flavonoids: antioxidants or signalling molecules?* Free Radic Biol Med 36, 838-849

3. **N. Llópiz, et al.** (2004) *Antigenotoxic Effect of Grape Seed Procyanidin Extract in Fao Cells Submitted to Oxidative Stress.* J Agric Food Chem 52, 1083-1087

4. **M. Dell'Agli, et al.** (2004) *Vascular effects of wine polyphenols*. Cardiovasc Res 63, 593-602

5. **M. Pinent, et al.** (2004) *Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines*. Endocrinology 145, 4985-4990

6. **A. W. Harmon and B. H. Joyce** (2001) *Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis.* Am J Physiol 280, C807-C813

7. **O. J. Park and Y.-J. Surh** (2004) *Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies*. Toxicol Letters 150, 43-56

8. **M. Mochizuki and N. Hasegaw**a (2004) *Stereospecific effects of catechin isomers on insulin induced lipogenesis in 3T3-L1 cells*. Phytother Res 18, 449-450.

9. **T. Tsuda, et al.** (2004) *Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes*. Biochem Biophys Res Commun 316, 149-157.

10. **A. Ardévol, et al.** (2000) *Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes*. Int J Obes 24, 319-324

11. **J. M. Ntambi and K. Young-Cheul** (2000) *Adipocyte differentiation and gene expression*. J Nutr 130, 3122S-3126S.

12. **O. M. Rosen, et al**. (1978) *Development of hormone receptors and hormone responsiveness in vitro. Effect of prolonged insulin treatment on hexose uptake in 3T3-L1 adipocytes.* J Biol Chem 253, 7579-7583

13. **T. Takamura, et al.** (2001) *Stage-specific effects of a thiazolidinedione on proliferation, differentiation and PPAR[gamma] mRNA expression in 3T3-L1 adipocytes*. Eur J Pharmacol 422, 23-29

14. **H. Muller and K. Helin** (2000) *The E2F transcription factors: key regulators of cell proliferation*. Biochim Biophys Acta - Reviews on Cancer 1470, M1-M12

15. **E. D. Rosen, et al.** (2000) *Transcriptional regulation of adipogenesis.* Genes Dev 14, 1293-1307

16. **D. Patsouris, et al**. (2004) *PPARalpha governs glycerol metabolism*. Journal Clin Invest 114, 94-103.

17. **G. Pearson, et al.** (2001) *Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.* Endocr Rev 22, 153-183

18. **J. B. Harp, et al.** (2001) *Differential Expression of Signal Transducers and Activators of Transcription during Human Adipogenesis*. Biochem Biophys Res Commun 281, 907-912

19. **H. S. Kim, et al.** (2001) *Inhibition of Preadipocyte Differentiation by Myostatin Treatment in 3T3-L1 Cultures*. Biochem Biophys Res Commun 281, 902-906

20. **F. Castro-Munozledo, et al.** (2003) *Commitment of 3T3-F442A cells to adipocyte differentiation takes place during the first 24-36 h after adipogenic stimulation: TNF-[alpha] inhibits commitment*. Exp Cell Res 284, 161-170

21. **D. Shao and M. A. Lazar** (1997) *Peroxisome Proliferator Activated Receptor , CCAAT/ Enhancer-binding Protein , and Cell Cycle Status Regulate the Commitment to Adipocyte Differentiation*. J Biol Chem 272, 21473 - 22147

22. **W. Yeh, et al.** (1995) *Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells*. Proc Natl Acad Sci USA 92, 11086-11090

23. **M. Pinent, et al.** *Intracellular mediators of procyanidin-induced lipolysis in 3T3-L1 adipocytes*. J Agric Food Chem IN PRESS

24. **L. S. Wise and H. Green** (1979) *Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells*. J Biol Chem 254, 273-275

25. **M. M. Bradford** (1976) *A rapid and sensitive method for quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding*. Anal Biochem 72, 248-254

26. **J. L. Ramirez-Zacarias, et al.** (1992) *Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O*. Histochemistry 97, 493-497.

27. **P. Hegde, et al.** (2000) *A concise guide to cDNA microarray analysis*. Biotechniques 29, 548-550, 552-544, 556 passim.

28. **S.-C. F. Pieler R, Hackl H, Thallinger GG, Trajanoski Z.** (2004) *ArrayNorm: comprehensive normalization and analysis of microarray data.* Bioinformatics 20, 1971-1973

29. **M. A. Harris, et al.** (2004) *The Gene Ontology (GO) database and informatics resource*. Nucleic Acids Res 32, D258-261.

30. **H. S. Sul, et al**. (2000) *Function of pref-1 as an inhibitor of adipocyte differentiation*. Int J Obes 24, S15-S19

31. **T. Kuzumaki, et al.** (1998) *Genistein induces p21Cip1/WAF1expression and blocks the G1 to S phase transition in mouse fibroblast and melanoma cells*. Biochem Biophys Res Commun 251, 291-295

32. **Y. H. Choi, et al.** (1998) *Genistein-induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells*. Int J Oncol 13, 391-396.

33. **J. N. Davis, et al.** (1998) *Genistein-induced upregulation of p21WAF1, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells*. Nutr Cancer 32, 123-131.

34. **M. Liberto and D. Cobrinik** (2000) *Growth factor-dependent induction of p21CIP1 by the green tea polyphenol, epigallocatechin gallate*. Cancer Lett 154, 151-161

35. **M. Notoya, et al.** (2004) *Quercetin, a flavonoid, inhibits the proliferation, differentiation, and mineralization of osteoblasts in vitro*. Eur J Pharmacol 485, 89-96

36. **A. Soukas, et al.** (2001) *Distinct Transcriptional Profiles of Adipogenesis in Vivo and in Vitro*. J Biol Chem 276, 34167

37. **L. Fajas, et al.** (1998) *Transcriptional control of adipogenesis*. Curr Opin Cell Biol 10, 165-173

38. **Q. Tong and G. S. Hotamisligil** (2001) *Molecular mechanisms of adipocyte differentiation*. Rev Endocr Metab Disord 2, 349-355.

39. **G. R. Burton and J. McGehee, Robert E.** (2004) *Identification of candidate genes involved in the regulation of adipocyte differentiation using microarray-based gene expression profiling*. Nutrition 20, 109-114

40. **G. R. Burton, et al.** (2004) *Microarray analysis of differentiation-specific gene expression during 3T3-L1 adipogenesis*. Gene 329, 167-185

41. **G. R. Burton, et al.** (2002) *Microarray analysis of gene expression during early adipocyte differentiation*. Gene 293, 21-31

42. **Z. Qiu, et al.** (2001) *DNA Synthesis and Mitotic Clonal Expansion Is Not a Required Step for 3T3-L1 Preadipocyte Differentiation into Adipocytes*. J Biol Chem 276, 11988-11995

43. **Q.-Q. Tang, Otto, T.C., and Lane, D.M.** (2003) *Mitotic clonal expansion: A synchronous process required for adipogenesis*. Proc Natl Acad Sci USA 100, 44-49

44. **Q.-Q. Tang, et al.** (2004) *Sequential gene promoter interactions by C/EBP[beta], C/EBP[alpha], and PPAR[gamma] during adipogenesis*. Biochem Biophys Res Commun 318, 213-218

45. **C. Agarwal, et al.** (2000) *A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468 cells by inhibiting mitogen-activated protein kinases activation and inducing G1 arrest and differentiation*. Clin Cancer Res 6, 2921-2930.

46. **F. Casagrande and J.-M. Darbon** (2001) *Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1*. Biochem Pharmacol 61, 1205-1215

47. **S.-M. Kuo** (1996) *Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells*. Cancer Lett 110, 41-48

48. **N. Ahmad, et al.** (1998) *Skin Cancer Chemopreventive Effects of a Flavonoid Antioxidant Silymarin Are Mediated via Impairment of Receptor Tyrosine Kinase Signaling and Perturbation in Cell Cycle Progression*. Biochem Biophys Res Commun 247, 294-301

49. **J. M. Darbon, et al.** (2000) *Distinct Chk2 activation pathways are triggered by genistein and DNA-damaging agents in human melanoma cells*. J Biol Chem 275, 15363-15369.

50. **N. Dyson** (1998) *The regulation of E2F by pRB-family proteins*. Genes Dev 12, 2245-2262.

51. **A. P. Bracken, et al.** (2004) *E2F target genes: unraveling the biology*. Trends Biochem Sci 29, 409-417

IV~ General Discussion

This thesis is directed at studying the effects of a grape-seed procyanidin extract (GSPE) on the adipocyte, taking as model of study the 3T3-L1 cell line. For some of the experiments, different doses of procyanidins were assayed, all showing a dose-response effect. Therefore, the dose chosen for the majority of the assays was the one that gave maximal response, previously described as non-toxical (1), in order to get a high response that would not lead to confusing results but clear answers to our questions.

This study was initiated following previous results that showed a lipolytic effect of GSPE in 3T3-L1 adipocytes (1), and the first objective was to find mechanisms involved in this GSPE-induced lipolysis.

As some flavonoids have been shown to stimulate lipolysis through the β-receptors (2), and since the β-receptor-stimulated pathways are of major importance in the control of lipolysis, an initial hypothesis was that the GSPE-lipolytic effects were mediated through the interaction with β-receptors. Present results rule out direct action of GSPE in the β-receptors, but its effects have some points in common with the adrenergic stimulation such as the increase in intracellular cAMP and the activation of protein kinase A (PKA). Major research and the characterization of lipolytic pathways has been carried out on short-term signals (3-6), and the central role of the protein kinase A in the control of short-term lipolysis is clearly established (3, 4). However, GSPE effects are long-term mediated, thus, GSPE might act through a usual means of long-term modulation which is modifying the gene expression. In fact, the activation of protein kinase A can be secondary to changes in gene expression, e.g. reduction of phosphodiesterase-3-B (PDE3B) mRNA (7). In this sense,

present results describe a reduction in hormone-sensitive lipase (HSL) and peroxisome proliferator-activated receptor-γ (PPARγ) mRNA. Decreased hormone-sensitive lipase expession was already found in previous results, in which a modification of cAMP levels was suggested to cause this reduction, due to a counter-regulatory mechanism (1). The present results support this hypothesis, since GSPE increases cAMP levels. However, a reduction in the gene expression does not necessarily mean a reduction in protein activity (8), and consequently the reduction in hormone-sensitive lipase expression could follow the activation of the lipase by GSPE. In any case, with the present results, it is not possible to ascribe GSPE-induced lipolysis to the hormone-sensitive lipase actions. In fact, recent theories suggest the existence of other lipases different from hormone-sensitive lipase (6, 9). Okazaki et al. (10) showed that stimulated lipolysis in the absence of the hormone-sensitive lipase was inhibited both by H89 (the protein kinase A inhibitor), and thiazolidinediones. And the present results show an inhibition of GSPE-stimulated lipolysis by the same factors, suggesting the involvement of other lipases in GSPE action. In fact, insulin, which has been shown to be lipolytic after chronic exposure in 3T3-L1, has been suggested to exert these effects through a putative non-HSL lipase (11). Thus, since GSPE shows several similarities with insulin action, it is likely that it also acted in this way.

On the other hand, the fact that GSPE-stimulated lipolysis was inhibited by thiazolidinediones, which are PPARγ ligands, suggests that PPARγ is another mediator of GSPE-induced lipolysis. PPARγ is a transcription factor that regulates several lipid-metabolism genes, especially during adipose differentiation, but its role in mature adipocytes remains controversial (12-14).

Further description of the PPARγ action in differentiated adipocytes would help to find out how it modulates GSPE-induced lipolysis. It is worth mentioning that Guan et al. recently suggested that the thiazolidinediones induce a 'futil cycle' involving the recycling of glycerol through an increase of the enzyme glycerol kinase, barely expressed in adipocytes (15), which would explain the recycling of fatty acids induced by the thiazolidinediones. However, afterwards, the hypothesis of Guan et al. has been questioned since in studies with humans, this 'futil cycle' was not induced by the thiazolidinediones (16); and more recent evidence suggests that the main action of thiazolidinediones is not the induction of glycerol kinase but the stimulation of glyceroneogenesis from other precursors (such as lactate, pyruvate and aminoacids) via the induction of the enzyme phosphoenol pyruvate carboxykinase-C (PEPCK-C) gene expression probably mediated by PPARγ activation (17-19).

In addition, GSPE induced a down-regulation of PPARγ, as did the thiazolidinediones. The thiazolidinediones had already been shown to down-regulate PPARγ expression after long-term treatment (20), following a timing in which they simultaneously activated PPARγ transcriptional activity (8). Liang et. al showed that some flavonoids also decreased PPARγ mRNA after binding (21). This decrease followed conformational changes that led to PPARγ activation; but not all the functions of the transcription factor were activated: flavonoid binding and activating PPARγ did not induce differentiation of mesenchymal stem cells to adipocytes, suggesting that flavonoids acted as effectors binding into a different site than the thiazolidinediones. Thus, the down-regulation of PPARγ by procyanidins

could also reflect direct binding to PPARγ which, after long term exposure, would decrease its mRNA levels.

It should be mentioned that high PPARγ levels are associated with obesity (22) and reductions of the PPARγ expression are associated with weight loss in humans (23); thus, despite the PPARγ role in mature adipocytes not being fully understood, the reduction in the level of PPARγ seems to be related with the improvement of obesity.

In this sense, the study of the effects of GSPE on differentiation showed a reduction in the differentiation process of the 3T3-L1 cells, most likely due to an impaired cell proliferation. Reduction in proliferation is a feature that has already been demonstrated for procyanidins, mainly in the context of the study of their potential as being preventive against cancer (24-27). Present results show that GSPE treatment at the induction of differentiation produces changes in the cell cycle-related gene expression that points out GSPE as an inhibitor of mitotic clonal expansion. Growth arrest is produced either by DNA damage or by a lack of mitogens, and it is controlled by cell cycle checkpoints that regulate the advance from one phase of the cell cycle to the other. Arrested cells induced to proliferate with mitogenic signals, progress through the cell cycle and reach a point in G1 in which cells are independent of mitogenic signals in order to proliferate (28). The fact that the present results show that GSPE interferes with intracellular signalling pathways suggests that procyandins might interfere with mitogenic signals counteracting their effects. Likewise, the monomer epigallocatechin-gallate has been shown to inhibit proliferation only when added at the induction of the cell cycle

progression, that is to say, during the phase in which cells are dependent on mitogens, suggesting that flavonoids interfere with the pathways of mitogens (29). Moreover, epigallocatechin-gallate (30) and genistein (31) added at the induction of differentiation reduced the cell number and the triacylglyceride content of differentiating 3T3-L1 preadipocytes treated with the hormonal cocktail, therefore the *in vitro* effect of those flavonoids on adipocytes may be mediated by the modulation of hormone-stimulated cell proliferation. The arrest of the proliferation by GSPE might be mediated by an increase of p21 cyclin dependent kinase-inhibitor, similarly to what has been shown for grape seed extracts in other cell types (24, 26), and also for other flavonoids in adipocytes (30, 32). However, the intracellular pathways that lead to such an increase in p21 may vary within cell types and flavonoid structure.

In any case, since a lack of mitotic clonal expansion in 3T3-L1 impedes differentiation (33, 34), GSPE seems to alter the adipose differentiation through inhibiting proliferation. Thus, the reduction of hyperplasia would be a potential beneficial effect against obesity.

Another aim of this thesis was to describe the interactions of GSPE with glucose metabolism. In this field, the present results show that GSPE acts both in a short-term and long-term way. Firstly, acute GSPE treatment has insulin-like effects, especially enhancing glucose uptake. Other flavonoids have been shown to modify glucose uptake in adipocytes, but with different results, since some of them inhibited glucose transport (35-37) and others activated it (38, 39), so the effects depend on their structure. Many of them modulated

transport through interaction with the insulin pathways at different levels. In the present results, the increase in glucose uptake is accompanied by glucose transporter GLUT4 translocation to the plasma membrane, thus increasing the number of available transporters. But insulin has been shown to act not only at the number of transporter levels, but also increasing their activity (40). Insulin modifies several intracellular signalling cascades to exert its effects. The phosphatidylinositol-3-kinase (PI3K)/ protein kinase B (PKB/Akt) pathway is the most studied and widely accepted as mediating insulin's signals. The present results show that GSPE also uses this pathway. Kamei et. al suggested that shikonin, another flavonoid, stimulated glucose uptake in adipocytes through a PI3K independent pathway (38). But in their studies, they found a partial inhibition (up to 40%) of glucose uptake by wortmannin, a PI3K inhibitor. In the present results, a higher percentage of inhibition (80%) by wortmannin was found than in Kamei's work, and despite not being total inhibition, the interpretation is that PI3K is at least in part involved in GSPE stimulation of glucose uptake. However, the effects of GSPE cannot be solely explained by PI3K pathway and GLUT4 translocation, and seem to involve other pathways such as p38 mitogen-activated protein kinase (p38 MAPK). The role of p38 MAPK in insulin action is not clear, but it has been related to the activation of glucose transporters (41, 42). It is therefore likely that GSPE activates glucose transport through p38 MAPK in addition or complementary to PI3K, though this might not be the only difference between insulin and GSPE pathways, since there are further differences in their effects.

The study of glucose fate within adipocytes after acute GSPE treatment showed anabolic effects similar to those of insulin. However, GSPE and insulin

effects differed on the glycogen synthesis. Insulin activates glycogen synthesis by stimulating glycogen synthase through several molecular mechanisms (protein dephosphorilation, allosteric activation and enzymatic translocation). The exact pathways through which insulin controls the glycogen-metabolyzing enymes remain unclear, but PI3K is largely involved in the regulation of those mechanisms (43-45). Thus, the GSPE stimulation of glycogen synthesis might at least in part be mediated by PI3K, despite the activation of enzymes down-stream of this kinase possibly differing. Instead, differences in glucose uptake and glycogen synthesis pathways are overcome in the final activation of lipid synthetizing enzyme given that lipogenesis is activated to the same extent with GSPE as with insulin. A hypothesis that should be further analysed is the involvement of PI3K-independent stimulation of PKB/Akt in GSPE acute anabolic effects, since other flavonoids have been shown to activate PKB/Akt independently of PI3K (38), and constitutively active PKB/Akt leads to a similar anabolic profile to GSPE (46). Altogether those results signal GSPE as a factor able to induce a similar response to that of insulin. In fact, results obtained *in vivo* after an acute high dose of GSPE support this hypothesis, as it reduced hyperglycaemia in streptozotocin-diabetic rats. Similar results were previously obtained with other flavonoids, such as myrycetin (47). However, the hypoglycemic effect of this flavonoid was attributed to its role in the glycogen metabolism in the liver, as well as muscle, where it increased glucose uptake. The study did not include the observation of the adipose tissue, despite previous results having shown myrycetin's capability to increase glucose uptake in this cell type (39). According to present results, GSPE antihyperglycemic effects are mediated by a delay in glucose absorption together with direct insulin-like effects on

insulin-sensitive tissues. Interaction of GSPE with the liver has not been assayed, but GSPE increased glucose uptake in muscle similarly to in adipocyte. However, due to the emerging importance of the role of adipose tissue in insulin resistant pathologies (48), it worth emphasize an important role of GSPE effects on adipocytes in glucose homeostasis.

One must bear in mind that the difficulties in quantifying procyanidin absorption to the organism, its transport into blood, concentrations in plasma and so on lead to controversial opinions regarding an accurate estimation of the length of procyanidin presence within the organism in a physiological situation (49-51). Therefore, long-term studies have also been carried out. Anabolic coincidences between GSPE and insulin are extended to long-term treatments. Long-term exposure to GSPE increased basal glucose uptake. However, the effects of GSPE on glucose metabolism showed more differences with the effects of insulin. Glucose uptake after GSPE stimulation was accompanied by lipogenesis and glycogen synthesis. Similarly to short-term effects, GSPE-stimulated glycogen synthesis was lower than with insulin. Instead, *de novo* lipogenesis was equally enhanced by GSPE as by insulin, while glycerol synthesis was more activated due to GSPE. This suggests that GSPE enhances triacylglyceride synthesis not only by increasing fatty acid synthesis but also by promoting reesterification of fatty acids, either from the intracellular pool or from enhanced fatty acid uptake. Insulin controls adipose metabolism by a late response regulation in addition to the described acute responses. Those late changes induced by insulin are mediated by changes in gene expression. E.g. fatty acid synthase, a key controller of *de novo* lipogenesis, has insulin responsive elements in its promoter, and sterol-

regulatory element-binding protein-1c (SREBP1c) together with an upstream stimulatory factor are its main regulators (52). It is likely that GSPE interaction with intracellular signalling transduction pathways leads to the modulation of gene expression through the regulation of transcription factors, thus similarities and differences with insulin pathways could be reflected in the the modulation of transcription factors.

Bringing together the present results, it is shown that long-term exposure to GSPE increases both lipolysis and triacylglyceride synthesis, together with glycogen synthesis. GSPE seems to induce a futile cycle in which lipid synthesis and degradation are enhanced, but no net triglyceride changes are observed. The meaning of this cycle remains poorly understood; however it helps to explain previous results that constitute the origin of this thesis: that GSPE enhances lipolysis without affecting net triacylglyceride accumulation (1). The present results show GSPE-enhanced triacylglyceride synthesis simultaneous to triacylglyceride degradation, in spite of the fact that previous results showed decreased glycerol-3-phosphate dehydrogenase activity by GSPE (1). A possible explanation involves glycerol biosynthesis through an alternative pathway. This is a peroxisomal pathway in which dihydroxyacetone phosphate (DHAP) is not reduced to glycerol-3-phosphate via glycerol-3-phosphate dehydrogenase (G3PDH), but acylated to acyl DHAP via dihydroxyacetone phosphate acyltransferase (DHAPAT) and then reduced to 1-acyl glycerol phosphate, which will finally constitute the glycerol portion of triacylglyceride. This acyl-DHAP pathway has been shown to contribute at least half of the triacylglyceride synthesized in 3T3-L1 adipocytes (53).

In summary, since triacylglycerides in adipocytes are in a constant turnover, long-term exposure to GSPE seems to enhance this natural triacilglyceride renewal, linked to decreased plasmatic glycemia. Since fatty acid release to the plasma is related to insulin resistance, stimulation of the fatty acid reesterification would impede a high increase in fatty acid release that could negatively influence other insulin-sensitive tissues. It has been suggested that insulin resistance originates in adipose tissue, and then it signals to other tissues as muscle or liver (48), so glucose transport in adipocytes plays a critical role in glucose homeostasis. Increased glucose uptake due to GSPE would ameliorate hyperglycemia preventing excessive insulin secretion. In addition, long-term exposure to GSPE does not reduce total uptake of glucose after acute insulin dose: despite it decreasing the response to acute insulin stimulation, GSPE enhances basal glucose uptake. The *in vivo* results reinforce the view of GSPE as being beneficial for diabetes, helping to prevent insulin resistance by several mechanisms: a delay in glucose absorption that leads to lower glucose plasmatic levels and thus reduced insulin secretion and an enhancement of glucose uptake in insulin sensitive tissues by a mechanism partially different from that of insulin. Those mechanisms would act in addition to the already described antioxidant properties of GSPE, which confers them beneficial properties against oxidative stress linked to insulin resistance (54-56).

What is surprising about the present results is that while GSPE in mature adipocytes behaves in an insulin-like way, in preadipocytes it does the opposite, since insulin is one of the main mitogens involved in 3T3-L1 differentiation and GSPE reduced differentiation. This difference could be

attributed to the supra-physiological concentrations of insulin needed to differentiate preadipocytes, higher than the insulin concentrations that lead to its metabolic responses. Another hypothesis would involve the platelet-derived growth factor (PDGF) pathway. This factor is the main mitogen of serum, and is necessary for 3T3-L1 differentiation. 3T3-L1 preadipocytes treated with the hormonal cocktail including insulin but in the absence of either serum or PDGF failed to differentiate (57). Red wine procyanidins have been shown to inhibit the PDGF receptor and the PDGF-dependent cellular responses, such as cell proliferation (58). However, it was assayed in vascular smooth muscle cells, therefore its involvement in 3T3-L1 proliferation should be further assayed.

Despite all, one must bear in mind that these results are not extapolable to *in vivo* situations or to human beings. Actually, the involvement of red wine procyanidins in the control of obesity or diabetes is not so clear. Studies *in vivo* addressing this question are currently been carried out. Preliminary results demonstrate that moderate red wine consumption in a model of rat with diet induced obesity reduced weight gain and epididimal fat tissue (**59**), and lean rats treated with oral gavage of grape seed procyanidins also showed a reduced weight gain (unpublished results). There are other i*n vivo* works showing body weight reduction by grape-seed procyanidins, explained in part through the reduction in food intake (60), a mechanism suggested also in humans (61). Therefore, it is likely that an increase in lipolytic rate and a reduced adipose differentiation contribute, at least in part, to those antiobesity effects of red wine procyanidins. However, further *in vivo* studies must be

carried out and more detailed GSPE effects should be assessed to better understand its role and mechanisms of action in the organism.

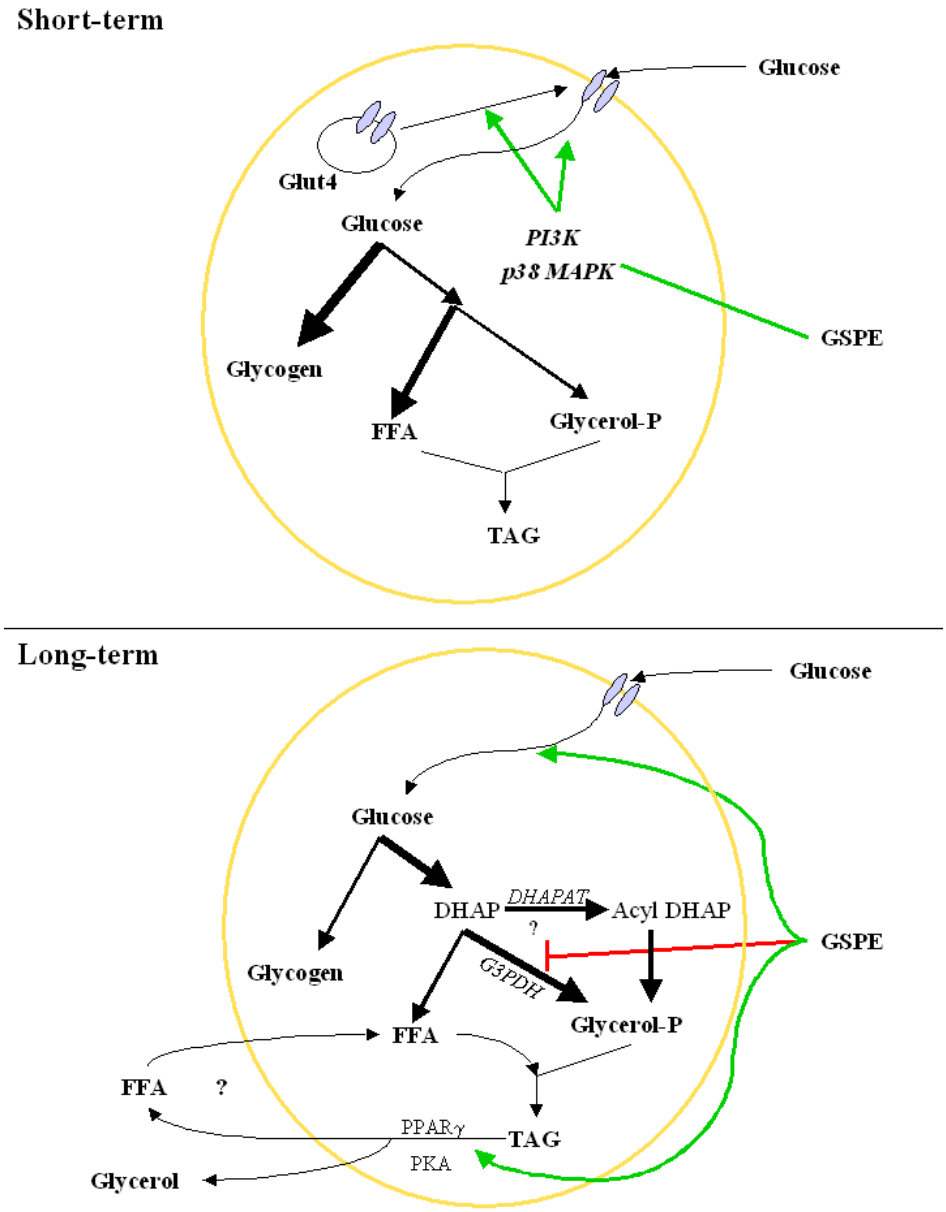

Diagram showing the summary of the major results of this thesis

GSPE: Grape-Seed Procyanidin Extract, PI3K: Phosphatidylinositol-3-Kinase, p38 MAPK: p38 Mitogen-Activated Protein Kinase, FFA: Free Fatty Acids, TAG: Triacylglycerides, DHAP: Dihydroxyacetone Phosphate, DHAPAT: Dihydroxyacetone Phosphate Acyltransferase, G3PDH: Glycerol-3-phosphate Dehydrogenase, PPARγ: Peroxisome Proliferator Activated Receptor-γ, PKA: Protein Kinase A.

References

1. **A. Ardévol, et al.** (2000) *Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes*. Int J Obes 24, 319-324

2. **U. R. Kuppusamy and N. P. Das** (1994) *Potentiation of β-adrenoreceptor agonist-mediated lipolysis by quercetin and fisetin in isolated rat adipocytes*. Biochem Pharmacol 47, 521-529

3. **C. Londos, et al.** (1999) *On the control of lipolysis in adipocytes*. Ann N Y Acad Sci 892, 155-168.

4. **C. Holm** (2003) *Molecular mechanisms regulating hormone-sensitive lipase and lipolysis*. Biochem Soc Trans 31, 1120-1124.

5. **S. J. Yeaman** (2004) *Hormone-sensitive lipase - new roles for an old enzyme*. Biochem J 379, 11-22

6. **V. W. Dolinsky, et al.** (2004) *Triacylglycerol hydrolase: role in intracellular lipid metabolism*. Cell Mol Life Sci 61, 1633-1651.

7. **T. Rahn-Landström, et al.** (2000) *Donwn-regulation of cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumor necrosis factor-alpha and cAMP*. Biochem J 346, 337-343

8. **M. Watanabe, et al.** (2003) *Regulation of PPAR[gamma] transcriptional activity in 3T3-L1 adipocytes*. Biochem Biophys Res Commun 300, 429-436

9. **V. W. Dolinsky, et al.** (2003) *Regulation of triacylglycerol hydrolase expression by dietary fatty acids and peroxisomal proliferator-activated receptors*. Biochim Biophys Acta 1635, 20-28

10. **H. Okazaki, et al.** (2002) *Lipolysis in the absence of Hormone-Sensitive Lipase*. Diabetes 51, 3368-3375

11. **M. Rosenstock, et al.** (2001) *Distinct long-term regulation of glycerol and non-esterified fatty acid release by insulin and TNF-alpha in 3T3-L1 adipocytes*. Diabetologia 44, 55-62

12. **D. E. Moller and J. P. Berger** (2003) *Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation*. Int J Obes Relat Metab Disord 27, S17-21.

13. **Y. Tamori, et al.** (2002) *Role of peroxisome proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3-L1 adipocytes*. Diabetes 51, 2045-2055.

14. **T. Takamura, et al.** (2001) *Stage-specific effects of a thiazolidinedione on proliferation, differentiation and PPAR[gamma] mRNA expression in 3T3-L1 adipocytes*. Eur J Pharmacol 422, 23-29

15. **H. P. Guan, et al.** (2002) *A futile metabolic cycle activated in adipocytes by antidiabetic agents*. Nat Med 8, 1122-1128.

16. **G. D. Tan, et al.** (2003) *A "futile cycle" induced by thiazolidinediones in human adipose tissue?* Nat Med 9, 811-812

17. **J. Tordjman, et al.** (2003) *Thiazolidinediones block fatty acid release by inducing glyceroneogenesis in fat cells*. J Biol Chem 278, 18785-18790

18. **L. Reshef, et al.** (2003) *Glyceroneogenesis and the triglyceride/fatty acid cycle*. J Biol Chem 278, 30413-30416

19. **C. Forest, et al.** (2003) *Fatty acid recycling in adipocytes: a role for glyceroneogenesis and phosphoenolpyruvate carboxykinase*. Biochem Soc Trans 31, 1125-1129.

20. **S. Perrey, et al.** (2001) *Thiazolidinedione and tumor necrosis factor alpha induced down-regulation of peroxisome proliferator activated receptor gamma mRNA in differentiated adipocytes*. Metabolism 50, 36-40

21. **Y.-C. Liang, et al.** (2001) *Suppression of inducible cyclooxigenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor-gamma by flavonoids in mouse macrophages*. FEBS Lett 496, 12-18

22. **A. J. Vidal-Puig, et al.** (1997) *Peroxisome proliferator-activated receptor gene expression in human tissues . Effects of obesity, weight loss, and regulation by insulin and glucocorticoids*. J Clin Invest 99, 2416-2422

23. **J. Ribot, et al.** (2001) *Weight loss reduces expression of SREBP1c/ADD1 and PPARgamma2 in adipose tissue of obese women*. Eur J Physiol 441, 498-505

24. **C. Agarwal, et al.** (2000) *A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468 cells by*

inhibiting mitogen-activated protein kinases activation and inducing G1 arrest and differentiation. Clin Cancer Res 6, 2921-2930.

25. **C. Laurent, et al.** (2004) *Grape seed extract affects proliferation and differentiation of human intestinal Caco-2 cells*. J Agric Food Chem 52, 3301-3308.

26. **C. Agarwal, et al.** (2000) *Anticarcinogenic effect of a polyphenolic fraction isolated from grape seeds in human prostate carcinoma DU145 cells: modulation of mitogenic signaling and cell-cycle regulators and induction of G1 arrest and apoptosis*. Mol Carcinog 28, 129-138.

27. **S. Carnesecchi, et al.** (2002) *Flavanols and procyanidins of cocoa and chocolate inhibit growth and polyamine biosynthesis of human colonic cancer cells*. Cancer Lett 175, 147-155

28. **M. Tessema, et al.** (2004) *Cell cycle and no end*. Virchows Arch 444, 313-323

29. **M. Liberto and D. Cobrinik** (2000) *Growth factor-dependent induction of p21CIP1 by the green tea polyphenol, epigallocatechin gallate*. Cancer Lett 154, 151-161

30. **Y. H. Kao, et al.** (2000) *Modulation of obesity by a green tea catechin*. Am J Clin Nutr 72, 1232-1234.

31. **A. W. Harmon and J. B. Harp** (2001) *Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis*. Am J Physiol Cell Physiol 280, C807-813

32. **T. Kuzumaki, et al.** (1998) *Genistein induces p21Cip1/WAF1expression and blocks the G1 to S phase transition in mouse fibroblast and melanoma cells*. Biochem Biophys Res Commun 251, 291-295

33. **W. Yeh, et al.** (1995) *Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells*. Proc Natl Acad Sci USA 92, 11086-11090

34. **Y. M. Patel and M. D. Lane** (2000) *Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27*. J Biol Chem 275, 17653-17660

35. **A. Abler, et al.** (1992) *Genistein differentially inhibits postreceptor effects of insulin in rat adipocytes without inhibiting the insulin receptor kinase*. J Biol Chem 267, 3946-3951

36. **P. Strobel, et al.** (2004) *Myricetin, quercetin and catechin-gallate inhibit glucose uptake in isolated rat adipocytes*. Biochem J 7, 7

37. **A. W. Harmon, Patel, Y.M.** (2003) *Naringenin inhibits phosphoinositide 3-kinase activity and glucose uptake in 3T3-L1 adipocytes.* Biochem Biophys Res Commun 305, 229-234

38. **R. Kamei, et al.** (2002) *Shikonin stimulates glucose uptake in 3T3-L1 adipocytes via an insulin-independent tyrosine kinase pathway*. Biochem Biophys Res Commun 292, 642-651

39. **K. C. Ong and H.-E. Khoo** (1996) *Insulinomimetic effects of myricetin on lipogenesis and glucose transport in rat adipocytes but not glucose transporter translocation.* Biochem Pharmacol 51, 423-429

40. **K. V. Kandror** (2003) *A long search for Glut4 activation*. Sci STKE 2003, PE5.

41. **R. Somwar, et al.** (2002) *A dominant-negative p38 MAPK mutant and novel selective inhibitors of p38 MAPK reduce insulin-stimulated glucose uptake in 3T3-L1 adipocytes without affecting GLUT4 translocation*. J Biol Chem 277, 50386-50395

42. **L. M. Furtado, et al.** (2002) *Activation of the glucose transporter GLUT4 by insulin*. Biochem Cell Biol 80, 569-578

43. **S. A. Summers, et al.** (1999) *The role of glycogen synthase kinase 3beta in insulin-stimulated glucose metabolism*. J Biol Chem 274, 17934-17940

44. **H. Ou, et al.** (2004) *Spatial reorganization of glycogen synthase upon activation in 3T3-L1 adipocytes*. Endocrinology, en.2004-1022

45. **T. Yamada, et al.** (2002) *Role of PDK1 in insulin-signaling pathway for glucose metabolism in 3T3-L1 adipocytes*. Am J Physiol Endocrinol Metab 282, E1385-1394

46. **A. D. Kohn, et al.** (1996) *Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation*. J Biol Chem 271, 31372-31378

47. **K. C. Ong and H.-E. Khoo** (2000) *Effects of myricetin on glycemia and glycogen metabolism in diabetic rats*. Life Sci 67, 1695-1705

48. **U. Smith** (2002) *Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance--is insulin resistance initiated in the adipose tissue?* Int J Obes Relat Metab Disord 26, 897-904.

49. **M.-P. Gonthier, et al.** (2003) *Metabolism of dietary procyanidins in rats*. Free Radic Biol Med 35, 837-844

50. **A. Scalbert** (2000) *Dietary intake and bioavailability of polyphenols*. J Nutr 130, 2073S-2085S

51. **S. A. Aherne and N. M. O'Brien** (2002) *Dietary flavonols: chemistry, food content, and metabolism*. Nutrition 18, 75-81

52. **Y. Wang, et al.** (2004) *The human fatty acid synthase gene and de novo lipogenesis are coordinately regulated in human adipose tissue*. J Nutr 134, 1032-1038

53. **A. K. Hajra, et al.** (2000) *Induction of the peroxisomal glycerolipid-synthesizing enzymes during differentiation of 3T3-L1 adipocytes. Role in triacylglycerol synthesis*. J Biol Chem 275, 9441-9446

54. **N. Landrault, et al.** (2003) *Effect of a polyphenols-enriched chardonnay white wine in diabetic rats*. J Agric Food Chem 51, 311-318.

55. **G. Caimi, et al.** (2003) *Diabetes mellitus: oxidative stress and wine*. Curr Med Res Opin 19, 581-586.

56. **A. Ceriello, et al.** (2001) *Red wine protects diabetic patients from meal-induced oxidative stress and thrombosis activation: a pleasant approach to the prevention of cardiovascular disease in diabetes*. Eur J Clin Invest 31, 322-328.

57. **H. Staiger, et al.** (2002) *Serum-free differentiation of 3T3-L1 preadipocytes is characterized by only transient expression of peroxisome proliferator-activated receptor-[gamma]*. Biochem Biophys Res Commun 296, 125-128

58. **S. Rosenkranz, et al.** (2002) *Inhibition of the PDGF receptor by red wine flavonoids provides a molecular explanation for the "French paradox"*. FASEB J 16, 1958-1960

59. **M. Vadillo, et al.** *Moderate red wine consumption prevents body weight gain in rats fed a hyperlipidic diet*. Int J Obes (Submitted)

60. **K. Tebib, et al.** (1996) *effects of dietary grape seed tannins on rat cecal fermentation and colonic bacterial enzymes*. Nutr Res 16, 105-110

61. **N. Vogels, et al.** (2004) *The effect of grape-seed extract on 24 h energy intake in humans*. Eur J Clin Nutr 58, 667-673.

V~ Conclusions

To sum up, with the present results progress has been made regarding the knowledge of the effects of grape-seed procyanidins on adipocytes, and more concretely the main conclusions of this thesis are:

1. Grape seed derived procyanidins (GSPE) have an antihyperglycemic effect on STZ-diabetic rats.

2. GSPE has insulinomimetic effects on adipocytes, both for short-term and long-term exposure: it increases glucose uptake, enhances glycogen synthesis and triacylglyceride synthesis. However there are some differences with the effects of insulin: procyanidins are less efficient at activating glycogen synthesis than insulin in both long-term and acute treatments; in long-term exposure it enhances glycerol synthesis more than insulin; and GSPE shows lower insulin resistance after long-term exposure.

3. GSPE shares some of the intracellular mediators of insulin signalling pathways to exert its insulinomimetic effects: PI3K and p38 MAPK, and also some of the insulin mechanisms such as Glut-4 translocation; but GSPE might use also some other complementary mechanisms.

4. The long-term lipolytic effect of GSPE requires the intracellular factors protein kinase A (PKA) and PPARγ.

5. GSPE interferes with the differentiation process of 3T3-L1 fibroblasts when added at the onset of differentiation. It reduces triglyceride accumulation, modifies adipose differentiation markers and induces great gene expression changes at this time point, suggesting that GSPE interferes with progression through the cell cycle which is necessary to fully differentiate.