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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Materials and reagents

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

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

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

2.2. Cell culture

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

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

2.3. Glucose uptake assay

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

2.4. Immunoblotting

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

2.5. Data analysis

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

3. Results

3.1. GSPE activates the insulin receptor

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

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

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

3.2. Akt and MAPK are mediators of GSPE effects

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

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

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

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

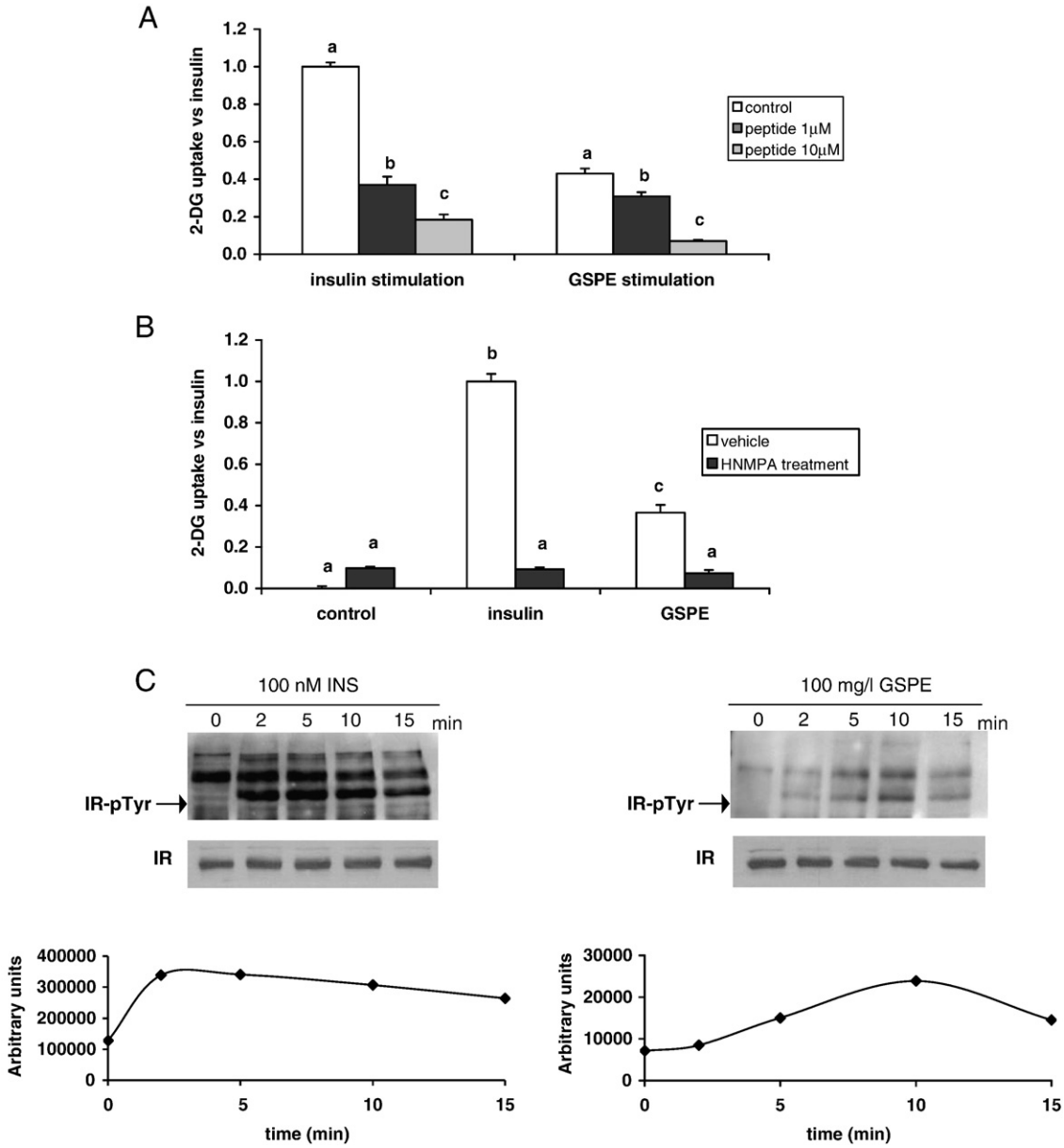

Fig. 1. Effects of GSPE on insulin receptor activation. Fully differentiated 3T3-L1 adipocytes were depleted with serum-free medium supplemented with Dulbecco's Modified Eagle's Medium containing 0.2% BSA for 2 h. Cells were preincubated for 1 h with (A) an insulin antagonist peptide at 1 and 10 μM,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

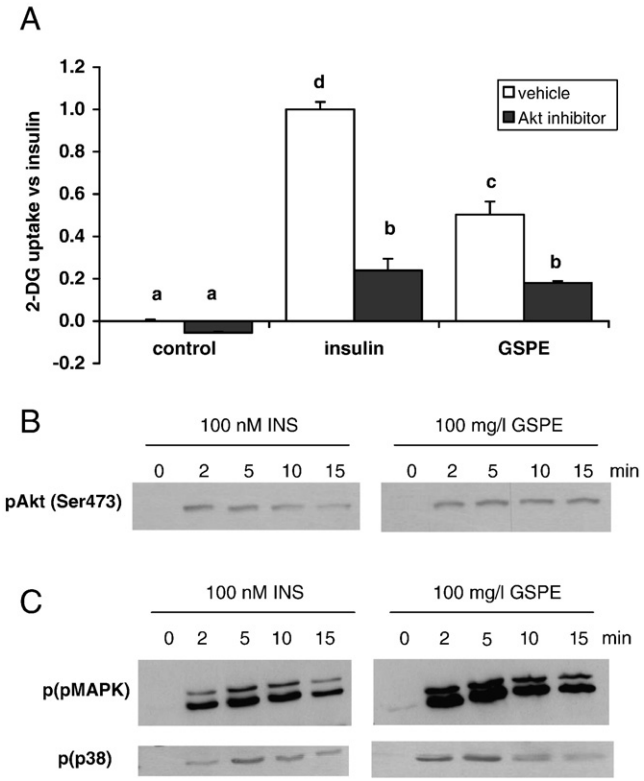

Fig. 2. Effects of GSPE on the downstream signaling insulin pathway. (A) Depleted 3T3-L1 adipocytes were incubated with 15 μM of Akt inhibitor VIII for 1 h. After treatment, cells were stimulated with 100 nM insulin or 100 mg/L GSPE for 30 min and 2-deoxyglucose uptake was assayed. Protein content was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

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

4. Discussion

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

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

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

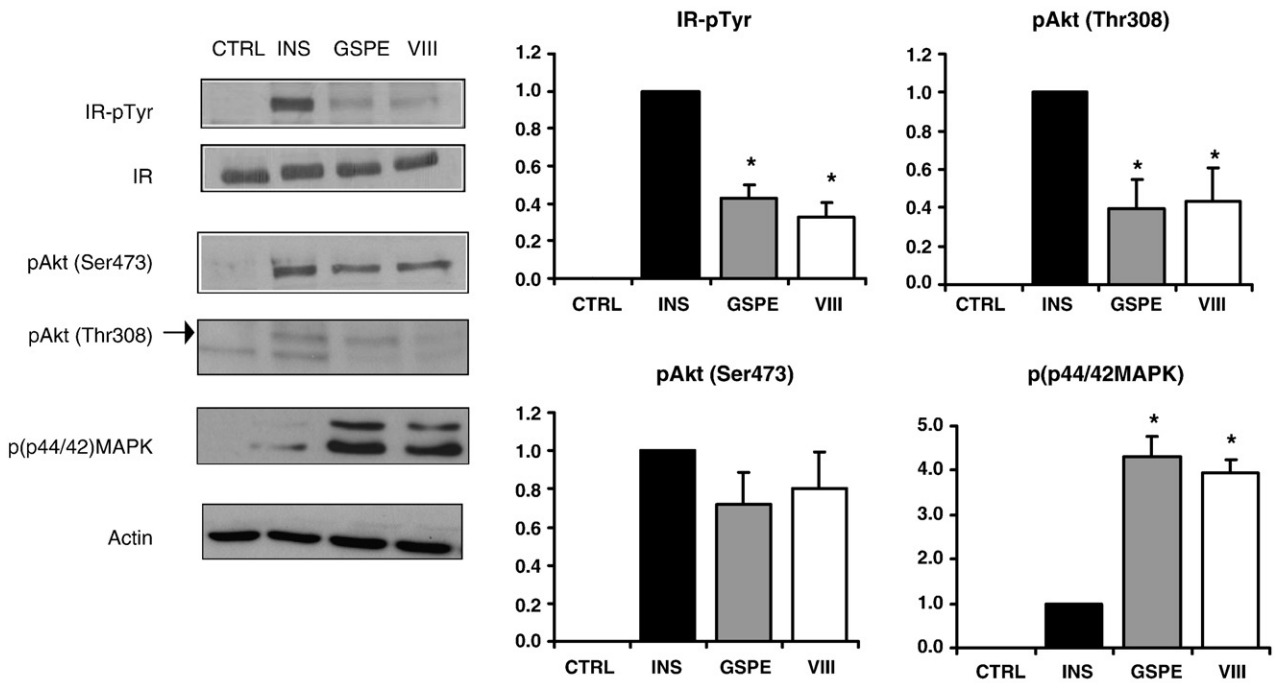

Fig. 3. Effects of fraction VIII on insulin receptor and on insulin signaling pathway. CHO-IR cells were treated with 100 nM insulin, 100 mg/L GSPE and 100 mg/L of fraction VIII for 5 min. Cells were lysed and proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes. Insulin receptor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

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

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

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

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

phosphorylation of these kinases. However, more studies must be carried out to clarify the detailed mechanism underlying GSPE-activated signaling mechanisms.

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

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References

[1] Alberti KG, Zimmet P, Shaw J. Metabolic syndrome—a new world-wide definition. A consensus statement from the International Diabetes Federation. Diabet Med 2006;23(5):469–80.
[2] Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 2001;414(6865):799–806.
[3] White MF. Insulin signaling in health and disease. Science 2003;302(5651): 1710–1, doi:10.1126/science.1092952.
[4] Bevan P. Insulin signalling. J Cell Sci 2001;114(8):1429–30.
[5] Harmon AW, Paul DS, Patel YM. MEK inhibitors impair insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 2004;287(4): E758–66, doi:10.1152/ajpendo.00581.2003.
[6] Williams RJ, Spencer JPE, Rice-Evans C. Flavonoids: antioxidants or signalling molecules? Free Radic Biol Med 2004;36(7):838–49.
[7] Liu IM, Tzeng TF, Liou SS, Lan TW. Myricetin, a naturally occurring flavonol, ameliorates insulin resistance induced by a high-fructose diet in rats. Life Sci 2007; 81(21-22):1479–88.
[8] Zanatta L, Rosso A, Folador P, et al. Insulinomimetic effect of kaempferol 3-Neohesperidoside on the rat soleus muscle. J Nat Prod 2008;71:532–5.
[9] Waltner-Law ME, Wang XL, Law BK, et al. Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. J Biol Chem 2002;277:34933–40.
[10] Lorenz M, Wessler S, Follmann E, et al. A constituent of green tea, epigallocatechin-3-gallate, activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMP-dependent protein kinase-, and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. J Biol Chem 2004;279:6190–5.
[11] Kim J-A, Formoso G, Li Y, et al. Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and fyn. J Biol Chem 2007;282 (18):13736–45.
[12] Potenza MA, Marasciulo FL, Tarquinio M, et al. EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. Am J Physiol Endocrinol Metab 2007;292(5):E1378–87.
[13] Nomura M, Takahashi T, Nagata N, et al. Inhibitory mechanisms of flavonoids on insulin-stimulated glucose uptake in MC3T3-G2/PA6 adipose cells. Biol Pharm Bull 2008;31(7):1403–9.
[14] Collins QF, Liu HY, Pi J, et al. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5'-AMP-activated protein kinase. J Biol Chem 2007:30143–9.
[15] Sugiyama H, et al. Oligomeric procyanidins in apple polyphenol are main active components for inhibition of pancreatic lipase and triglyceride absorption. J Agric Food Chem 2007;55:4604–9.
[16] Schäfer A, Högger P. Oligomeric procyanidins of French maritime pine bark extract (Pycnogenol®) effectively inhibit [alpha]-glucosidase. Diabetes Res Clin Pract 2007;77(1):41–6.
[17] Muthusamy VS, et al. Tannins present in *Cichorium intybus* enhance glucose uptake and inhibit adipogenesis in 3T3-L1 adipocytes through PTP1B inhibition. Chem Biol Interact 2008;174(1):69–78.
[18] Klein G, et al. Antidiabetes and anti-obesity activity of *Lagerstroemia speciosa*. eCAM 2007;4(4):401–7.
[19] Kwon DY, et al. The isoflavonoid aglycone-rich fractions of *Chungkookjang*, fermented unsalted soybeans, enhance insulin signaling and peroxisome proliferator-activated receptor-gamma activity in vitro. BioFactors 2006;26(4): 245–58.
[20] Cao H, Polansky MM, Anderson RA. Cinnamon extract and polyphenols affect the expression of tristetraprolin, insulin receptor, and glucose transporter 4 in mouse 3T3-L1 adipocytes. Arch Biochem Biophys 2007;459(2):214–22.
[21] Li W, et al. Antihyperglycemic effect of *Cephalotaxus sinensis* leaves and GLUT-4 translocation facilitating activity of its flavonoid constituents. Biol Pharm Bull 2007;30(6):1123–9.
[22] Pinent M, et al. Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. Endocrinology 2004;145(11):4985–90.
[23] Ardévol A, et al. Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes. Int J Obes 2000;24:319–24.
[24] Bradford M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–54.
[25] Onnockx S, De Schutter J, Blockmans M, Xie J, Jacobs C, Vanderwinden JM, Erneux C, Pirson I. The association between the SH2-containing inositol polyphosphate 5-phosphatase 2 (SHIP2) and the adaptor protein APS has an impact on biochemical properties of both partners. J Cell Physiol 2008;214: 260–72.
[26] Schäffer L, et al. Assembly of high-affinity insulin receptor agonists and antagonists from peptide building blocks. Proc Natl Acad Sci U S A 2003;100(8): 4435–9.
[27] Li Y, et al. Natural anti-diabetic compound 1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose binds to insulin receptor and activates insulin-mediated glucose transport signaling pathway. Biochem Biophys Res Commun 2005;336(2):430–7.
[28] Terra X, Valls J, Vitrac X, et al. Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. J Agric Food Chem 2007;55:4357–65.
[29] Moller DE. New drug targets for type 2 diabetes and the metabolic syndrome. Nature 2001;414:821–7.
[30] Pinent M, et al. Grape-seed derived procyanidins interfere with adipogenesis of 3T3-L1 cells at the onset of differentiation. Int J Obes Relat Metab Disord 2005;29 (8):934–41.
[31] Pinent M, Blade MC, Salvado MJ, et al. Metabolic fate of glucose on 3T3-L1 adipocytes treated with grape seed-derived procyanidin extract (GSPE). Comparison with the Effects of Insulin. J Agric Food Chem 2005;53:5932–5.
[32] Wada T, et al. Characteristics of signalling properties mediated by long-acting insulin analogue glargine and detemir in target cells of insulin. Diab Res Clin Pract 2008.
[33] Jensen M, et al. Activation of the insulin receptor by insulin and a synthetic peptide leads to divergent metabolic and mitogenic signaling and responses. J Biol Chem 2007;282(48):35179–86.
[34] Zhang J. Resveratrol inhibits insulin responses in a SirT1-independent pathway. Biochem J 2006;397(3):519–27.
[35] Thornton TM, Pedraza-Alva G, Deng B, et al. Phosphorylation by p38 MAPK as an alternative pathway for GSK3{beta} inactivation. Science 2008;320:667–70.
[36] Kumar A, Harris TE, Keller SR, et al. Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances basal glycogen synthase activity. Molec Cell Biol 2008;28:61–70.
[37] Hansen BF, et al. Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. Biochem J 1996; 315(1):271–9.
[38] Kurtzhals P, et al. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. Diabetes 2000;49(6): 999–1005.
[39] Uhles S, et al. Selective gene activation by spatial segregation of insulin receptor B signaling. FASEB J 2007;21(7):1609–21.
[40] Hamer I, et al. An arginine to cysteine252 mutation in insulin receptors from a patient with severe insulin resistance inhibits receptor internalisation but preserves signalling events. Diabetologia 2002;45(5):657–67.