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Procyanidins modify insulinemia by affecting insulin production and degradation☆

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Abstract

Previous studies from our research group have suggested that procyanidins modify glycemia and insulinemia. The aim of this work was to evaluate the effects of procyanidins on β-cell functionality in a nonpathological system. Four groups of healthy rats were studied. The animals were given daily acute doses of grape seed procyanidin extract (GSPE) for different time periods and at different daily amounts. A β-cell line (INS-1E) was treated with 25 mg GSPE/L for 24 h to identify possible mechanisms of action for the procyanidins. *In vivo* experiments showed that different doses of GSPE affected insulinemia in different ways by modifying β-cell functionality and/or insulin degradation. The islets isolated from rats that were treated with 25 mg GSPE/kg of body weight for 45 days exhibited a limited response to glucose stimulation. In addition, insulin gene expression, insulin synthesis and expression of genes related to insulin secretion were all down-regulated. *In vitro* studies revealed that GSPE decreased the ability of β-cells to secrete insulin in response to glucose. GSPE increased glucose uptake in β-cells under high-glucose conditions but impaired glucose-induced mitochondrial hyperpolarization, decreased adenosine triphosphate (ATP) synthesis and altered cellular membrane potentials. GSPE also modified Glut2, glucokinase and Ucp2 gene expression as well as altered the expression of hepatic insulin-degrading enzyme (Ide), thereby altering insulin degradation. At some doses, procyanidins changed β-cell functionality by modifying insulin synthesis, secretion and degradation under nonpathological conditions. Membrane potentials and Ide provide putative targets for procyanidins to induce these effects.

1. Introduction

It is now generally accepted that food can have health-promoting properties beyond its traditional nutritional value [1]. Procyanidins are a class of bioactive compounds that are usually found in fruits and other plant organs and are widely consumed. Procyanidins can protect against coronary heart diseases and atherosclerosis as well as act on several metabolic processes that are associated with the development of these disorders [2].

Most of the studies describing the beneficial effects of procyanidins have shown the peripheral activity of these molecules [3–7]. However, there are little data addressing whether procyanidins have central effects on the endocrine pancreas, a key organ of metabolic control [8]. Hanhineva et al. demonstrated that water extract from *Eriobotrya japonica* increased the insulin secretion of INS-1E cells, but treatment with procyanidin B-2 isolated from the extract decreased insulin secretion [9]. Previously, pretreatment and a daily administration of proanthocyanidins for 72 h were shown to protect β-cell function in alloxan-diabetic rats, suggesting a protective effect against the generation of reactive oxygen species [10].

The importance of the endocrine pancreas in whole-body nutrient equilibrium is highlighted by the emergence of several pathologies of nutrient metabolism, such as type 1 and 2 diabetes, that involve pancreatic cell deregulation. In addition, the pancreas is exposed to bioactive compounds immediately after their enteric absorption, suggesting that bioactive absorbed flavonoids can achieve high concentrations in this organ [11]. Therefore, the pancreas may be a target for procyanidins and their effects on metabolic processes.

Procyanidins act positively on glucose metabolism [7] by modifying both glycemia and insulinemia. We have previously described the peripheral targets of procyanidins that partially account for these effects [12,13]. However, the question remains whether procyanidins affect β-cell functionality. To address this question, healthy rats were treated with different doses of grape seed procyanidin extract (GSPE). In addition, a β-cell line (INS-1E) was treated with GSPE to gain a better understanding of potential mechanisms of action, and work done in this cell line focused on the central pathways that regulate glucose-driven insulin secretion.

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Table 1
Summary of animal experimental procedures

Animal group	Group A	Group B	Group C	Group D
Sex	Female	Male	Female	Female
Weight (g)	150–175	130–150	225–250	175–200
Doses GSPE (mg/kg of bw)	0, 2.5, 5, 10, 25, 50	0, 5, 15, 25, 50	0, 25	0, 1000
Treatment period	36 days	21 days	45 days	1 h
Doses GSPE (mg/kg of bw*days of treatment)	0, 90, 180, 360, 900, 1800	0, 105, 315, 525, 1050	0, 1125	0, 1000
Vehicle	Sweetened condensed milk	Sweetened condensed milk	Sweetened condensed milk diluted 1:6 with water	Tap water
Fasting period before sacrifice	5 h	Overnight	Overnight	Overnight
Last dose time	9 a.m.	9 a.m.	8 p.m. day before	9 a.m.
Sacrifice time	2 p.m.	12 p.m.	9 a.m.	10 a.m.
Anesthetic (mg/kg of bw)	Ketamine (70) and xylazine (5)	Ketamine (70) and xylazine (5)	Pentobarbital sodium (75)	Pentobarbital sodium (75)

2. Materials and methods

2.1. Chemicals

According to the manufacturer, GSPE (*Les Dérives Résiniques et Terpéniques*, Dax, France) contained monomeric (16.6%), dimeric (18.8%), trimeric (16.0%), tetrameric (9.3%) and oligomeric procyanidins (5–13 units, 35.7%) as well as phenolic acids (4.2%).

2.2. Cell culture

INS-1E cells were kindly provided by Prof. Pierre Maechler, University of Geneva [14]. The cell line was cultured as previously described [15]. Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium).

2.3. Animal procedures

Four groups of Wistar rats were studied. All animals were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22°C with a 12-h light, 12-h dark cycle. Treatment began after 1 week in quarantine, as detailed in Table 1. Pancreatic islets were isolated from groups that underwent treatments C and D (Table 1). Blood was collected from all of the animal groups using heparin. Tissues from all of the animal groups were excised, frozen immediately in liquid nitrogen and stored at −80°C until analysis. All procedures were approved by the Experimental Animals Ethics Committee of the Universitat Rovira i Virgili. Insulin and C-peptide plasma levels were measured by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden) following the manufacturer's instructions. Glucose plasma levels were determined using an enzymatic colorimetric kit (GOD-PAP method from QCA, Amposta, Spain).

2.4. Islet isolation

Islets from animals in groups C and D were prepared by collagenase digestion as described previously [16]. Briefly, the rats were anesthetized, and the pancreas was infused with 7 ml of ice-cold collagenase P (Roche, Barcelona, Spain) solution (1 mg/ml) before removal. After the pancreas was removed, it was incubated at 37°C for 15 min. Islets were purified on a Histopaque gradient (Sigma, Madrid, Spain) and handpicked until a population of pure islets was obtained.

2.5. Glucose-stimulated insulin secretion (GSIS)

Secretory responses to glucose were tested in INS-1E cells as previously described [15]. GSIS and cellular insulin contents were measured by radioimmunoassay (RIA) using rat insulin as a standard [17]. GSIS was tested in islets from rats in groups C and D. Islets were maintained for 24 h in RPMI-supplemented medium. The islets were then washed twice and incubated for 1 h at 37°C in Krebs–Ringer bicarbonate HEPES buffer (KRBH) with 2.8 mM glucose (basal) or 16.8 mM glucose (stimulated). Insulin secretion was measured using the Insulin ELISA Kit (Mercodia, Uppsala, Sweden). The islet protein content for each sample was measured using the Bradford method [18].

2.6. Glucose uptake

Glucose transport was determined by measuring the uptake of 2-deoxy-D-[^{3}H] glucose in INS-1E cells cultured in 24-well plates using a methodology adapted from Ref. [19]. Briefly, pancreatic cells were maintained for 30 min at 37°C in glucose-free KRBH. The cells were then incubated for 10 min in a KRBH transport solution containing 2.5 mM or 15 mM glucose and 0.75 μCi 2-deoxy-D-[^{3}H]glucose. Uptake was halted by adding 100 mM glucose, and cells were disrupted by adding 0.1 M NaOH/0.1% phosphate-buffered saline. Glucose uptake was assessed by scintillation counting, and protein content was used to normalize the glucose transport values. The protein content was determined by the Bradford method [18].

2.7. Mitochondrial membrane potential ($\Delta\Psi_m$) and cellular membrane potential measurements

The $\Delta\Psi_m$ and cellular membrane potential were measured as described previously [14].

2.8. Cytosolic adenosine triphosphate (ATP) levels

The cytosolic ATP levels were monitored in cells expressing an ATP-sensitive bioluminescent luciferase probe 1 day after transduction. Pancreatic β-cells were maintained for 2 h in glucose-free culture medium and then stimulated with 15 mM glucose in the presence of 200 μM luciferin. Finally, 2 mM NaN_3 was added as a mitochondrial poison [14].

2.9. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from INS-1E cells grown in six-well plates was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA), and 2 μg of RNA was converted into cDNA [20]. Total RNA was extracted from the pancreas and the liver of animals in groups A and B using TRIzol reagent following the manufacturer's instructions, and cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RNA was extracted from the islets using the miRNeasy Mini Kit (Qiagen, Barcelona, Spain), and cDNA was generated using a kit from Applied Biosystems. The cDNA from all the experiments was subjected to quantitative RT-PCR amplification using the TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA). Specific TaqMan probes (Applied Biosystems, Foster City, CA, USA) were used for different genes: Rn01774648-g1 for insulin, Rn00755591-m1 for pancreatic duodenal homeobox 1 (Pdx1), Rn00561265-m1 for glucokinase, Rn00563565-m1 for Glut2, Rn00565839-m1 for insulin-degrading enzyme (Ide) and Rn01754856-m1 for uncoupling protein 2 (Ucp2). β-Actin was used as the reference gene (Rn00667869-m1). The reactions were run on a quantitative RT-PCR 7300 System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

2.10. Calculations and statistical analysis

The results are expressed as the mean±S.E.M. The effects were assessed by the Student's *t* test. All calculations were performed with the SPSS software.

Fig. 1. Effects of procyanidin on insulinemia after different GSPE treatments. Animals (five or six per group) were sacrificed, and their plasma and tissues were collected according to Table 1. (a) Plasma insulin levels and the HOMA index vs. control for different doses of GSPE (seeTable 1). Glucose and insulin levels were analyzed by colorimetric and ELISA methods, respectively. (b) Pancreatic insulin and Pdx1 gene expression vs. control for different doses of GSPE (seeTable 1). mRNA levels were determined by quantitative RT-PCR. (c) The ratio between plasma insulin and insulin gene expression after treatment with different doses of GSPE for 21 days. (d) Plasma C-peptide levels after a treatment with 25 mg GSPE/kg of bw for 21 days (525 mg GSPE/kg of bw*days of treatment). The plasma C-peptide levels were quantified using ELISA methodology. (e) Liver Ide gene expression after treatment with 15 mg of GSPE/kg of bw for 21 days (315 mg GSPE/kg of bw*days of treatment). The data are presented as the mean±S.E.M. *P<.05 vs. the control; #P<.1 vs. the control.

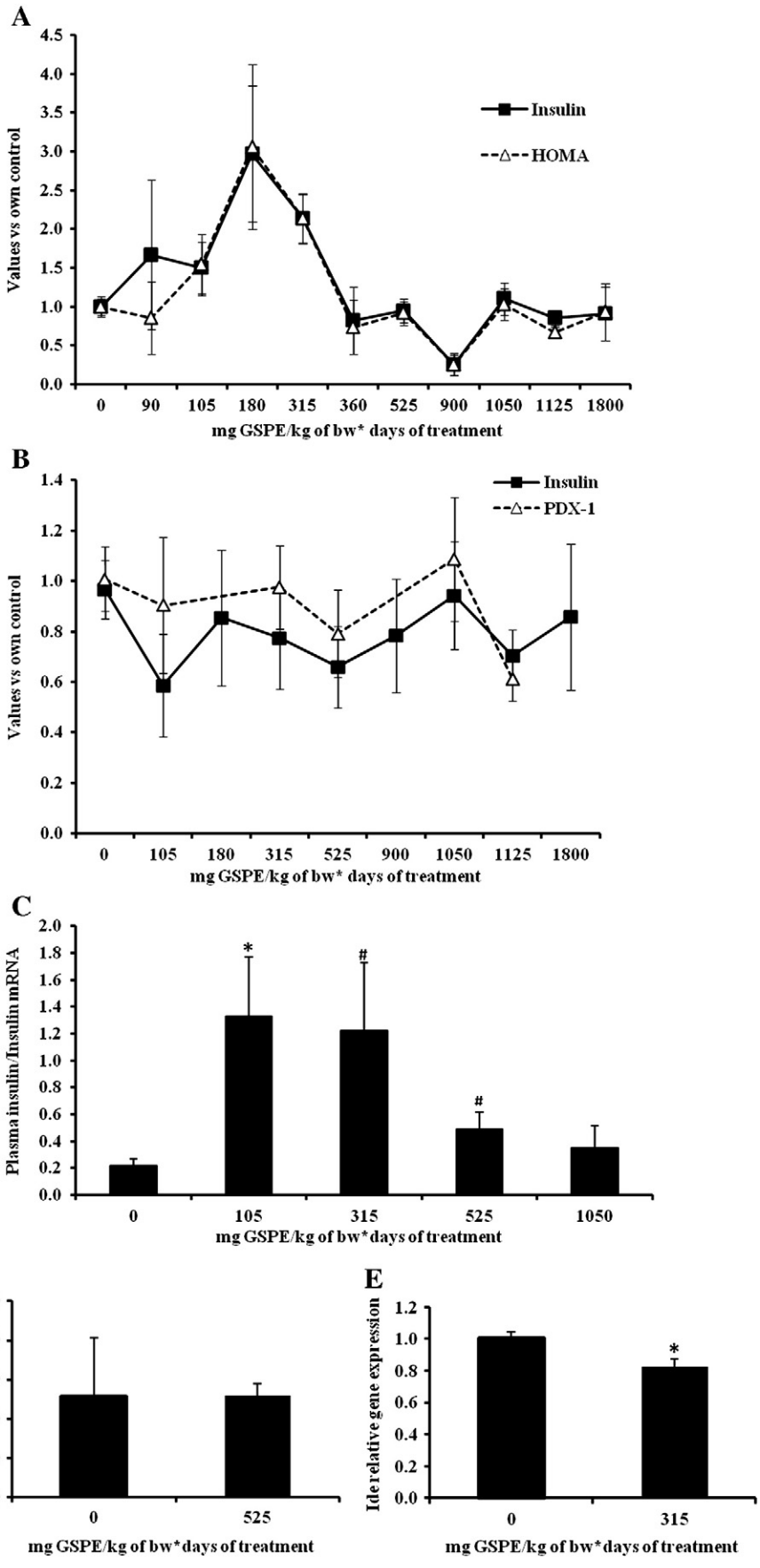
A
Values vs own control
Insulin
HOMA
mg GSPE/kg of bw* days of treatment
B
Values vs own control
Insulin
PDX-1
mg GSPE/kg of bw* days of treatment
C
Plasma insulin/Insulin mRNA
mg GSPE/kg of bw*days of treatment
D
C-peptide (pM)
mg GSPE/kg of bw*days of treatment
E
Ide relative gene expression
mg GSPE/kg of bw*days of treatment

Table 2
Statistical results of the effects on plasma insulin, HOMA index, insulin mRNA and Pdx1 mRNA after a treatment with different doses of GSPE

GSPE doses (mg GSPE/kg of bw*days)	90	105	180	315	360	525	900	1050	1125	1800
Plasma insulin vs. control	NS	NS	NS	$P<.05$	NS	NS	$P<.05$	NS	NS	NS
HOMA vs. control	NS	NS	NS	$P<.05$	NS	NS	$P<.06$	NS	$P<.05$	NS
Insulin mRNA vs. control	–	$P<.1$	NS	NS	–	$P<.1$	NS	NS	$P<.1$	–
Pdx-1 mRNA vs. control	–	NS	–	NS	–	NS	–	NS	$P<.05$	–

NS, not significant.

3. Results and discussion

3.1. Procyanidins affect insulinemia due to their effects on insulin synthesis and degradation

Procyanidins have been shown to have beneficial effects on glucose homeostasis [7], but most of these studies focused on the bioactivity of procyanidins in the liver and adipose tissues. The present study shows that a daily acute administration of GSPE to healthy rats at different concentrations and for different time periods results in a peculiar effect on insulinemia. To better compare the GSPE effects between different animal studies, the effects of each treatment are shown relative to its own control group [insulin reference values for each group (μg/L): A, 0.47±0.2; B, 0.24±0.0; C, 0.90±0.1]. Fig. 1 shows that the lowest doses of GPSE did not affect insulinemia, and statistically significant results were found at moderate doses (summarized in Table 2). Treatments at 5 mg and 15 mg GSPE/kg of body weight (bw) for 21 to 36 days (180 mg and 315 mg GSPE/kg of bw*days of treatment) increased insulinemia, whereas treatments at 25 mg/kg of bw for 36 days (900 mg GSPE/kg of bw*days of treatment) decreased insulinemia. Higher doses did not show any effect. The homeostasis model assessment (HOMA) index [21] for these treatment groups exhibited a similar pattern (Fig. 1a) and showed that the changes in insulin did not provoke significant changes in glycemia (as seen in Table 2) (HOMA reference values for each group: A, 9.49±4.1; B, 1.61±0.3; C, 10.24±2.2). These results agree with our previous results that suggested that procyanidins alter insulinemia, although the relationship between the dose and the effect was unclear [12]. In a retrospective review, we highlighted that the efficacy of procyanidins or procyanidin extracts depends on the dose and the metabolic situation [7].

To better understand this procyanidin effect, insulin production was analyzed. Fig. 1b shows that the insulin gene expression profile in these animals exhibits the tendency for lower insulin mRNA levels at lower doses. The same tendency was found for Pdx1 mRNA (Fig. 1b), a key controller in insulin synthesis. Fig. 1c shows the relationship between plasma insulin and insulin mRNA at different GSPE concentrations. Insulin mRNA levels reflect the amount of insulin synthesis. Insulin plasma levels reflect the amount of insulin from pancreatic production and the clearance of this hormone in different tissues. At some GSPE doses, the plasma insulin protein levels were higher than the pancreatic mRNA levels. These ratios may reflect modifications in insulin production (synthesis and/or secretion) and/or in insulin removal. Fig. 1b showed no effect on insulin mRNA synthesis at these doses. To determine if insulin secretion was altered, the C-peptide levels were analyzed. Fig. 1d shows that GSPE treatment did not modify pancreatic insulin secretion. Therefore, insulin removal was analyzed. Because Ide is responsible for the removal of insulin and Ide activity is high in the liver [22], liver Ide gene expression was measured in the treatment group that showed significantly increased insulinemia: 15 mg GSPE/kg of bw*21 days (315 mg GSPE/kg of bw*days of treatment). At this dose, procyanidins decreased Ide mRNA levels (Fig. 1e),

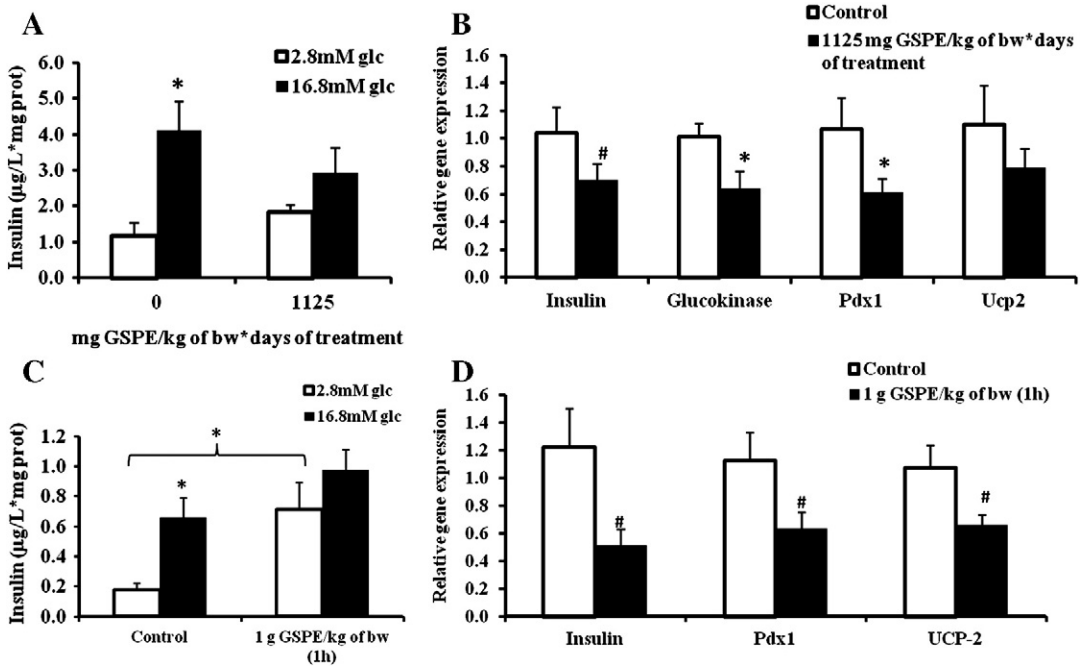

Fig. 2. Effects of GSPE treatment on rat islets. (a and b) The rats were treated with 25 mg GSPE/kg of bw for 45 days. (a) GSIS measurements and (b) insulin, glucokinase, Pdx1 and Ucp2 gene expression. (c and d) The rats were treated acutely with 1000 mg GSPE/kg of bw. (c) GSIS measurements and (d) insulin, Pdx1 and Ucp2 gene expression. To analyze the glucose response in both experiments, isolated islets were maintained for 24 h in RPMI-supplemented medium and then cultured for 1 h at low (2.8 mM) or high (16.8 mM) glucose concentrations. Insulin levels were quantified by ELISA methodology, and mRNA levels were determined by quantitative RT-PCR. The data are presented as the mean±S.E.M. *$P<.05$ vs. the control; #$P<.1$ vs. the control.

suggesting that these animals have limited insulin degradation activity, which could explain their increased insulinemia. These data suggest that Ide is a target for procyanidins, and to our knowledge, this is the first data describing the effect of procyanidins on Ide. The transcriptional regulation of Ide and its effect on insulin homeostasis are still not well understood, and there are little data describing the factors that regulate Ide gene expression. Insulin increases Ide gene expression in HepG2 cells but only under high-glucose conditions [23]. Concerning other tissues, Du et al. showed that peroxisome proliferator-activated receptor-γ (PPARγ) plays an important role in regulating Ide gene expression in rat primary neurons through its interaction with a functional peroxisome proliferator-response element on the Ide promoter, thereby activating Ide gene transcription [24]. We do not exclude that the effect of GSPE on Ide expression could involve PPARγ regulation since chronic GSPE treatment down-regulates PPARγ expression in 3T3-L1 adipocytes [25].

Therefore, the effects of procyanidins on plasma insulin could be related to their bioactivity on Ide as well as their effects on β-cells.

3.2. Islets from GSPE-treated animals show decreased responsiveness to glucose

To directly test whether the islets of Langerhans are the targets of procyanidins, islets from rats treated with 25 mg GSPE/kg of bw for 45 days were isolated, and their response to glucose stimulation was measured after 24 h in culture. Fig. 2a shows that there were a higher basal level of insulin production and a clearly limited response to glucose stimulation in islets from GSPE-treated animals (GSIS rate for the control group, 4.94±1.32, was significantly different from the GSIS rate for GSPE-treated animals, 1.45±0.43). At the mRNA level (Fig. 2b), there was a decrease in both insulin and Pdx1 mRNA levels. Glucokinase gene expression was significantly down-regulated, and there was also a small, but not statistically significant, decrease in Ucp2 mRNA levels. Taken together, these data suggest that procyanidins modify islet functionality by decreasing their sensitivity to glucose and by modifying mRNA expression levels.

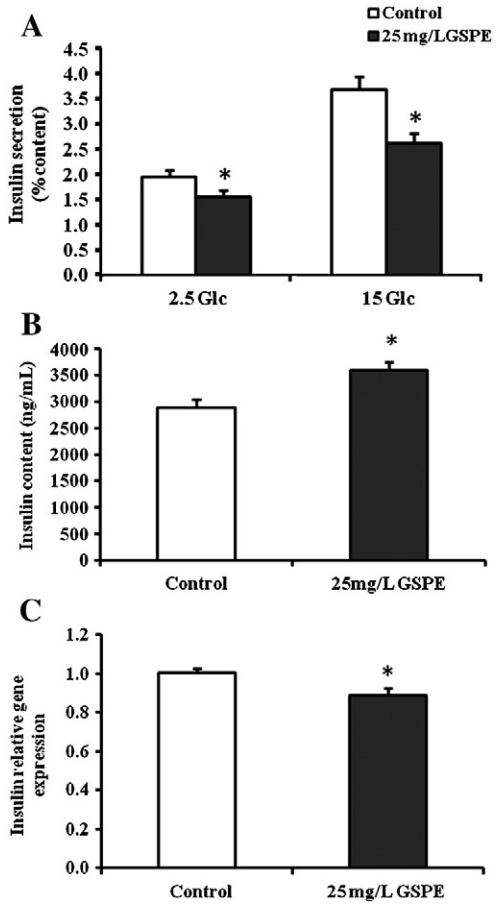

Fig. 3. Effects of procyanidin treatment on insulin synthesis and secretion in INS-1E cells. INS-1E cells were treated with 25 mg/L of GSPE for 24 h. After 2 h of starvation in RPMI without glucose medium, cells were cultured in medium with basal (2.5 mM) or stimulated (15 mM) glucose levels. (a) GSIS measurements and (b) insulin content in the cells were determined from an acid-ethanol extract. Insulin was measured by insulin RIA. (c) Insulin mRNA levels were measured by quantitative RT-PCR. The data are presented as the mean±S.E.M. *P<.05 vs. the control.

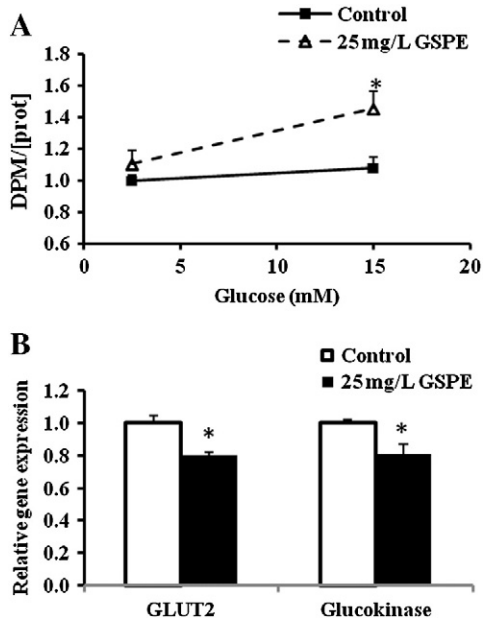

Fig. 4. Effects of a 24-h GSPE treatment (25 mg/L) on glucose entry in INS-1E cells. The cells were treated as indicated in Fig. 3. (a) Glucose uptake was determined by measuring 2-deoxy-D-[^{3}H]glucose uptake by scintillation counting. (b) Glut2 and glucokinase gene expression was analyzed by quantitative RT-PCR. The data are presented as the mean±S.E.M. *P<.05 vs. the control.

Next, we tested whether the same effects could be induced by a similar amount of procyanidins in an acute dose. Healthy female rats were treated with an acute dose of 1000 mg/kg of bw. After 1 h, which corresponded to peak procyanidin levels in the blood [26], islets from these animals were isolated and cultured as described above, and their response to glucose stimulation was measured. Fig. 2c shows that this treatment led to higher basal levels of insulin production, which limited islet sensitivity to glucose stimulation. Similar to islets from animals in the 45-day treatment group, these islets exhibited lower insulin, Pdx1 and Ucp2 mRNA levels (Fig. 2d), and there was no effect on glycemia (control: 6.22±0.31 mM, GSPE treatment: 6.11±0.28 mM).

Similar effects were produced *in vitro* after long-term fatty acid treatment [27,28] or after hyperglycemia [29]. The molecules that are used as antidiabetic drugs also produced similar effects. Chronic treatment of islets with glibenclamide, a sulfonylurea, inhibited proinsulin biosynthesis at basal and intermediate glucose concentrations and promoted insulin secretion independently of glucose concentration [30].

Surprisingly, when two different administrations of the same total amount of procyanidins were compared, we found very similar effects. Both treatments limited glucose sensitivity in the islets that were removed from GSPE-treated animals and cultured for 24 h. These effects did not correlate with plasma procyanidin levels in either treatment group. In the acute treatment group, dimeric

procyanidins reached 0.5 nM, and trimeric procyanidins reached 2.5 nM [26]. In the chronic treatment group, dimeric procyanidins reached 11.5±1.25 nM, but there was no measurable amount of trimeric procyanidins. These results suggest that a minimum amount of dimeric procyanidins (approximately 0.5 nM) is necessary to induce the described effects. However, unpublished results from our research group, working with pure structures, do not support this conclusion. We expected that other components from the extract that we were not able to identify could play a role in eliciting these effects. However, measuring the bioavailability of different molecules in plant extracts is beyond the scope of this paper [8,15,31]. We included this information to highlight the relationship between two very different procyanidin administrations, both in their pancreatic effects and in the amount of procyanidins achieved in plasma.

3.3. GSPE limits mitochondrial function

To better understand how procyanidins modify β-cell insulin secretion, the study was carried out using the INS-1 β-cell line. The cells were treated for 24 h with 25 mg GSPE/L (Fig. 3a), and we found that insulin secretion decreased under basal glucose conditions, and this effect was even stronger under stimulated glucose conditions. In addition, the amount of insulin content was higher (Fig. 3b) and insulin gene expression was lower (Fig. 3c) in cells treated with 25 mg GPSE/L.

Because insulin secretion depends on cell energetics, key pathways in cell energetics from glucose entry to insulin secretion [32] were analyzed to identify possible targets of procyanidins that could limit insulin secretion and/or insulin synthesis.

Fig. 4a shows that GSPE administration led to an increase in glucose uptake in β-cells under high-glucose conditions. In contrast, procyanidins lowered mRNA levels for the Glut2 glucose transporter and glucokinase, which are key effectors of glucose uptake (Fig. 4b). After glucose enters the cell and is metabolized through the glycolytic pathway, it reaches the mitochondria and enters the Krebs cycle. At the mRNA level, there was no effect on citrate synthase enzyme levels (1.03±0.04 vs. control 1.00±0.03). In contrast, there was a clear effect on the $\Delta\Psi_m$. Fig. 5a shows that INS-1E cells treated with 25 mg GSPE/L for 24 h exhibited a decrease in glucose-induced mitochondrial hyperpolarization (~5%). The total $\Delta\Psi_m$ revealed by *p*-trifluoromethoxyphenylhydrazone (FCCP) was reduced by GSPE treatment compared with the control (Fig. 5b). A possible cause for this uncoupling could be the increase in Ucp2 expression (Fig. 5c). Uncoupling protein-2 is thought to catalyze a mitochondrial inner-membrane H^+ leak that bypasses ATP synthase, thereby reducing the cellular ATP content [33]. These data suggest that although there was an increased entry of glucose under high-glucose conditions, coupling with the mitochondria was altered and resulted in lower levels of ATP synthesis. Fig. 6a shows that the GSPE strongly inhibits cytosolic ATP production after glucose stimulation, which may lower the ability of the GSPE-treated cells to close ATP-sensitive potassium (K^+_{ATP}) channels as suggested in Fig. 6b. GSPE treatment for 24 h did not affect the cellular membrane potential after glucose stimulation, but addition of 30 mM KCl in the presence of 25 mg GSPE/L resulted in an increased depolarization. These results suggest that absolute cellular membrane potential levels are lower in INS-1E cells treated with the highest GSPE dose than in control cells. Therefore, GPSE decreases the ability of these cells to secrete insulin in response to glucose entry by uncoupling the entire process.

Thus, our results indicate that procyanidins limit insulin secretion through modifying membrane permeability and the glucose-stimulated insulin secretion pathway, which lead to an increase in insulin content in β-cells. Accumulation of insulin in the cell could be responsible for the inhibition of insulin mRNA levels, as there is

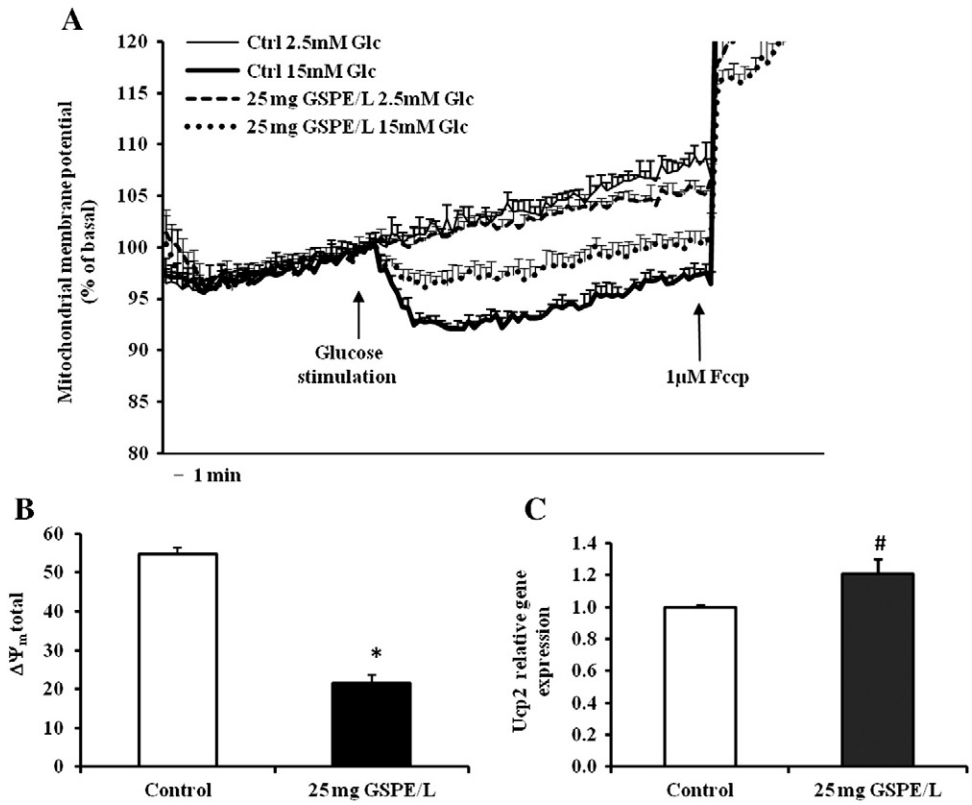

Fig. 5. Effects of a 24-h GSPE treatment (25 mg/L) on mitochondrial function in INS-1E cells. The cells were treated as indicated in Fig. 3. (a) The $\Delta\Psi_m$ was monitored by rhodamine 123 fluorescence. Hyperpolarization of $\Delta\Psi_m$ was induced with 15 mM glucose, and after 10 min of glucose stimulation, depolarization was induced by FCCP. (b) Total $\Delta\Psi_m$ revealed by FCCP. (c) Ucp2 mRNA levels were measured by quantitative RT-PCR. The data are presented as the mean±S.E.M. *P<.05 vs. the control; #P<.06 vs. the control.

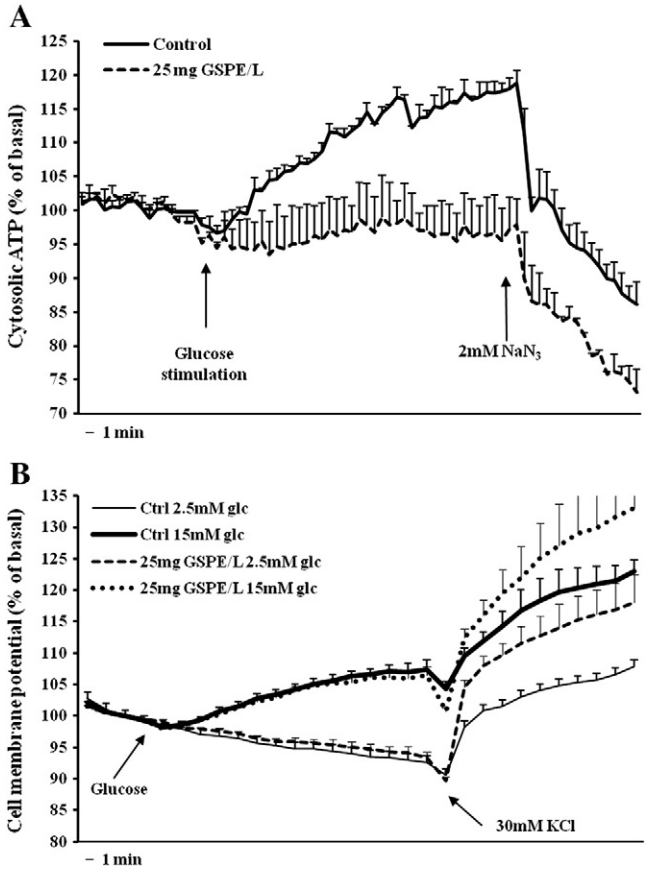

Fig. 6. Effects of a 24-h GSPE treatment (25 mg/L) on ATP generation and cellular membrane potential in INS-1E cells. (a) Cytosolic ATP levels were monitored in cells expressing luciferase. The cells were stimulated by raising glucose levels from 2.5 mM to 15 mM. After 10 min of stimulation, 2 mM NaN_3 was added as a mitochondrial poison. (b) The cellular membrane potential was monitored by bisoxonol fluorescence. Depolarization of cellular membranes was induced with 15 mM glucose for 10 min, followed by control depolarization, which was induced by 30 mM KCl. The data are presented as the mean±S.E.M.

sufficient insulin protein synthesized. However, according to published data [34], it seems not to be responsible to explain the effects on insulin biosynthesis; therefore, GSPE could also act directly at the insulin promoter to inhibit gene transcription. We do not exclude this possibility because we found it in all the models we have assayed.

A recently published study demonstrated that treating INS-1E cells with resveratrol, another phenolic compound, for 24 h promoted GSIS by increasing glucose oxidation, ATP production and mitochondrial oxygen consumption [35]. Vetterli et al. have also shown that resveratrol up-regulates key genes in β-cell function, such as Glut2, glucokinase, Pdx1, hepatocyte nuclear factor 1 homeobox A and mitochondrial transcription factor A. The differences between this result and our results could be due to the differences in chemical structure between procyanidins and resveratrol; in fact, there have been several controversial results reported for the effects of different flavonoids on β-cells [8].

Alternatively, acute treatment with pioglitazone (Pio), a thiazolidinedione (TZD), in INS 832/13 cells and in isolated rat islets produced effects similar to those for GSPE treatment. Pio reduced the GSIS in β-cells at intermediate glucose concentrations, which altered ATP content and inhibited glucose-induced mitochondrial membrane hyperpolarization. A previous study published by Kim et al. showed that chronic treatment with other TZD molecules, such as rosiglitazone, stimulated insulin release and synthesis. These molecules upregulated Glut2 and glucokinase gene expression after a 24-h treatment period through PPARγ activation [36]. The results from this study and other studies suggest that the effects of TZDs, which are PPARγ agonists, on pancreatic β-cells remain controversial, and the effect depends on the dose and treatment period of antidiabetic agents, similar to our results with procyanidins.

We speculate that during chronic treatment, GSPE can act as a PPARγ antagonist in β-cells; this is similar to its effect on adipocytes, where procyanidins limit adipogenesis during the induction of differentiation [12,25]. In β-cells, GSPE limited glucose-induced insulin secretion by uncoupling the process and down-regulated the expression of genes that act directly on insulin synthesis and secretion. Furthermore, Moibi et al. showed that PPARγ induces Pdx1 expression and, consequently, induces the expression of Glut2, glucokinase and insulin [37]. The present study showed that the expression of these genes decreased after GSPE treatment at different doses and at different treatment periods. However, the precise role of PPARγ in the molecular mechanisms by which GSPE alters β-cell functionality remains elusive.

In conclusion, we showed that procyanidins play an important role in β-cell function by limiting glucose sensitivity and insulin biosynthesis. GPSE treatment altered the $\Delta\Psi_m$, ATP production and the cellular membrane potential. Various *in vivo* experiments corroborate this procyanidin effect. Both acute and chronic treatment reduced glucose-induced insulin secretion and down-regulated insulin and Pdx1 mRNA levels, the β-cell master gene, in rat islets. Moreover, our results demonstrated that low doses of procyanidins increased the plasma insulin levels and inhibited insulin gene expression, which led to reduced Pdx1 mRNA levels in the pancreas and reduced hepatic Ide gene expression.

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