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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

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In this paper we investigate the effects of a grape seed procyanidin extract (GSPE) on the metabolic fate of glucose in adipocytes. Differentiated 3T3-L1 cells were treated with 140 mg/L GSPE or 100 nM insulin for a short period (1 h, acute treatment) or for a long period (15 h, chronic treatment). 2-Deoxy-[1-^{3}H]glucose uptake and [1-^{14}C]glucose incorporation into cells, glycogen, and lipid were measured. We found that GSPE mimicked the anabolic effects of insulin but there were several important differences. GSPE stimulated glycogen synthesis less than insulin. After chronic exposure, GSPE induced a higher incorporation of glucose into lipid, mainly due to the increase in glucose directed to glycerol synthesis. Our main conclusions, therefore, are that GSPE has insulinomimetic properties and activates glycogen and lipid synthesis. However, the differences between the effects of GSPE and the effects of insulin indicate that GSPE uses mechanisms complementary to those of insulin signaling 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 have beneficial effects on human health, being cardioprotective, antioxidant, antigenotoxic, antiinflammatory, 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 (*3*, *4*). In fact, most of their beneficial effects have so far been attributed to their antioxidant properties (*2*, *4*, *5*). It has recently been suggested, however, that they also act on cells by modifying or interacting with specific proteins of important intracellular signaling pathways (*6*, *7*), and in fact their role in improving hyperglycemia in streptozotocin-induced diabetes in rats has been reported (*8*). 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 in insulin-sensitive cell lines (3T3-L1 adipocytes and L6E9 myotubes) (*9*). 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 phosphatidylinositol 3-kinase (PI3K) and a p38 mitogen-activated protein kinase (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. (*10*) showed that catechin has a hypoglycemic action due to an increase in glycogenesis and a decrease in glycogenolysis in the liver. Ahmad et al. (*11*) 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 analyze 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 and compare these effects with those of insulin. We worked with the higher, nontoxic doses of GSPE (140 mg/L) and 100 nM insulin to obtain a clearly visible effect.

MATERIALS AND METHODS

Cells, Reagents, and Materials. 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 Science Group, Hercules, CA). 2-Deoxy-[1-^{3}H]glucose was from Amersham Biosciences (Buckinghamshire, England). ARC-120-D-[1-^{14}C]Glucose was from American Radiolabeled Chemicals (St Louis, MO).

Cell Culture and Differentiation. 3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (*12*). Briefly, confluent preadipocytes were treated with 0.25 μmol/L

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dexamethasone, 0.5 mmol/L 3-isobutylmethylxanthine, and 5 μg/mL insulin for 2 days in 10% fetal bovine serum (FBS) containing Dulbecco's modified Eagle's medium (DMEM). The cells were switched to 10% FBS/DMEM containing only insulin for 2 more days and then switched to 10% FBS/DMEM 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 37 °C with serum-free supplemented DMEM containing 0.2% BSA (depletion medium) for 2 h. During the last 60 min 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 h without previous depletion.

For experiments on glucose uptake there was only the chronic GSPE treatment. The cells were treated with GSPE for 15 h, then GSPE was removed, and the cells were incubated with depletion medium for 2 h. Finally, during the last 30 min of this depletion, the cells were treated with insulin.

2-Deoxyglucose Uptake. Glucose transport was determined by measuring the uptake of 2-deoxy-D-[^{3}H]glucose. The cells were cultured on 6- or 12-well plates. The transport assay was initiated by washing the cells twice in a transport solution (20 mmol/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 $CaCl_2$, and 2 mmol/L pyruvate, pH 7.4). The cells were then incubated for 7 min in the transport solution, which contained 0.1 mmol/L 2-deoxy-D-glucose and 1 μCi of 2-deoxy-D-[^{3}H]glucose (10 mCi/mmol). Glucose uptake was stopped by adding 2 volumes of ice-cold 50 mmol/L glucose in PBS and washing twice in the same solution. Cells were disrupted by adding 0.1 mol/L NaOH in 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 (*13*). Each condition was run in triplicate.

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

To determine the incorporation into glycogen, homogenate was hydrolyzed by incubating it at 100 °C with 50% KOH for 15 min. Carrier glycogen (5 mg) was also added to the sample during this boiling time. Glycogen was then precipitated with 95% cold ethanol and incubated for 30 min at −20 °C. After centrifugation (30 min, 2000*g*), the pellet was resuspended in 1 mL of hot water and washed again with ethanol. The final glycogen precipitated was resuspended in 1 mL of 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 of 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 50 °C for 4 h to saponify the lipids. The solution was then acidified and fatty acids were extracted into 2 mL of 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 triacylglycerol.

Calculations and Statistical Analysis. Results are expressed as the mean ± SEM. Effects were assessed by Student's *t*-test. All calculations were performed with SPSS software.

RESULTS

Metabolic Fate of Glucose in GSPE-Treated Adipocytes. We previously showed that an acute GSPE treatment stimulated 2-deoxyglucose uptake by up to 7 times (*9*). 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. However, 2-deoxyglucose only allows measurements of coupled transport and phosphorylation, since 2-deoxyglucose is phosphorylated but not further metabolized by cells (*14*). To assay the metabolic fate of this uptaken glucose, we measured the radiolabeled glucose incorporated into the cells and into the glycogen and lipids. **Table 1** shows that after an acute treatment of 140 mg/L GSPE for 60 min, total glucose incorporated into the cell increased ∼6-fold over the base situation. This effect was similar to that induced on 2-deoxyglucose uptake (*9*). **Table 1** also shows two metabolic fates of this glucose: glycogen synthesis and lipid synthesis. The same methodological approach was carried out after a chronic treatment of 140 mg/L GSPE for 15 h. Total glucose incorporated into the cell increased (1.83 ± 0.24)-fold (**Table 2**), which was similar to the stimulation of 2-deoxyglucose uptake. In the chronic treatment, glycogen synthesis and lipid synthesis were again metabolic fates of this glucose. There were two main differences between the metabolic fates of glucose after acute and chronic GSPE treatments. With the acute treatment, the stimulation of glycogen synthesis was higher than the stimulation of lipid synthesis; with the chronic treatment, the stimulation of lipid synthesis was higher than the stimulation of glycogen synthesis.

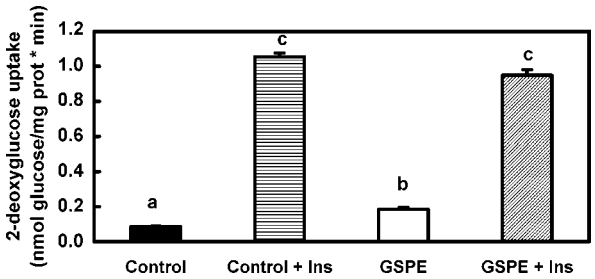

Figure 1. Effects of GSPE pretreatment on insulin-stimulated 2-deoxyglucose uptake. 3T3-L1 adipocytes were treated for 15 h with 140 mg/L GSPE. This was followed by 2-h depletion (cells without pretreatment are called controls). For the last 30 min, acute insulin stimulation (100 nM) was performed (bars 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, and c indicate statistically significant differences between treatments.

Table 1. Radiolabeled Glucose Incorporation after an Acute GSPE or Insulin Treatment in 3T3-L1 Adipocytes[a]

glucose incorporation (nmols/mg prot*h)	treatment		
	control	GSPE	insulin
total	1.95 ± 0.3a	9.64 ± 0.9b	11.15 ± 2.2b
into glycogen	0.04 ± 0.0a	1.89 ± 0.2b	3.93 ± 0.7c
into lipid	0.26 ± 0.1a	2.80 ± 0.5b	3.09 ± 0.7b

[a] Fully differentiated adipocytes were treated with 140 mg/L GSPE or 100 nM insulin for 1 h, and D-[1-^{14}C]glucose incorporation was assayed as described under Materials and Methods. First row, total glucose incorporation into the cells; second row, glucose incorporated into glycogen; third row, total glucose incorporated into lipid. The data are the means ± SEM of three independent experiments. a, b, and c indicate statistically significant differences between treatments.

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

Table 2. Radiolabeled Glucose Incorporation after a Chronic GSPE or Insulin Treatment in 3T3-L1 Adipocytes[a]

glucose incorporation (nmols/mg prot*h)	treatment		
	control	GSPE	insulin
total	3.64 ± 0.48a	6.16 ± 0.30b	5.36 ± 0.07c
into glycogen	0.18 ± 0.01a	0.30 ± 0.03b	0.92 ± 0.07c
into lipid	0.33 ± 0.05a	1.09 ± 0.05b	0.85 ± 0.06c

[a] Fully differentiated adipocytes were treated with 140 mg/L GSPE or 100 nM insulin for 15 h, and D-[1-^{14}C]glucose incorporation was assayed as described under Materials and Methods. First row, total glucose incorporation into the cells; second row, glucose incorporated into glycogen; third row, total glucose incorporated into lipid. The data are the means ± SEM of three independent experiments. a, b, and c indicate statistically significant differences between treatments.

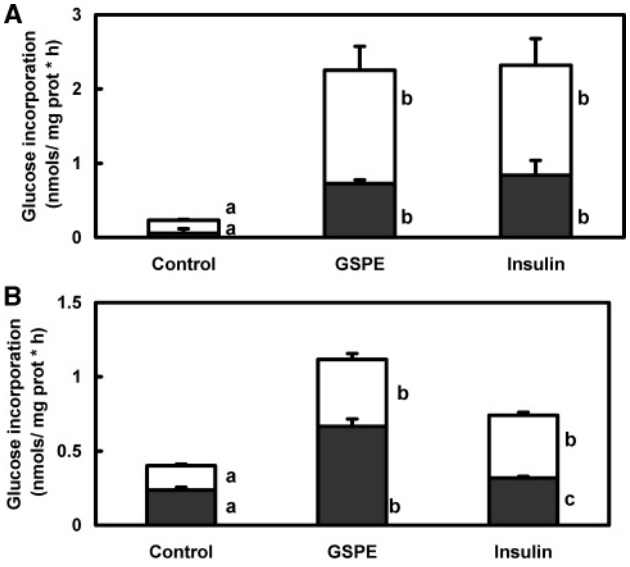

Figure 2. Distribution of D-[1-^{14}C]glucose incorporated into lipids after GSPE or insulin treatment in 3T3-L1 adipocytes. Fully differentiated adipocytes were treated with 140 mg/L GSPE or 100 nM insulin for 1 h **(A)** or 15 h **(B),** and D-[1-^{14}C]glucose incorporation into the cells was performed. 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, and c indicate statistically significant differences between treatments.

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 (**Table 1)** increased the total glucose incorporated into the cell by similar amounts, but GSPE activated glycogen synthesis less than insulin. 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 or as fractionated lipid components (**Figure 2A**). The differences between the treatments were greater after the chronic treatment. **Table 2** 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 (**Table 2)**. GSPE activated the synthesis of glycogen to a lesser extent than insulin but directed a higher amount of glucose toward lipid synthesis. Specifically, most of this glucose was directed toward the synthesis of glycerol (**Figure 2B**).

DISCUSSION

We previously showed that an acute treatment with GSPE had an antihyperglycemic effect in streptozotocin-diabetic animals (*9*). Similarly, Al-Awwadi et al. (*8*) showed that a polyphenol extract from a red wine, chronically administered to streptozotocin-diabetic animals, also induced a reduction in glycemia. 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 signaling that control glucose uptake in insulin-sensitive cells (*9*). 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 toward glycerol synthesis. This effect helps to explain the previously described effects of GSPE on adipose cells; that is, GSPE increases glycerol release to the medium after a chronic treatment (*12*) without changing the triglyceride contents in the adipocytes (*7*). Since glycerol kinase (*15*) 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 toward 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 (*10*) and epicatechin (*11*). These studies showed that these monomeric catechins increase glycogen synthesis in the liver (*10*) and in the diaphragm (*11*) 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-signaling pathways to exert their effects on glucose uptake (*9*). Nevertheless, in that study we observed that there were some differences between the effects of GSPE and insulin on 2-deoxyglucose uptake. There was an additive effect (20%) after simultaneous treatment of procyanidins with a saturating dose of insulin. Also, two different specific kinase inhibitors acted differentially on the stimulation by those agents: GSPE-stimulated 2-deoxyglucose uptake was 20% less inhibited than that of insulin by the PI3K inhibitor, while it was 20% more inhibited by a p38 MAPK inhibitor. In this paper we also show that the responsiveness to an acute dose of insulin after chronic pretreatment with GSPE was higher than the responsiveness after chronic pretreatment with insulin. Several reports have shown that prolonged exposure to insulin induces insulin resistance (*16*, *17*). The degree of insulin resistance depends on the insulin doses for pretreatment 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 the effects 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. A similar anabolic profile was obtained by Kohn et al. (*18*) with a constitutively active Akt, a kinase involved in the insulin-signaling pathways. In fact, all these differences between GSPE and insulin effects suggest that these agents use complementary mechanisms and we do not discard a possible point of convergence between the stimulation by the two agents, as has been suggested by other flavonoids (*19*). A further study of the exact mechanisms used by GSPE will help to better understand the anabolic effects described here.

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-signaling pathway mediators, suggest that GSPE uses a mechanism complementary to that of insulin to induce its insulinomimetic properties in 3T3-L1 adipocytes.

ABBREVIATIONS

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

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