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Steviol glucuronide, a metabolite of steviol glycosides, potently stimulates insulin secretion from isolated mouse islets: Studies in vitro

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Abstract

Aims: Steviol glycosides are the sweet components extracted from medicinal plant *Stevia rebaudiana* Bertoni, which have antihyperglycaemic effects. Steviol glucuronide (SVG) is the metabolite excreted in human urine after oral administration of steviol glycosides. We aimed to clarify whether SVG exerts direct insulin stimulation from pancreatic islets and to explore its mode of action.

Materials and Methods: Insulin secretion was measured after 60 minutes static incubation of isolated mouse islets with (a) 10^{-9}-10^{-5} mol/L SVG at 16.7 mmol/L glucose and (b) 10^{-7} mol/L SVG at 3.3-16.7 mmol/L glucose. Islets were perifused with 3.3 or 16.7 mmol/L glucose in the presence or absence of 10^{-7} mol/L SVG. Gene transcription was measured after 72 hours incubation in the presence or absence of 10^{-7} mol/L SVG.

Results: SVG dose-dependently increased insulin secretion from mouse islets with 10^{-7} mol/L exerting the maximum effect in the presence of 16.7 mmol/L glucose ($P < .001$). The insulinotropic effect of SVG was critically dependent on the prevailing glucose concentration, and SVG (10^{-7} mol/L) enhanced insulin secretion at or above 11.1 mmol/L glucose ($P < .001$) and showed no effect at lower glucose concentrations. During perifusion of islets, SVG (10^{-7} mol/L) had a long-acting and apparently reversible insulinotropic effect in the presence of 16.7 mmol/L glucose ($P < .05$). Gene-transcript levels of B2m and Gcgr were markedly altered.

Conclusion: This is the first report to demonstrate that SVG stimulates insulin secretion in a dose- and glucose-dependent manner from isolated mouse islets of Langerhans. SVG may be the main active metabolite after oral intake of steviol glycosides.

KEYWORDS
insulin secretion, mouse pancreatic islets, steviol glucuronide, steviol glycosides

1 | INTRODUCTION

Steviol glycosides are a group of sweet diterpene glycosides found in the leaves of *Stevia rebaudiana* Bertoni, a scrub plant native to the subtropical regions of South America. To date, more than 40 steviol glycosides have been identified from stevia leaf extracts, of which stevioside and rebaudioside A are the most abundant.[1,2] Highly purified steviol glycosides have been permitted for use as food sweetener in numerous countries and regions, including Japan, the European Union and the United States. Besides the sweet property, steviol glycosides also possess potential therapeutic benefits. A multitude of preclinical and clinical studies have demonstrated their antihyperglycaemic effects, including lowering postprandial blood glucose,[3,4] enhancing insulin secretion[5-11] and improving insulin sensitivity.[12,13]

Steviol glycosides share steviol (ent-13-hydroxykaur-16-en-18-oic acid) as the aglycone core structure and only differ in the number and type of sugar molecules attached (at R1 and R2), see Figure 1A Moreover, all steviol glycosides found in stevia leaf extracts share the same metabolic pathway.[2,14] As previously unanimously described,[1,14-16] enzymes and acid present in the upper gastrointestinal tract are not able to hydrolyse the glycosidic bonds in steviol glycosides. However, the colonic microflora is able to degrade steviol glycosides to steviol. Most of the steviol is absorbed quickly from the intestine and transported to the liver; the rest is excreted in the faeces. In the liver, steviol is conjugated with glucuronic acid to form SVG (Figure 1B), which is excreted in the urine in humans and in faeces in rats. The difference in excretion between species is due to the lower molecular weight threshold for biliary excretion in rats than in humans.[17] Steviol glycoside derivatives do not accumulate in the body. Minor differences in the metabolism of steviol glycosides exist.[1] Intake of rebaudioside A results in slightly lower SVG concentrations (59%) than following stevioside (62%) consumption.[18]

After oral administration of steviol glycosides in humans, SVG is the main metabolite (no free steviol can be detected) in plasma and no other derivatives could be found except SVG in the urine.[19] Hence, it is highly relevant to investigate whether SVG is the active metabolite after oral administration of steviol glycosides. Unfortunately, there is no research available on the bioactive properties of SVG concerning insulin secretory capacity. We hypothesize that SVG has insulinotropic effects. The purpose of the present in vitro experiments was to clarify whether SVG stimulates insulin secretion from normal mouse islets of Langerhans and to test the transcription of key insulin regulatory genes to reveal the mode of action.

2 | MATERIALS AND METHODS

2.1 | Purification of SVG

SVG was purified from human urine as previously described.[19,20] In brief, stevioside (250-mg capsules) was given thrice daily for 3 days to 10 healthy volunteers; thereafter, a 24-hour urine sample was collected, between 1124 and 2494 mL from each volunteer. Total urine fraction was applied for column chromatography with an Amberlite XAD-2 column (Sigma). The column bed volume was 200 mL, being sufficient for the adsorption of amphipathic molecules of a 24-hour urine. About 600 mg of urine residue isolated from the prior purification step was dissolved in MeOH and adsorbed to silica gel by evaporating the solvent. The silica gel-bound sample was applied onto the top of the column (Machery-Nagel Silica gel 60 (0.063-0.2 mm)) and was eluted with a solvent mixture of ethyl acetate, ethanol and water. Fractions were collected, and samples of each fraction were analysed by TLC and compared with SVG as a reference compound. 20 μL samples of each fraction were evaporated. Acetate buffer (pH 5) and *β*-glucuronidase/sulfatase were added. After enzymatic reaction, the reaction mixture was freeze-dried followed by derivatization reaction with 4-(bromomethyl)-7-methocoumarin. All fractions containing enzyme-sensitive steviol conjugates eluted as a single large peak from the silica gel column, indicating the presence of only one steviol conjugate in the urine samples. All fractions containing this steviol conjugate were pooled, and the solvent was evaporated. The residue was subjected to preparative TLC, and around 10 mg purified, white crystalline SVG was obtained.
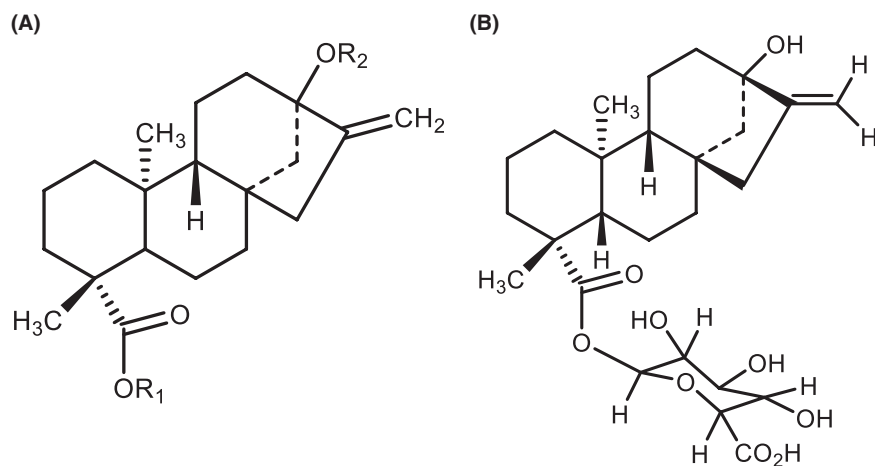

FIGURE 1 Chemical structures of steviol glycosides (A) and steviol glucuronide (B)

The melting point was measured on a Thermovar 9200 (type 300429) apparatus of Reichert-Jung (VWR). Mass spectral analysis of SVG was carried out using the LCQ Advantage ion trap mass spectrometer of Thermo Finnigan (San Jose, CA) in positive and negative ESI electrospray ionization modes. MS/MS spectra were generated by collision-induced decomposition of the $[M + 2NA – H]^+$ adduct ion formed by positive ESI and of the $[M – H]^-$ molecular ion obtained by negative ESI.

Record of ^1H and ^{13}C NMR spectra of SVG was conducted on a Bruker AMX 400 spectrometer equipped with an inverse ^1H multinuclei probe (Bruker).

Purity of SVG: >95%. SVG was dissolved in sterile water at a stock concentration of 10^{-2} mol/L.

2.2 | Isolation of islets

Pancreatic islets were isolated from adult female NMRI mice (Taconic) weighing 22-25 g by collagenase digestion technique, as described previously.[21-23] In brief, the mice were anaesthetized with pentobarbital intraperitoneally (50 mg/kg). A midline laparotomy was applied, and the distal end of the common bile duct was clamped at the papilla Vateri, whereafter the hepatic duct was cannulated and ~3 mL of ice-cold Hanks' Balanced Salts Solution (HBSS; Sigma Chemical) containing 0.3 mg/mL of Collagenase P (Boehringer Mannheim GmbH) was injected into the duct system of the pancreas. The pancreas was subsequently removed and placed in a test tube in water bath at 37°C for 19 minutes. After being rinsed for 3 times with HBSS, the islets were hand-picked under a stereomicroscope and immediately transferred for an overnight incubation in PRMI 1640 medium. The medium contained 11 mmol/L glucose and supplemented with 10% foetal calf serum, 5 μmol/L β-mercaptoethanol (Sigma-Aldrich), 100 IU/mL penicillin and 100 μg/mL streptomycin (all obtained from GIBCO BRL, Paisley, UK, if not stated otherwise). The incubation condition was 37°C and 95% normal atmosphere/5% CO_2. Islets for static incubation and perifusion studies were obtained from 12 to 20 mice to compensate for interindividual differences.

2.3 | Static incubation and perifusion of islets

After overnight culture, the NMRI female mice islets were rinsed twice with a modified Krebs-Ringer bicarbonate buffer, supplemented with 3.3 mmol/L glucose. The Krebs-Ringer bicarbonate buffer contained 125 mmol/L NaCl, 5.9 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 1.28 mmol/L $CaCl_2$, 25 mmol/L HEPES and 0.1% albumin from bovine serum (pH 7.4; all Sigma). After preincubation for 30 minutes in normal atmosphere, single islets were hand-picked and incubated in 100 μL of Krebs-Ringer bicarbonate buffer containing glucose (3.3- and 16.7-mmol/L) and glucose (16.7 mmol/L) in the presence of SVG (10^{-5}, 10^{-7}, 10^{-9} or 10^{-11} mol/L) for dose-dependent assay and 3.3, 6.6, 11.1 or 16.7 mmol/L glucose with and without 10^{-7} mol/L SVG for glucose-dependent assay. After incubation for 1 hour, 50 μL of the medium was frozen for analysis of insulin.

In the perifusion experiment, 30 pre-incubated (room temperature) islets were transferred to each of the chambers of the perifusion system (Suprafusion 2 500, Brandel).[22] The experiments were designed as follows: (a) 10-minute preperifusion at 3.3 mmol/L glucose, (b) 20-minute perifusion at 3.3 mmol/L glucose with/without SVG (10^{-7} mol/L), (c) 40-minute washout at 3.3 mmol/L glucose, (d) 20-minute perifusion at 16.7 mmol/L glucose with/without SVG (10^{-7} mol/L), (e) 40-minute washout at 3.3 mmol/L glucose and (f) 20-minute perifusion at 16.7 mmol/L glucose with 0.1 mmol/L carbamylcholine (sigma). The flow rate was 75 μL/min. Fractions were collected every two minutes.

2.4 | Insulin assay

Insulin was analysed by radioimmunoassay using a guinea pig anti-porcine insulin antibody, mono-^{125}I-(Tyr A14)-labelled human insulin as tracer and rat insulin as standard (all from Novo Nordisk). The separation of bound and free radioactivity was performed using ethanol, and the inter- and intra-assay variation coefficients were both <5%.

2.5 | Purification of RNA

After overnight incubation (37°C), mouse pancreatic islets were incubated (37°C) for 72 hours in RPMI 1640 with 11.1 mmol/L glucose in the presence or absence of 10^{-7} mol/L SVG. Thereafter, 100-120 islets for each sample were pooled in 1 mL of Trizol reagent (Ambion, Life Technologies) before RNA purification. Total RNA was isolated according to local laboratory protocol. RNA quality was analysed on a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific). The concentration of RNA was normalized to 50 ng/μL, and all samples were kept at −80°C until cDNA reaction.

2.6 | Quantitative real-time RT-PCR

Reverse transcription reaction, preamplification, quantitative real-time RT-PCR and related calculations were performed by BioXpedia, Aarhus, Denmark. Complementary DNA (cDNA) was prepared by reverse transcription of 100 ng of isolated RNAs in 20 μL final reaction volumes using TaqMan MicroRNA Reverse Transcription Kit. The reactions were performed according to the manufacturer's protocol. Reverse transcription was performed using 96-well Thermal Cycler (Veriti 384, Thermo Fisher Scientific). The reaction conditions were as following: 25°C for 10 min and 37°C for 120 min, following enzyme inactivation at 85°C for 5 seconds. All cDNA samples were stored at −20°C until preamplification. Following the reverse transcription, a preamplification reaction was performed on the cDNA samples using the TaqMan PreAmp Master Mix 2X (Thermo Fisher) and a Primer Pool 0.2X each primer. The reactions were performed in a 384-well Thermal Cycler (Veriti 384, Thermo Fisher) according to the manufacturer's protocol and enzyme activation at 95°C for 600 seconds, followed by 14 cycles with 95°C for 15 seconds and 60°C for 240 seconds.

The amplified cDNA was analysed on a 96.96 IFC on the Biomark HD Real-Time PCR system (Fluidigm) using the standard Gene Expression Protocol and thermal mixing and enzyme activation at 95°C for 600 seconds, followed by 15 seconds at 95°C, and finally 30 cycles at 60°C for 60 seconds. All samples were measured in quadruplicates. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene transcription using eukaryotic 18S rRNA (TaqMan assay ID: Hs99999901_s1), hypoxanthine phosphoribosyltransferase 1 (TaqMan assay ID: Rn01527840_m1) and actin, beta (TaqMan assay ID: Mm02619580_g1) as reference. The samples were analysed for transcription of different gene transcripts using mouse-specific TaqMan assays, Table 1.

2.7 | Statistical analysis

All data analysis was performed with GraphPad Prism Software Version 7.0 (GraphPad Software). Data are presented as the mean ± standard error of the mean (SEM), and statistical comparisons between two groups were evaluated using unpaired Student's t test. P-values ≤0.05 were considered statistically significant.

3 | RESULTS

3.1 | Effects of SVG on glucose-stimulated insulin secretion

SVG (10^{-5}-10^{-11} mol/L) potentiated glucose (16.7 mmol/L)-stimulated insulin secretion with a maximal effect obtained in the presence of 10^{-7} mol/L SVG, and the increase was more than sevenfold compared with 16.7 mmol/L glucose alone ($P < .001$). The stimulatory effect of SVG was found only at concentrations above 10^{-9} mol/L, with a lower effect at a concentration of 10^{-5} mol/L (Figure 2). To examine whether the action of SVG is glucose-dependent, the influence of the maximal stimulatory concentration of 10^{-7} mol/L was studied at 3.3, 6.6, 11.1. or 16.7 mmol/L glucose, respectively, As illustrated in Figure 3, SVG potentiated insulin secretion at glucose levels of 11.1 mmol/L and higher ($P < .001$), whereas no effect was detected at 3.3 or 6.6 mmol/L glucose.

3.2 | Effects of SVG on dynamic insulin release from perifused mouse islets

In the presence of 3.3 mmol/L glucose, the addition of SVG did not change basal insulin secretion from perifused islets. As expected, the increase in glucose level from 3.3 to 16.7 mmol/L caused a monophasic insulin response. In the presence of 16.7 mmol/L glucose, 10^{-7} mol/L SVG elicited a pronounced and sustained monophasic increase in insulin release, and at 70-120 min, the insulin AUC (area under the curve) was significantly increased by twofold in the SVG group ($P < .01$) compared with control group. At 130-150 min, insulin release showed a clear upturn in both groups with carbamylcholine

TABLE 1 List of genes investigated for transcription from mouse pancreatic islets with TaqMan assay ID

Gene symbol	TaqMan assay ID	Name
Ins1	Mm01259683_g1	Insulin I
Ins2	Mm00731595_gH	Insulin II
Insr	Mm00439693_m1	Insulin receptor
Irs1	Mm01278327_m1	Insulin receptor substrate 1
Akt1	Mm01331626_m1	Thymoma viral proto-onco-gene 1
Mafb	Mm00627481_s1	v-maf musculoaponeurotic fi-brosarcoma oncogene family, protein B (avian)
Sirt 1	Mm01168521_m1	Sirtuin 1
Pdx1	Mm00435565_m1	Pancreatic and duodenal homeobox 1
Beta2/ Neurod1	Mm01946604_s1	Neurogenic differentiation 1
Pax6	Mm00443081_m1	Paired box 6
Nkx2-2	Mm00839794_m1	NK2 homeobox 2
Nkx6.1	Mm00454961_m1	NK6 homeobox 1
Slc2a2	Mm00446229_m1	Solute carrier family 2 (facilitated glucose transporter), member 2
Gcg	Mm00801714_m1	Glucagon
Gcgr	Mm00433546_m1	Glucagon receptor
Pdk1	Mm00554300_m1	Pyruvate dehydrogenase kinase, isoenzyme 1
B2m	Mm00437762_m1	Beta-2 microglobulin

added in as a positive control (Figure 4). More details are provided in Table 2.

3.3 | Effects of 72-h exposure to SVG on gene transcription in mouse islets

To gain further insight into the effects of SVG on insulin secretion, transcript levels of a number of selected genes were examined in mouse islets (Figure 5). After 72 hours of intervention, SVG (10^{-7} mol/L) significantly decreased transcript level of beta-2-microglobulin (B2m, $P < .05$, Figure 5N) and significantly increased transcript level of glucagon receptor by 102% (Gcgr, $P = .05$, Figure 5M). Moreover, SVG (10^{-7} mol/L) markedly upregulated transcript level of pyruvate dehydrogenase kinase, isoenzyme 1 (Pdk1, $P = .06$, Figure 5E), nearly reached a significant level. As depicted at Figure 5F, 5I, 5J, 5P and 5Q, islets from the SVG group enhanced transcript levels of NK2 homeobox 2 (Nkx2-2, 164%, $P = .12$), pancreatic and duodenal homeobox 1 (Pdx1, 45%, $P = .21$), NK6 homeobox 1 (Nkx6.1, 59%, $P = .21$), thymoma viral proto-oncogene 1 (Akt1, 40%, $P = .2$) and insulin receptor (Insr, 25%, $P = .18$) compared with islets from the control group.

FIGURE 2 Effects of steviol glucuronide (10^{-5}-10^{-11} mol/L) on glucose (16.7 mmol/L)-stimulated insulin secretion from isolated mouse islets. Single mouse islets were incubated at 37°C for 1 h. Each bar represents the mean ± SEM from 24 incubations of single islets. *$P < .001$
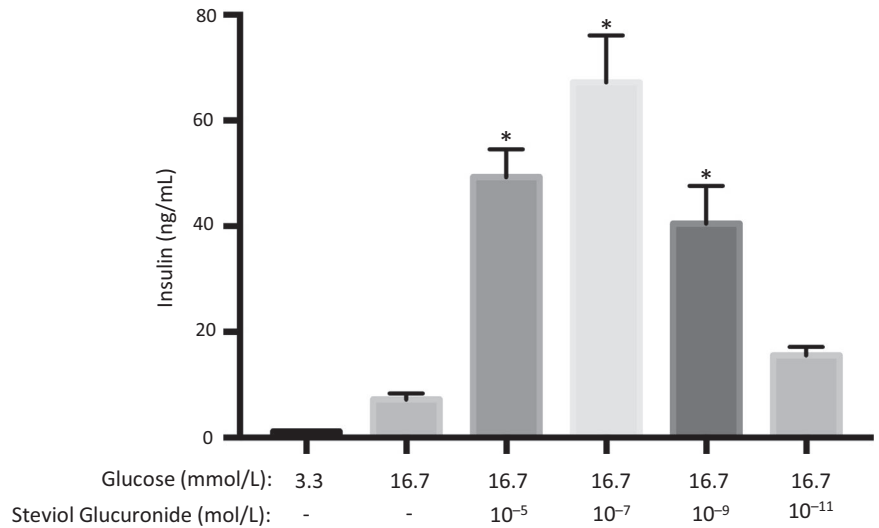
| Glucose (mmol/L): | 3.3 | 16.7 | 16.7 | 16.7 | 16.7 | 16.7 |
| Steviol Glucuronide (mol/L): | - | - | 10^{-5} | 10^{-7} | 10^{-9} | 10^{-11} |

FIGURE 3 Effects of steviol glucuronide (10^{-7} mol/L) on insulin secretion from isolated mouse islets incubated (37°C) for 1 h at glucose concentrations varying between 0 and 16.7 mmol/L. Each column represents the mean ± SEM from 24 incubations of single islets. *$P < .001$
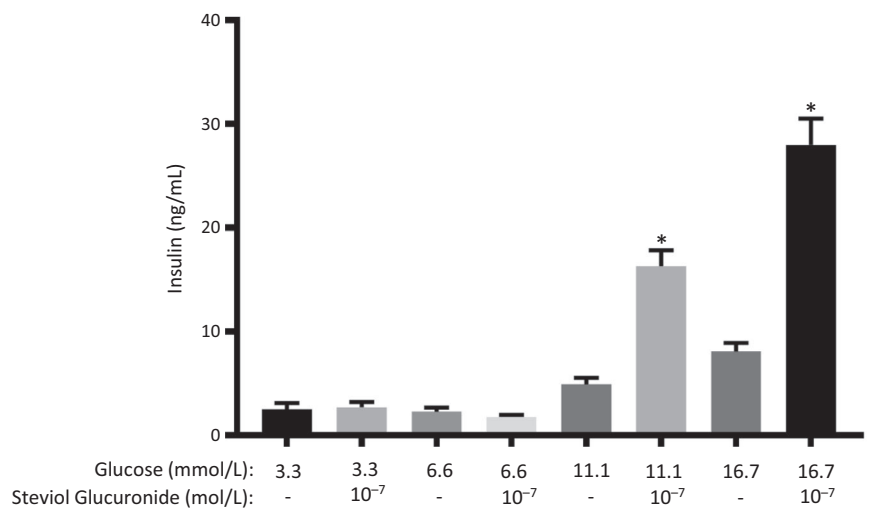
| Glucose (mmol/L): | 3.3 | 3.3 | 6.6 | 6.6 | 11.1 | 11.1 | 16.7 | 16.7 |
| Steviol Glucuronide (mol/L): | - | 10^{-7} | - | 10^{-7} | - | 10^{-7} | - | 10^{-7} |

FIGURE 4 Insulin secretion from mouse islets in a perifusion setup in absence (control ○) or presence (intervention ●) of 10^{-7} mol/L SVG at 3.3 mmol/L and 16.7 mmol/L glucose. Each curve represents mean ± SEM from 6 perifusion chambers each containing 30 islets. Experiments are finished by perifusion with carbamylcholine (0.1 mmol/L) as a positive control at 16.7 mmol/L glucose. For statistical significance, see Table 2
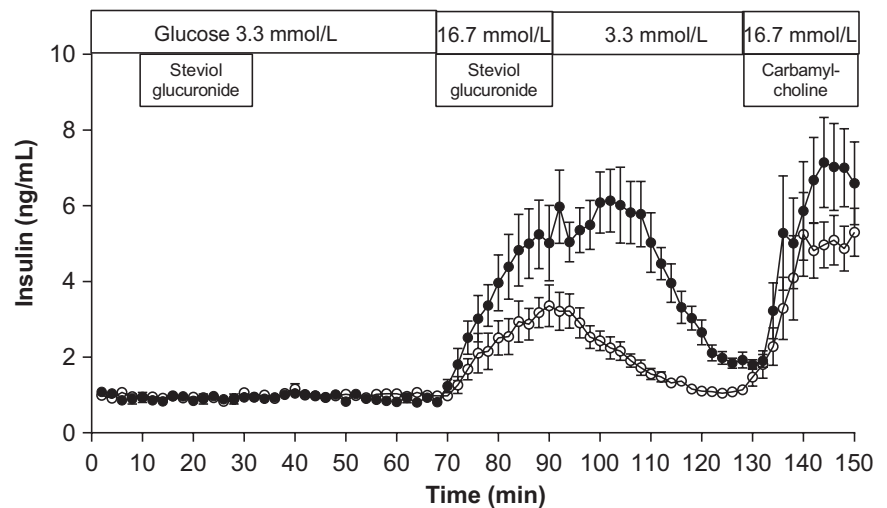
4 | DISCUSSION

Type 2 diabetes (T2D) is a metabolic disease characterized by persistent hyperglycaemia secondary to inadequate insulin secretion, insulin resistance, excessive glucagon secretion and/or a combination.

The epidemic increase in the prevalence of T2D is most pronounced in lower-income countries.[24] Due to the high price of the newer antidiabetic drugs as well as the risk of severe side effects of some of the current T2D therapies, it is important to continue the search of more effective, safer and cost-effective antidiabetic agents. Hundreds of

Time intervals (min)	Average insulin AUC (ng*min/mL)		Unpaired t test	Ratio
	Control	Steviol glucuronide		
0-10	9.7 ± 0.5	9.4 ± 0.8	P = .74	0.97
10-30	20.6 ± 0.9	20.0 ± 2.2	P = .81	0.97
70-120	111.9 ± 12.8	229.9 ± 33.1	P = .0077	2.06
130-150	85.0 ± 13.7	113.2 ± 20.0	P = .27	1.33

TABLE 2 Average insulin AUC (ng*min/mL) from control and intervention group at different time intervals from the perifusion study on steviol glucuronide

Note: Average insulin AUC is compared in ratio and with unpaired Student's *t* test. Glucose concentrations are depicted at Figure 4.

substances originating from plants, for example Stevia *rebaudiana Bertoni*, have antihyperglycaemic properties and are parts of modern diabetes treatment strategy.[25-27]

As mentioned, the effective compounds extracted from stevia, steviol glycosides, are acknowledged to have antihyperglycaemic effects. Our group previously demonstrated that stevioside reduces postprandial blood glucose levels in type 2 diabetic subjects,[4] and suppresses glucose response to a glucose tolerance test, increases insulin secretion, reduces glucagon release and lowers blood pressure in type 2 diabetic Goto-Kakizaki rats.[7,11] It is important to stress that stevioside is unlikely to increase the risk of hypoglycaemia, since insulin stimulation disappeared at normal or low circulating glucose concentrations. When supplemented to soy-based diet, stevioside shows beneficial effects on the development of the metabolic syndrome with hyperglycaemia, hypertension and dyslipidaemia.[10,28]

Furthermore, we have disclosed that stevioside, rebaudioside A, steviol (steviol glycosides' aglucon) and isosteviol (steviol glycosides' derivative) have the capability to enhance insulin secretion from isolated mouse islets in a dose- and glucose-dependent manner.[8,9,13,23] In the present study, we have found that the metabolite, SVG, possesses the same properties. In static incubation studies, the insulinotropic effect of SVG was present in a broad concentration range, with the maximal effect at 10^{-7} mol/L. Despite the potent insulin release at glucose concentrations 11.1 and 16.7 mmol/L, it is important to note that no insulinotropic action of SVG was present at lower glucose levels of 3.3 and 6.6 mmol/L. This may indicate that the risk of hypoglycaemia in vivo is eliminated.

In perifusion experiments, we applied the maximal effective concentration (10^{-7} mol/L) and showed the dynamic effects of SVG on insulin secretion. SVG elicits a typical monophasic insulin response at high glucose of 16.7 mmol/L but had no insulinotropic effect at low glucose (3.3 mmol/L). During the washout period at 90-130 min, the effect of SVG showed a 'long tail', with its effect fading gradually towards prestimulatory level rather than disappear immediately. It indicates that (a) the insulin stimulatory effect of SVG is reversible and (b) SVG may affect a certain receptor (eg, a TRPM5-related receptor[5]) on islet cells that remain bound to some extent during the washout period. The responsiveness of islets was confirmed by the prompt insulin secretory response to carbamylcholine (0.1 mmol/L) at the end of the experiment. The dynamic insulin response found in the perifusion experiment corroborates with our previous studies with stevioside, rebaudioside A, steviol and isosteviol.[8,9,13,23]

These results emphasize that steviol glycosides have a promising potential in the treatment of T2D, since the insulinotropic action of their metabolic end product, SVG, only operates at high glucose level, but disappears at low glucose concentration, which is indicative of improved glycaemic control with a good safety profile.[29] The risk of hypoglycaemia in T2D patients treated with antidiabetic medications, such as sulfonylureas, is a major safety issue that can be fatal.[30] In addition to imposing a risk of hypoglycaemia, long-term use of oral hypoglycaemic agents induces desensitization to metabolic stimuli in patients with T2D.[31] In mice, stevioside has been shown to counteract the suppressed glucose-stimulated insulin induced by the sulfonylurea, glyburide.[32] Thus, steviol glycosides seem to exhibit a vital function in potentiating the insulinotropic action only at high glucose concentrations without possessing the risk of hypoglycaemia in T2D. Furthermore, they may counteract the suppressed glucose-stimulated insulin release induced by certain oral antidiabetic medications.

We have now demonstrated that steviol glycosides and the derivative, isosteviol, as well as their glucuronide(s) share a common capability to stimulate insulin secretion. In view of their chemical structures, it can be speculated that the functional group may originate in the shared bone structure, steviol or the glucuronide bond.

The mechanism underlying the blood glucose-lowering effect of steviol glycosides has been investigated in some detail. Our group have demonstrated that stevioside acts directly on pancreatic β-cells to stimulate insulin secretion and the actions are independent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive K+-channel activity.[8] The effect of rebaudioside A is critically dependent on the presence of extracellular Ca^{2+}.[32] Isosteviol improves glucose and insulin sensitivity by up-regulating the gene transcription of key insulin regulatory genes and insulin transcription factors.[13] Recent research by Philippaert et al suggests that steviol glycosides enhance glucose-induced insulin secretion by potentiating the activity of TRPM5. TRPM5 is a Ca^{2+}-dependent cation channel expressed in both type II taste receptor cells and pancreatic β-cells.[5] In the current study, we explored the mode of action underlying the insulinotropic effect of SVG by studying the transcription of seventeen genes related to the regulation of glucose-stimulated insulin secretion and to the signalling pathways of glucagon/insulin secretion. Only the relative mRNA level of B2m and Gcgr was found to be significantly altered in islets treated with SVG (10^{-7} mol/L). Even though B2m is present on all nucleated cells, this small subunit of the major
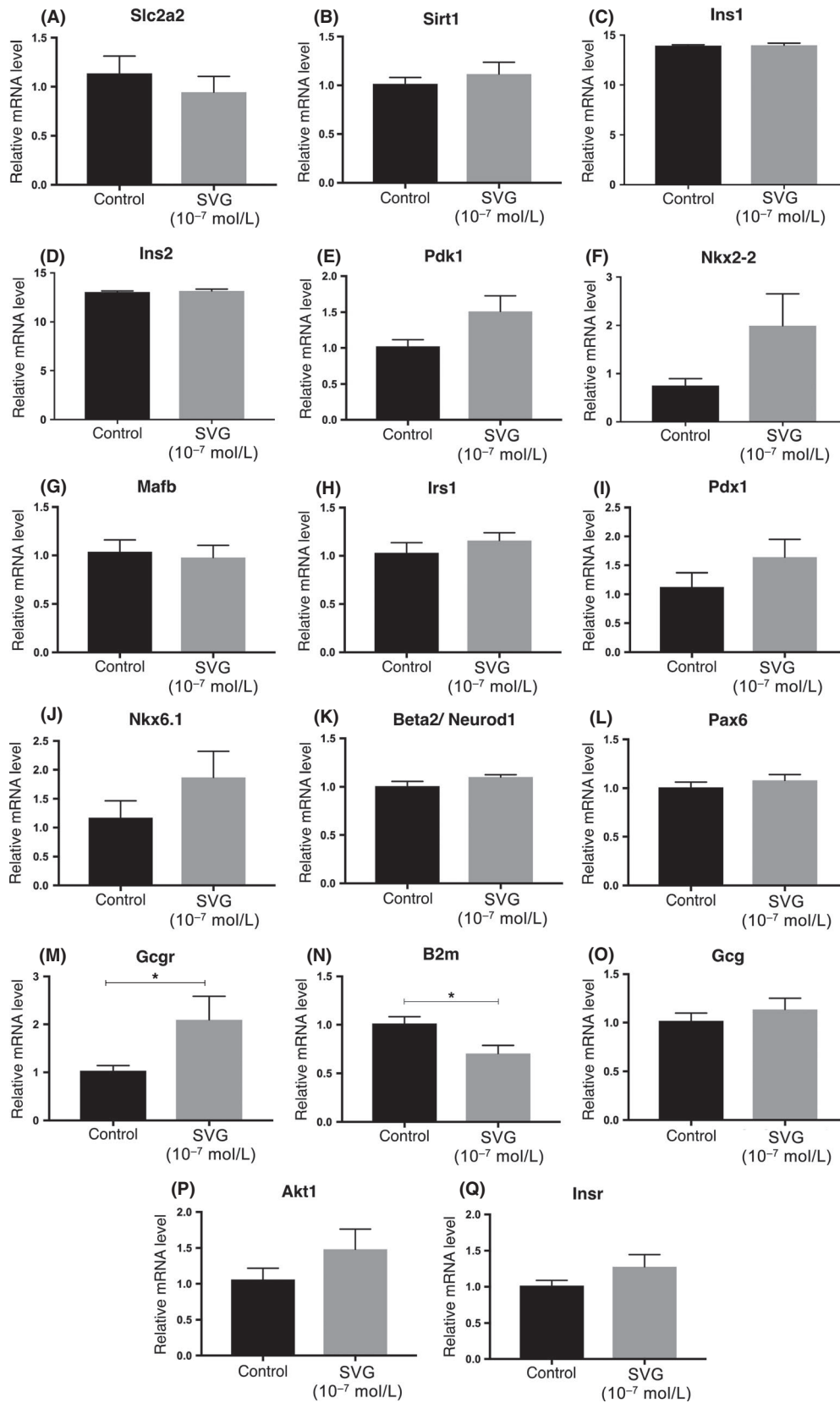
FIGURE 5 Effects of 72-h incubation (37°C) at 11.1 mmol/L glucose with or without 10^{-7} mol/L steviol glucuronide on isolated mouse islets gene transcription. Transcript abundance of 17 specific genes was studied by real-time RT-PCR using TaqMan assays. Seven samples were obtained in each treatment, and the samples were measured in quadruplicates. Data are presented as mean ± SEM. *$P \leq .05$

histocompatibility (MHC) class I molecule has been found to be positively correlated with diabetes duration.[33] In addition, Pavlovic et al found elevated glucose levels to increase MHC class I mRNA levels twofold in both rat and human islet cell preparations, suggesting that a hyperglycaemic state is able to increase expression in islet β-cells.[34] Javadi et al reported a significant increase in B2m in STZ-induced diabetic rabbits. Following STZ injection, serum glucose level gradually increased, while the B2m level rose rapidly two days after injection.[35] In this light, it is interesting that SVG has the ability to downregulate the gene transcription of B2m and may thereby potentially decrease the incidences of microvascular and macrovascular complications such as atherosclerosis in untreated T2D subjects.

Glucagon is secreted from pancreatic α-cells that, as a major counterregulatory hormone to insulin, is essential for maintaining glucose homeostasis. Glucagon receptors are abundantly expressed on pancreatic β-cells and coupled to stimulation of insulin secretion.[36-38] Svendsen et al examined glucagon-induced insulin secretion using mouse models with different ways of disrupting glucagon signalling confirming that insulin secretion depends on intra-islet glucagon activity, and glucagon receptor, along with GLP-1 receptor, is crucial for β-cell secretory responses.[38] SVG upregulated the transcription of Gcgr gene, indicating the potentiated insulin release from β-cells, which is corroborated by the results from our static incubation and perifusion studies.

A limitation of current gene study is the relatively small sample size. Meanwhile, mouse islets contain other types of hormone-secreting cells. These may explain the lack of statistical significance of SVG increasing the gene transcription of Pdk1, Akt1, Insr, Pdx1, Nkx2-2 and Nkx6.1. Pdk1, Akt1 and Insr are components of the insulin signalling pathway. Pdx1, Nkx2-2 and Nkx6.1 are transcription factors regulating insulin expression and β-cell function. Upregulation of these genes indicates enhanced insulin release.

5 | CONCLUSION

To our knowledge, this is the first demonstration of a direct effect of SVG on insulin secretion. Our hypothesis was confirmed in the present study that SVG is insulinotropic. SVG dose- and glucose-dependently potentiates insulin release from isolated mouse islets and shows a dynamic insulin stimulatory effect during perifusion. RT-PCR results indicate that SVG may exert its effects through inhibiting of the gene transcription of B2m and increasing expression of Gcgr. Further research is required to confirm the mode of action of this compound. Our study suggests that SVG is the active metabolite after oral administration of steviol glycosides and possesses the potential as an antidiabetic agent.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and conduct of the protocol, or collection and interpretation of data, and preparation of this manuscript. All authors gave their approval of the final version to be published.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All experiments were performed in accordance with Directive 2010/63/EU. The mice used in this study did not suffer unnecessarily.

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