Grape-seed procyanidins modulate cellular membrane potential and nutrient-induced GLP-1 secretion in STC-1 cells

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Grape-seed procyanidins modulate cellular membrane potential and nutrient-induced glucose homeostasis in STC-1 cells. Procyanidins are phenolic compounds present in fruits and vegetables. We have previously reported that a grape-seed procyanidin extract (GSPE) modulates glucose homeostasis, and it was suggested that GSPE may achieve this by enhancing the secretion of incretin hormones such as glucagon-like peptide-1 (GLP-1). Therefore, the aim of the present study is to examine in detail the effects of GSPE on intestinal endocrine cells (STC-1). GSPE was found to modulate plasma membrane potential in enteroendocrine cells, inducing depolarization at low concentrations (0.05 mg/l) and hyperpolarization at high concentrations (50 mg/l), and surprisingly this was also accompanied by suppressed GLP-1 secretion. Furthermore, how GSPE affects STC-1 cells under nutrient-stimulated conditions (i.e., glucose, linoleic acid, and L-proline) was also explored, and we found that the higher GSPE concentration was effective in limiting membrane depolarization and reducing GLP-1 secretion. Next, it was also examined whether GSPE affected mitochondrial membrane potential, and it was found that this too is altered by GSPE; however, this does not appear to explain the observed effects on plasma membrane potential and GLP-1 secretion. In conclusion, our results show that grape-seed procyanidins modulate cellular membrane potential and nutrient-induced enteroendocrine hormone secretion in STC-1 cells.

Grape-seed procyanidins; cellular membrane potential; GLP-1; enteroendocrine cell

PROCYANIDINS ARE PHENOLIC compounds present in fruits and vegetables. We have previously reported that a grape-seed procyanidin extract (GSPE) modulates glucose homeostasis (33), and several mechanisms have been proposed to explain this effect, such as its action as an insulin mimetic in insulin-sensitive tissues (29, 32). Besides, GSPE has also been found to increase the insulin/glucose ratio after an oral glucose gavage, suggesting a modulation of the incretin system (12), and the fact that incretin production could be modulated at the gastrointestinal tract opens the door to evaluate if the ingested extract might directly modulate this enteroendocrine system, which is considered the largest endocrine organ in the body (41). Usually, the identification of the tissues directly reached by the components of a natural extract is difficult since the components of the extract are absorbed and highly metabolized before entering the body (27), but enteroendocrine cells are exposed to ingested food without needing absorption.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone mainly released from the L cells of the distal intestine (ileum and colon) (7, 9). GLP-1 enhances the responsiveness of the β-cells to glucose (17) and also improves β-cell mass by enhancing proliferation, inhibiting apoptosis, and increasing β-cell differentiation (reviewed in Ref. 1). It has been reported that GLP-1 secretion is impaired in type 2 diabetic patients (45, 48) and exogenous GLP-1 infusion improves glycemia (26, 30), indicating a potential for developing new antiadipic therapies that enhance GLP-1 secretion. However, even though the key role of entoehormone secretion in glucose homeostasis regulation, whether the bioactive extract of grape-seed procyanidins modulates incretin secretion has not previously been studied.

Several nutrients have been reported to stimulate GLP-1 release by intestinal L cells in vitro and in vivo, such as glucose (14, 37), fatty acids (46), intact dietary proteins (10), or amino acids (13, 38, 51), all of these involving cellular membrane depolarization, that leads to calcium entry and calcium-dependent GLP-1 release (15). However, the nonnutritive components in food, such as bioactive natural compounds, which can also reach these enteroendocrine cells, could therefore interfere with the mechanisms that regulate GLP-1 secretion. In this sense, polyphenols have been reported to modulate cellular membrane potential in aortic endothelial RAEC cells (2) and Yurkat cells (T lymphoblasts) (25), as well as in liposomes (47). Previous studies performed in our group also showed that GSPE modulates cellular membrane potential in pancreatic β-cells (4). Besides, some reports showed modulation of GLP-1 levels by some natural compounds. Chlorogenic acid, a major phenol found in coffee, was shown to improve plasma GLP-1 levels (20) and increase GLP-1 secretion and production in STC-1 cells, a murine enteroendocrine cell line (36). Berberine, a major active constituent of <i>Rhizoma coptidis</i>, has been reported to increase portal active GLP-1 levels in healthy and streptozotocin-induced diabetic rats (STZ) and enhance GLP-1 secretion and biosynthesis in NCI-H716 cells, a human enteroendocrine cell line (24, 52). Genistein and daidzen isoflavonoids, derived from soybean fermentation, have been reported to increase GLP-1 secretion from NCI-H716 cells (22), and glycocelins, phytocelaxes derived from daidzen in soybean with fungi infection, showed the same effect in vitro (31). Resveratrol, a polyphenolic compound produced by fruits such as red grapes or berries, was found to increase portal active GLP-1 levels and intestinal biosynthesis in high-fat-diet-fed rats (5). Similarly, cinnamminin B2, a tretameric procyanidin, was reported to increase GLP-1 secretion in vivo (49). Moreover, a recent article has reported that curcumin, a phenolic compound isolated from the rhizomes of <i>Curcuma</i>...
longa L., is able to increase GLP-1 secretion in the murine enteroendocrine cell line GLUTag (44).

The present study was therefore undertaken to evaluate whether GSPE modulates the mechanisms that affect enteroendocrine secretion in STC-1 cells and focused on GLP-1 due to its key role in glucose homeostasis.

RESEARCH DESIGN AND METHODS

Reagents. The GSPE used in these studies was obtained from Les Dérivés Résiniques et Terpéniques (Dax, France) and has been characterized in detail by our research group (35).

Linoleic acid (LA), L-proline (L-Pro), methyl α-d-glucopyranoside, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma-Aldrich (Madrid, Spain). LA was prepared as 100 mM stock in absolute ethanol and CCCP stock was prepared in DMSO to a final concentration of 10 mM. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC4(3)] was obtained from Biotium (Verviers, Belgium) and incubated in a 5% CO2-humidified atmosphere at 37 °C. At least three replicates using different cell passage numbers were performed for each experiment, including at least three wells of each condition in every replicate. Cells were used between passage numbers 30–50.

Cell culture. The STC-1 clonal cell line was received as a kind gift from Dr. B. Wice (Washington University of St. Louis) with the permission of Dr. D. Hanahan (University of California, San Francisco, CA). This enteroendocrine cell line was originated from a double-transgenic mouse tumor (40). Cells were cultured in DMEM with GlutaMAX containing 4.5 g/l D-glucose, without sodium pyruvate (GIBCO, Madrid, Spain), supplemented with 17.5% fetal bovine serum, 100 U/ml penicillin, and 100 mg/l streptomycin (BioWhittaker, Verviers, Belgium) and incubated in a 5% CO2-humidified atmosphere at 37 °C. At least three replicates using different cell passage numbers were performed for each experiment, including at least three wells of each condition in every replicate. Cells were used between passage numbers 30–50.

GLP-1 secretion. For secretion experiments, 1.8–2 × 10⁶ cells were seeded in 12-well culture plates and allowed to reach 70–90% confluence. On the day of the experiment, the cells were incubated for 2 h in HEPES buffer (20 mM HEPES, 140 mM NaCl, 4.5 mM KCl, 1.2 mM CaCl₂, and 1.2 mM MgCl₂ at pH 7.4). After preincubation period, buffer was removed and cells were incubated with 0.05, 0.5, 5, or 50 mg of GSPE/l for 3 h in different conditions: 1) basal conditions: 2.5 mM glucose in HEPES buffer; 2) glucose stimulation: 10 or 20 mM glucose in HEPES buffer; 3) fatty acid stimulation: 5 mM L-Pro in HEPES buffer with 2.5 mM glucose. At the end of the 3 h-treatments, the medium was collected and centrifuged to remove any cellular debris. Supernatants were stored at −80 °C until analyses.

Active GLP-1 levels were determined using a glucagon-like peptide-1 (active) ELISA kit (Millipore, Madrid, Spain).

Cellular membrane potential. The cellular membrane potential (∆Ψₘ) was measured using the fluorescent probe DiBAC₄(3) as previously reported (25). Then, 50–60,000 cells were seeded in 96-well culture plates and allowed to reach 70–90% confluence. On the day of the experiment, cells were preincubated for 2 h with HEPES buffer. Then, cells were labeled with 10 μM DiBAC₄(3) (diluted in HEPES with 2.5 or 10 mM glucose) for 30 min at 37 °C. After labeling, GSPE was added to a final concentration of 0.05, 0.5, 5, or 50 mg/l and the ∆Ψₘ was monitored every 15 s until end of experiment, with excitation and emission filters set at 493 nm and 516 nm, respectively. After 3 min in basal conditions, cells were stimulated with 10 or 20 mM glucose, 30 μM LA, 5 mM L-Pro, or 100 mM methyl α-d-glucopyranoside. Five minutes later, total cellular membrane depolarization was induced by adding KCl to a final concentration of 75 mM. ∆Ψₘ was expressed as ∆F/F₀ calculated as the difference of fluorescence between the peaks after stimulation (F, fluorescence at 195 s) and basal (F₀, fluorescence at 180 s).

Mitochondrial membrane potential. The mitochondrial membrane potential (∆Ψₘ) was determined using the fluorescent probe rhodamine 123. Cells were treated as described for ∆Ψₘ measurement and labeled with 10 μg/ml of rhodamine 123. ∆Ψₘ was monitored with excitation and emission filters set at 485 nm and 520 nm, respectively. Total mitochondrial membrane depolarization was induced by adding CCCP to a final concentration of 24 μM.

Data analyses. Results are expressed as the means ± SE. The effects were assessed by ANOVA and Student’s t-test. All calculations were performed with SPSS software (SPSS, Chicago, IL).

RESULTS

GSPE modulates basal and nutrient-induced cell membrane potential. To analyze whether GSPE modulates cellular membrane potential, we stimulated STC-1 cells with a range of GSPE concentrations and measured cellular membrane potential changes using the fluorescent probe DiBAC₄(3). The increase on the fluorescent signal is associated to membrane depolarization, while the decrease indicates membrane hyperpolarization. Cellular membrane depolarization was induced after stimulation with 0.05 and 0.5 mg of GSPE/l, while 5 mg of GSPE/l did not change membrane potential. Contrarily, 50 mg of GSPE/l caused membrane hyperpolarization (Fig. 1).

Fig. 1. Grape-seed procyanidin extract (GSPE) effects on cellular membrane potential. Effects of GSPE on cellular membrane potential expressed as %F/F₀ (A) and relative ∆F/F₀ (normalized to the control cells) (B), where F is fluorescence at 195 s and F₀ is basal fluorescence at 180 s. The data are displayed as the means ± SE. a,b,c Statistically significant differences at P < 0.05.
We then assessed whether GSPE alters cellular membrane changes induced by different nutrients, including a sugar, an amino acid, and a fatty acid. First, the effects of 10 and 20 mM glucose, 5 mM L-proline, and 30 μM linoleic acid on cellular membrane potential expressed as Δ(ΔF/F₀), where F is fluorescence at 195 s and F₀ is basal fluorescence at 180 s, and on glucagon-like peptide-1 (GLP-1) levels, normalized to the basal condition (2.5 mM glucose for all the treatments and 10 mM glucose for the linoleic acid). *Statistically significant differences at P < 0.05.

Table 1. Effect of nutrients on cellular membrane potential and GLP-1 levels

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Relative Δ(ΔF/F₀)</th>
<th>Relative GLP-1 Levels</th>
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<tbody>
<tr>
<td>10 mM glucose</td>
<td>1.52 ± 0.25</td>
<td>1.52 ± 0.13*</td>
</tr>
<tr>
<td>5 mM L-proline</td>
<td>2.71 ± 0.22</td>
<td>1.44 ± 0.31</td>
</tr>
<tr>
<td>20 mM glucose</td>
<td>3.12 ± 0.47</td>
<td>1.23 ± 0.32</td>
</tr>
<tr>
<td>30 μM linoleic acid</td>
<td>4.40 ± 0.28*</td>
<td>0.70 ± 0.08*</td>
</tr>
</tbody>
</table>

Data are displayed as the means ± SE. Effect of 10 and 20 mM glucose, 5 mM L-proline, and 30 μM linoleic acid on cellular membrane potential expressed as Δ(ΔF/F₀), where F is fluorescence at 195 s and F₀ is basal fluorescence at 180 s, and on glucagon-like peptide-1 (GLP-1) levels, normalized to the basal condition (2.5 mM glucose for all the treatments and 10 mM glucose for the linoleic acid). *Statistically significant differences at P < 0.05.

GSPE modulates GLP-1 secretion. To assess if GSPE effects on cell membrane modulates GLP-1 secretion, we determined active GLP-1 levels secreted into the media by STC-1 cells after 3 h of treatment. As shown in Fig. 3, the dose of 50 mg of GSPE/l significantly decreased GLP-1 secretion around 40%.

To check whether GSPE also modulates the nutrient-induced GLP-1 secretion, we first quantified GLP-1 levels in the medium after treatment with the three nutrients previously used (glucose, L-Pro, and LA). Table 1 shows that only glucose led to a significant accumulation of GLP-1 in the medium, while LA treatment reduced it. We also analyzed the effects of GSPE on nutrient-triggered cellular membrane depolarization, showing that GSPE reduced the depolarization induced by all the agents (Fig. 2).
together with the three agents and, as shown in Fig. 4A, 50 mg of GSPE/l caused a reduction of around 20% on GLP-1 secretion in glucose-stimulated cells. The same dose also decreased GLP-1 in the medium of proline-stimulated cells (Fig. 4B). Also, in LA-stimulated cells, 50 mg of GSPE/l tended to further decrease GLP-1 secretion, but it was not statistically significant (Fig. 4C).

**GSPE interaction on pathways involving glucose metabolism.**

To evaluate whether GSPE affects cellular membrane potential by modulating glucose metabolism, we treated STC-1 cells with different concentrations of GSPE in the presence or absence of glucose stimulus and measured mitochondrial membrane potential. In basal conditions, 50 mg of GSPE/l significantly hyperpolarized the mitochondrial membrane (Fig. 5, A and B). This effect was not observed after stimulation with 20 mM glucose (Figs. 5, C and D).

To further discard the involvement of glucose metabolism on GSPE modulation of glucose-triggered membrane depolarization, the nonmetabolizable glucose analog methyl-α-D-glucopyranoside was used to stimulate STC-1 cells, simultaneously with GSPE, and cellular membrane potential was measured. As shown in Fig. 6, 100 mM methyl-α-D-glucopyranoside triggered membrane depolarization and both GSPE treatments (5 and 50 mg of GSPE/l) significantly inhibited this depolarization.

**DISCUSSION**

Oral intake of GSPE leads to a direct exposure of the intestinal endocrine cells to the components of the extract, and it has been demonstrated to affect membrane potential in pancreatic endocrine β-cells and modulate insulin secretion (4). However, whether GSPE also affects the mechanisms involved in hormone secretion from intestinal endocrine cells has not been previously investigated. The current study used an in vitro approach to assess whether an extract of procyanidins from grape-seed modulates cellular membrane potential in enteroendocrine cells, as well as whether it influences the secretion of GLP-1.

In vitro studies using several enteroendocrine cell line models have found that cellular membrane depolarization is determinant for nutrient-induced hormone secretion (11, 14, 37, 38, 43, 51). Therefore, we analyzed the effect of GSPE on the cell membrane potential of the enteroendocrine STC-1 cells, and GSPE showed differential effects depending on the dose: at low concentrations (0.05 and 0.5 mg/l) it triggers cellular membrane depolarization, while at a higher (50 mg/l) concentration it leads to hyperpolarization. This observation is interesting because previous studies actually found GSPE to produce opposite effects depending on the dose, for example, when affecting insulin plasma responses and HOMA-IR in rats (4). Furthermore, it is well described that some flavonoids can act as either antioxidant or prooxidant depending on their concentration (34).

To further understand the mechanisms by which GSPE exerts such effects we focused on the pathways that modulate cellular membrane potential. Two different general pathways have been described to induce cell membrane depolarization in enteroendocrine cells. The first is a mechanism for glucose-induced hormone secretion analogous to that found in pancreatic β-cells, involving glucose uptake, glycolysis, and mitochondrial oxidation, which generates a rise in the ATP/ADP ratio, stimulating ATP-sensitive K^+ (K_ATP) channel closure and cellular membrane depolarization (42). The second mechanism is a rise in intracellular Na^+, which could be achieved either by nutrient-Na⁺ cotransporters, such as SGLT-1 for glucose (6) and amino acid cotransporters (51), or by Na⁺ intake via the cation channel TRPM5 that responds to the release of Ca²⁺ from the intracellular stores activated by G-protein coupled taste receptors (43, 50). Our results suggest that the first mechanism, that is, glucose metabolism in the mitochondria, is not involved in GSPE effects. We found that the highest concentration of GSPE hyperpolarizes mitochondrial membrane, but it does not lead to the depolarization of the cell membrane that would be expected, as occurs in pancreatic β-cells (4). In fact, cell membrane depolarization was observed, at low GSPE doses, but then we found no effects on mitochondrial membrane. Furthermore, no effects on mitochondrial membrane potential were found in high-glucose conditions. Therefore, the GSPE mechanisms in enteroendocrine cells are very different from those observed in pancreatic β-cells, where GSPE inhibits glucose-induced mitochondrial membrane hyperpolarization, lowering absolute cell membrane potential and limiting insulin release (4). In fact, there is a controversy regarding to the actual role of mitochondrial glu-
cose metabolism on enteroendocrine cells, which is sometimes related to the cell line used for these studies (8, 37, 39). Actually, methyl-α-D-glucopyranoside, a nonmetabolizable glucose analog, has been reported to also induce GLP-1 secretion (14, 39), so we tested it. We found that GSPE limits the membrane depolarization induced by methyl-α-D-glucopyranoside, in a clear dose-dependent manner, further discarding the requirement of glucose metabolism for the effects of GSPE. As this glucose analog is a specific substrate for SGLT-1, these results suggest that GSPE might modulate cell membrane potential through modulating Na\(^+\)/H\(^+\)-entrance-dependent mechanisms.

According to this hypothesis we studied the GSPE effects in the presence of nutrients that involve Na\(^+\)/H\(^+\) entrance for their intracellular signaling: glucose, LA, and L-Pro. We selected different treatment conditions for the different compounds according to previous studies in the same cell line, when it was possible, or in GLUTag cells (14, 43, 51). Our results point out that both the effects of low GSPE concentrations (that induced cell depolarization) and high GSPE concentrations (that had the opposite effect) might be achieved by some mechanism common to the three agents, since we observed no differential effect depending on the agent used. We found that, as expected, all these agents led to cell membrane depolarization,
but cotreatment with low GSPE doses did not show additive effects. At the moment there are insufficient studies in STC-1 cells to show that a common pathway is used by these three nutrients, but some studies in other enteroendocrine cell line models suggested that glucose might use the TRPM5 channel to lead to membrane depolarization (18, 21), as well as fatty acids in STC-1 cells (43). Despite L-Pro being shown to act, in part, through a Na\(^+\) cotransporter in STC-1 cells (51), the existence of other amino acid receptors and transporters in enteroendocrine cells (19) suggests that a possible involvement of TRMP5 should not be discounted. Thus more information on the STC-1 cell model could help to elucidate whether low doses of GSPE are directly or indirectly activating TRMP5 to lead membrane depolarization.

We also observed a common trend with higher GSPE concentrations and found that this limited the cell membrane depolarization induced by glucose, LA, and L-Pro. Interestingly GSPE also limited the strongest depolarizing stimulus, the one achieved by methyl-o-D-glucopyranoside, which is likely mediated by SGLT-1 transport (14), indicating that the higher concentrations of GSPE may be inhibiting Na\(^+\) entrance into the cell but independently on its action on specific transporters. Such inhibition could be performed at several levels, such as Na\(^+\) chelation. However, despite the fact that metal chelation capacity by flavonoids is well described (16), there are no reports demonstrating their capacity to chelate sodium cations. Another mechanism that might explain such inhibition is that procyanidins affect membrane fluidity, as has been found for procyanidin dimers and trimers isolated from peanuts and cocoa and other polyphenols such as epigallocatechin gallate, quercetin, and curcumin, which modulate membrane fluidity in a concentration-dependent manner, thus changing membrane potential (25, 47).

Thus, our results show that GSPE alters the cell membrane potential in STC-1 cells in a manner that is different to exposures in pancreatic \(\beta\)-cells. The GSPE concentration is influential, and concomitant effects with nutrients are important since these bioactive compounds are usually ingested together with nutrients in the diet. The obvious question is whether this effect leads to modulation of enterohormone secretion. Previous studies from our group suggested a modulation on glucose homeostasis and insulin levels by GSPE (3, 28); therefore, our main interest was on GLP-1, a well-described insulin secretagogue that is secreted by STC-1 cells (10, 36). Our results surprisingly showed that, under our experimental conditions, there is no relationship between cell membrane potential and GLP-1 secretion in STC-1 cells but there seems to be a threshold (a relative depolarization vs. control around 1.5) linked to GLP-1 secretion, while a broader depolarization does not lead to GLP-1 secretion, in fact, it appears to suppress secretion. Several studies reported that glucose triggers cellular membrane depolarization, stimulating GLP-1 secretion in primary cultures and GLUTag cells (14, 37), and we also found increased GLP-1 secretion in STC-1 cells. Fatty acids (23, 46) and amino acids (38) have also been shown to induce GLP-1 secretion in several enteroendocrine cell lines, and a few studies on STC-1 cells have shown cell membrane depolarization (43, 51), but their influence on GLP-1 secretion has not previously been studied in this cell model. Our results support the different sensitivity to nutrients by the different enteroendocrine cell line models and also reinforce that caution must be taken when extrapolating the effects on cellular membrane potential to describe hormone, at least GLP-1, secretion by enteroendocrine cells.

Finally, it is remarkable that regardless of the effect of the different nutrients, the highest GSPE dose limited GLP-1 secretion in all cases. A few studies showed that some phenolic compounds induce GLP-1 secretion, although at very different ranges of concentrations: genistin (at 5 \(\mu\)M ~ 1 mg/l), curcumin (at 25 \(\mu\)M ~ 9 mg/l), daidzein (at 100 \(\mu\)M ~ 25 mg/l), and chlorogenic acid (at 20 mM ~ 7 g/l) (22, 36, 44). However, this is the first time that GSPE (at 50 \(\mu\)M ~ 50 mg/l) has been shown to modulate nutrient-induced GLP-1 secretion. Taking together our results highlight the importance of the GSPE dose used, in accordance with previous reports from our group that found different effects on glucose homeostasis depending on the dose of procyanidins, among other factors (4).

In summary, this study indicates that grape-seed procyanidins modulate basal and nutrient-stimulated cell membrane potential in enteroendocrine STC-1 cells in a dose-dependent manner. We also show that surprisingly GSPE suppresses GLP-1 secretion from STC-1 cells. Finally, we describe the influence that a number of nutrients have on cell membrane potential and GLP-1 secretion in the enteroendocrine cell line STC-1. Further work is required to understand all the physiological effects that dietary consumption of grape seed procyanidins may have.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: N.G.-A., B.D.G., M.P., and A.A. conception and design of research; N.G.-A., B.D.G., M.P., and A.A. execution of research; N.G.-A., B.D.G., M.P., and A.A. interpretation of the results of research; N.G.-A., B.D.G., M.P., and A.A. writing the manuscript; N.G.-A., B.D.G., M.P., and A.A. critical revision of the manuscript for important intellectual content; N.G.-A., B.D.G., M.P., and A.A. final approval of the manuscript.

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