Antihyperglycemic and Blood Pressure-Reducing Effects of Stevioside in the Diabetic Goto-Kakizaki Rat

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Stevioside, a glycoside present in the leaves of the plant, *Stevia rebaudiana Bertoni* (SrB), has acute insulinotropic effects in vitro. Its potential antihyperglycemic and blood pressure–lowering effects were examined in a long-term study in the type 2 diabetic Goto-Kakizaki (GK) rat. Rats were fed 0.025 g · kg^{-1} · d^{-1} of stevioside (purity > 99.6%) for 6 weeks. An intra-arterial catheter was inserted into the rats after 5 weeks, and conscious rats were subjected to arterial glucose tolerance test (2.0 g · kg^{-1}) during week 6. Stevioside had an antihyperglycemic effect (incremental area under the glucose response curve [IAUC]): 985 ± 20 (stevioside) versus 1,575 ± 21 (control) mmol/L × 180 minutes, ($P < .05$), it enhanced the first-phase insulin response (IAUC: 343 ± 33 [stevioside] *v* 136 ± 24 [control] μU/mL insulin × 30 minutes, $P < .05$) and concomitantly suppressed the glucagon levels (total AUC: 2,026 ± 234 [stevioside] *v* 3,535 ± 282 [control] pg/mL × 180 minutes, $P < .05$). In addition, stevioside caused a pronounced suppression of both the systolic (135 ± 2 *v* 153 ± 5 mm Hg; $P < .001$) and the diastolic blood pressure (74 ± 1 *v* 83 ± 1 mm Hg; $P < .001$). Bolus injections of stevioside (0.025 g · kg^{-1}) did not induce hypoglycemia. Stevioside augmented the insulin content in the β-cell line, INS-1. Stevioside may increase the insulin secretion, in part, by induction of genes involved in glycolysis. It may also improve the nutrient-sensing mechanisms, increase cytosolic long-chain fatty acyl-coenzyme A (CoA), and downregulate phosphodiesterase 1 (PDE1) estimated by the microarray gene chip technology. In conclusion, stevioside enjoys a dual positive effect by acting as an antihyperglycemic and a blood pressure–lowering substance; effects that may have therapeutic potential in the treatment of type 2 diabetes and the metabolic syndrome.

AN UNDERSTANDING OF the pathophysiology of type 2 diabetes lies at the heart of its successful treatment. Type 2 diabetes is a chronic metabolic disorder that results from reduced first phase insulin secretion, α-cell dysfunction with relative glucagon excess, as well as insulin resistance. Its long-term prognosis is tied mainly to microvascular and macrovascular complications. The determinants of these complications include chronic hyperglycemia, hypertension, dyslipidemia, obesity, and smoking. It has been demonstrated that control of hypertension is at least as effective a means of preventing diabetic macrovascular complications as control of hyperglycemia.[1,2]

It is notable that a multifactorial approach combining blood pressure, blood glucose, and lipid lowering is most effective in preventing diabetic complications.[3] Consequently, therapeutic agents with diversified actions, eg, combined antihyperglycemic and blood pressure–lowering effects are in great demand.

Extracts of the leaves of the plant, *Stevia rebaudiana Bertoni* (SrB), have been used for many years in traditional South American treatment of diabetes. Oral intake of SrB extracts slightly suppresses plasma glucose during an oral glucose tolerance test in healthy subjects.[4] A 35% reduction in blood glucose is also observed in diabetic subjects after oral intake of SrB extracts.[5] However, we only have limited knowledge of which substance(s) are active. A possible candidate is the glycoside stevioside, a white crystalline material with the elemental composition $C_{38}H_{60}O_{18}$, which makes up the largest proportion of the dry matter of SrB. Thus stevioside in vitro exerts a direct insulinotropic action in both isolated mouse islets and the clonal β-cell line, INS-1.[6,7] Furthermore, we demonstrated in a pilot study in vivo that an acute bolus injection of stevioside had antihyperglycemic, insulinotropic, and glucagonostatic effects in the anesthetized, diabetic Goto-Kakizaki (GK) rats.[8] This is a non-obese animal model of type 2 diabetes characterized by a deficient insulin response to glucose in vivo and in vitro, as well as insulin resistance.[9-12] Interestingly, it has been found that stevioside induces blood pressure reduction in hypertensive nondiabetic rats[13] and also in nondiabetic hypertensive subjects.[14]

The aims of the present study were to test if long-term administration of stevioside possesses dual beneficial qualities, ie, antihyperglycemic effects and blood pressure suppression in type 2 diabetes. We specifically tested whether stevioside (1) has antihyperglycemic properties and lowers the blood pressure in the diabetic GK rat after long-term oral treatment and (2) elicits hypoglycemia in the fasting state in the GK and Wistar rats when given as a bolus injection. Furthermore, we used the high-density oligonucleotide microarray technology to explore the mechanisms underlying the insulinotropic effect of stevioside and the aglucon, steviol, by using the clonal β-cell line, INS-1.

MATERIALS AND METHODS

Animals

We used adult male GK (originally obtained from Takeda Chemical, Tokyo, Japan and bred locally; age, 20 weeks; weight, 250 to 320 g)

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and male Wistar rats (Bomholtgaard Breeding and Research Centre, Ry, Denmark; age, 15 weeks; weight, 250 to 350 g). The animals were kept on a standard pellet diet and had tap water ad libitum and a light cycle of 12 hours/12 hours. Before the experiments, food was withheld from the rats for 12 hours. The experiments were performed in accordance with the guidelines of the Danish Council on Animal Care.

Intra-Arterial Glucose Tolerance Test Performed in GK Rats After Long-Term Stevioside Treatment

The glucoside stevioside (19-O-β-glucopyranosyl-13-O [β-glucopyranosyl (1-2)]-β -glucopyranosyl-steviol (purity $>$ 99.6%) (Sigma Chemical, St Louis, MO) was added to the tap water and given to the GK rats (n = 20) at a dose of 0.025 $g \cdot kg^{-1} \cdot d^{-1}$ for a period of 6 weeks. The stevioside molecule is composed of steviol, a diterpenic carboxylic alcohol, and 3 D-glucose molecules. The control group received the same molecular amount of glucose as content in the stevioside group (0.0167 $g \cdot kg^{-1} \cdot d^{-1}$). At week 5, the GK rats were anesthetized by subcutaneous (SC) injection (1 $mL \cdot kg^{-1}$ rat) of a mixture consisting of 0.08 mg/mL phentanyl citrate, 2.5 mg/mL phluanisone (Janssen Pharmaceutica, Beerse, Belgium) and 1.25 mg/mL midazolame (Dumex-Alpharma, Oslo, Norway). In the frontal part of the neck, a midline incision was performed and the right carotic artery was isolated. The cephalic part of the artery was closed by suture and the catheter was held in position by ligatures. A hole in the artery was made caudal to the closing suture and a catheter (Tygon Microbore Tubing, Norton Performance Plastics [Akron, OH]; inner diameter [id] 0.40 mm, outer diameter [od] 0.78 mm) was inserted 30 mm into the artery and held in position by ligatures. The free end of the catheter was exteriorised at the neck leaving 2.5 cm of the catheter outside the animal. The catheter was filled with 0.9% saline containing 10 U/mL heparin (Løvens Kemiske Fabrik, Ballerup, Denmark) and closed with a fishing line inserted into the lumen. After surgery, the animals were given 0.08 mg Naloxone intramuscularly (IM) as antidote (DuPont Pharmaceuticals, Hertfordshire, UK). Animals were caged individually with free access to food and water. The catheters were flushed daily with 0.2 mL saline/heparin to keep them open. After 6 days' recovery, an intra-arterial glucose tolerance test (IAGTT) (2.0 $g \cdot kg^{-1}$) was performed. Food was withheld from the animals for 12 hours (water ad libitum) before the experiments starting at 8 PM. The animals were freely moving in separate plastic cylinders. The intra-arterial catheter was connected to a polyethylene tube system (PE20, Intramedic, Becton Dickinson, Sparks, MD) allowing blood sampling and infusion. Blood samples were drawn at time points from −15 to 180 minutes. Immediately after the 0 minute sample D-glucose (2 $g \cdot kg^{-1}$ dissolved in 0.9% saline) was administered as a bolus injection over 20 seconds. Blood samples (300 μL) were drawn in chilled tubes containing 3 μL aprotinin/heparin mix (7.7 mg/mL aprotinin and 2,300 IU/mL heparin), samples were then centrifuged (10 minutes, 4°C, 4,000 rpm), and plasma was frozen for subsequent analysis of insulin, glucose, and glucagon. Blood was replaced by isotonic saline to avoid volume depletion.[15]

Measurements of Tail Blood Pressure During Long-Term Treatment With Stevioside

The tail-blood pressure was determined every week during the 6-week period using the TRN005 Kent Instrumentation Amplifier (Scandidact, Kvistgaard, Denmark) to measure the diastolic and systolic blood pressure.

Intra-Arterial Bolus Injection of Stevioside in Conscious Wistar and GK Rats in the Fasting State

Acute experiments with intra-arterial infusion of stevioside alone were performed in the fasting state in normal Wistar (n = 22) and diabetic GK rats (n = 12) having undergone the same surgical procedure as described above. No oral treatment with stevioside was given before the experiments. Stevioside (0.2 $g \cdot kg^{-1}$) alone was given as a bolus injection 1 week after the day of catheter insertion and after 12 hours of fasting. Saline infusion served as control.

Gene Expression Profiles and Insulin Content of INS-1 Cells

The mechanism of action of stevioside and steviol was examined by culturing the clonal pancreatic β-cell line, INS-1, cells in the presence or absence of 1 μmol/L stevioside or steviol at 11 mmol/L glucose for 24 hours. INS-1 cells (passage 48 to 50) were cultured in RPMI 1640 supplemented with HEPES (10 mmol/L), sodium pyruvate (1 mmol/L), and β-mercaptoethanol (5 μmol/L).[16] The cells were collected for insulin content determination and RNA extraction. To ensure that the samples were representative and to reduce preanalysis variation, 3 extracted total RNA samples were pooled from the same culture conditions in an equal amount and used for further investigation of the gene expression profile. The methods were identical to those described previously.[16] Briefly, investigation of the expression of 8,740 genes or expressed sequence tags (ESTs) proceeded through the following steps: (1) 100 μg total RNA was used for mRNA preparation. Poly (A)+ RNA was isolated by an oligo-dT selection step (Oligotex kit, Qiagen, Valencia, CA). (2) A total of 1 μg mRNA was used as starting material for the cDNA preparation. Labeled cRNA was prepared using the MEGAscript In Vitro transcription (IVT) kit (Ambion, Austin, TX). (3) A total of 10 μg cRNA were fragmented and loaded onto the Affymetrix probe array cartridge rat U34A gene chip. (4) The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate. (5) The probe arrays were scanned at 560 nm by a scanning confocal microscope with an argon ion laser as the excitation source (made for Affymetrix by Hewlett Packard). (6) The readings from the quantitative scanning were analyzed by the Affymetrix gene chips analysis software. Two chips were done per sample. For each gene or EST, approximately 20 pairs of probes (complemented to different parts of mRNA of a gene) were used to measure the level of expression. One pair of probes consists of a perfect match (PM) probe and mismatch (MM) probe (1 MM nucleotide in the middle of sequence). The latter is used to determine the background signal after hybridization. A probe pair is considered positive when the intensity of PM minus the intensity of MM and the ratio of the intensity of PM to the intensity of MM are greater than or equal to the statistical gene expression. The difference of expression was measured by comparing the difference in intensity of all pairs of probes between samples for each gene.[16] The number of genes that changed expression depended on the cut-off values set (significant level). The cut-off values are arbitrary. The larger the numerical sort score (SS; a value based on both fold change and average difference change), the more significant or reliable is the measured difference in expression of gene between the experimental group and the control group. The genes that had changed in expression $\geq$ 3-fold and EST with an SS of 0.65 or more to stevioside and steviol were presented.

Assays

Blood samples were centrifuged (10 minutes, 4°C, 4,000 rpm), and glucose was determined using the GLU MPR Glucose/GOD-PAP method (Boehringer Mannheim GmbH, Mannheim, Germany). Plasma insulin and glucagon were analyzed by radioimmunoassay (RIA) kits (Linco Research, St Charles, MO). The antibody in the glucagon kit is specific for pancreatic glucagon and does not cross react with other islet polypeptides. The sensitivity of the glucagon assay is 20 pg/mL. For the insulin secretion study, insulin was analyzed by RIA using a guinea pig antiporcine insulin antibody (Novo Nordisk, Bagsvaerd, Denmark) and mono-^{125}I(Tyr A14)-labeled human insulin (Novo Nordisk) as

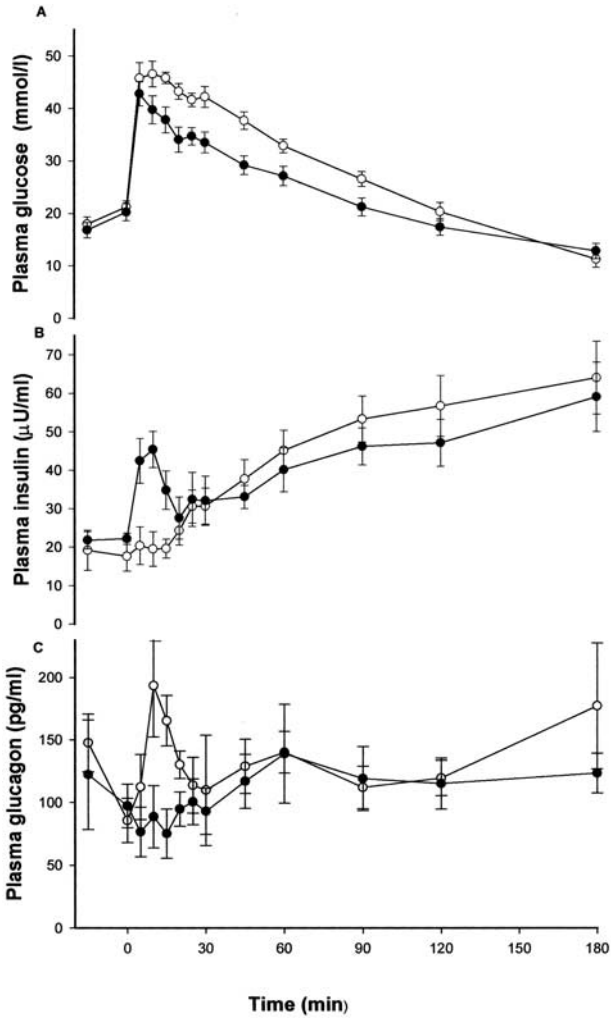

Fig 1. Effect of 6 weeks of treatment with stevioside (0.025 g · kg^{-1} · d^{-1}) on (A) plasma glucose, (B) insulin, and (C) glucagon during an IAGTT (2.0 g · kg^{-1}) in conscious GK rats. (○) control group (n = 10) and (●) stevioside group (n = 10). Data given as mean ± SEM.

tracer and porcine insulin as standard (Novo Nordisk). The separation of bound and free radioactivity was performed using ethanol.

Statistical Analysis

Statistical analysis was performed by Student's unpaired *t* test. Differences were considered significant at $P < .05$. If not indicated elsewhere, data are presented as mean ± SEM. The incremental area was calculated as the area under the curve above basal (IAUC). Total area under the curve (TAUC) was calculated as the area above zero.

RESULTS

Effects of Long-Term Treatment With Stevioside on Intra-Arterial Glucose Tolerance Tests in Type 2 Diabetic GK Rats

Figure 1 shows that there was no significant difference in fasting plasma glucose or plasma insulin levels between the stevioside and control group (n = 10) of conscious GK rats before glucose injection (2.0 g · kg^{-1}) (Fig 1A). Plasma glucose was suppressed after injection in the stevioside-treated group (IAUC: 985 ± 20 *v* 1,575 ± 21 mmol/L × 180 minutes, $P < .05$). The stevioside-fed animals had a higher first phase insulin response than the control group (IAUC: 343 ± 33 [stevioside] *v* 136 ± 24 [control] μU/mL insulin × 30 min, $P < .05$) (Fig 1B) and persistent increase in the second phase of the insulin responses during the entire observation period, which did not differ significantly between the 2 groups (IAUC: 4,798 ± 234 [control] *v* 3,776 ± 229 [stevioside] μU/mL × 150 min, $P < .38$). Interestingly, the preceding stevioside treatment caused a concomitant suppression of glucagon corresponding to the first phase insulin response (TAUC: 2,026 ± 234 [stevioside] *v* 3,535 ± 282 [control] pg · mL × 180 minutes, $P < .05$) (Fig 1C).

Effects of Long-Term Treatment With Stevioside on Blood Pressure and Body Weight in Type 2 Diabetic GK Rats

As illustrated in Fig 2, initial systolic and diastolic blood pressures were similar in the 2 groups: systolic/diastolic blood pressure. After the 6-week study period, a significant difference in systolic/diastolic blood pressure was observed: (mean ±

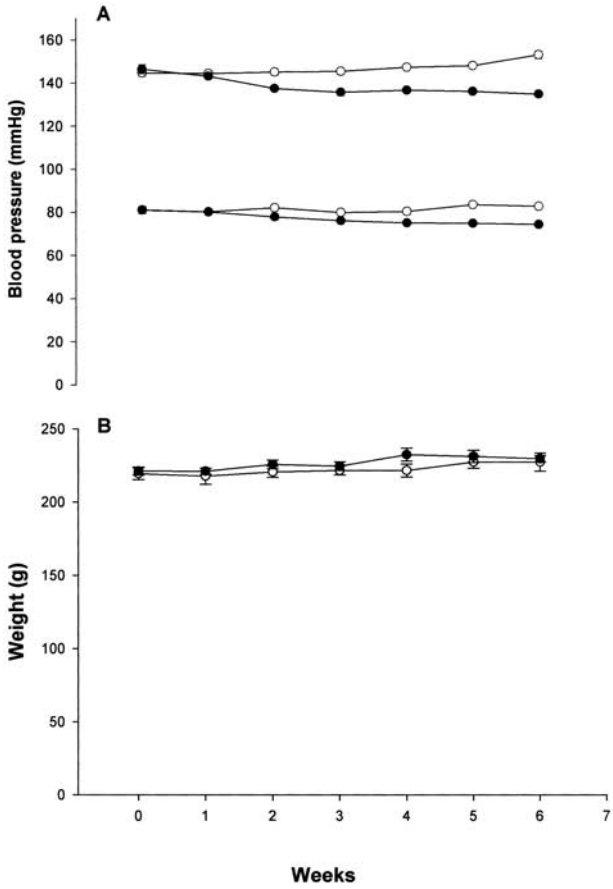

Fig 2. (A) Effect of stevioside treatment on diastolic and systolic blood pressure. (○) control group (n = 10) and (●) stevioside-treated group (n = 10). (B) Effect of stevioside treatment on body weight. (○) control group (n = 10) and (●) stevioside group (n = 10). Data given as mean ± SEM.

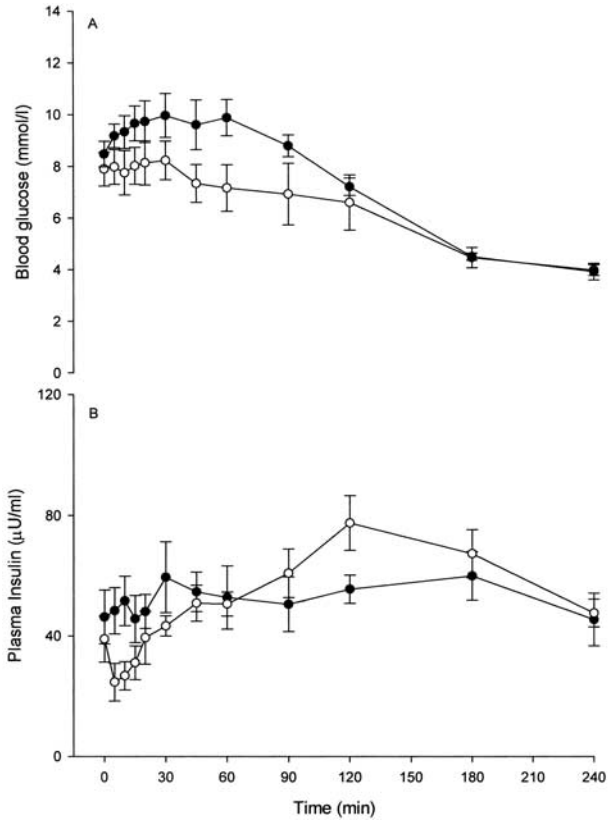

Fig 3. Blood glucose and plasma insulin levels after bolus injection of stevioside (0.2 g · kg^{-1}) in the conscious diabetic GK rat after a12-hour fast. (○) 0.9% saline alone (n = 6) and (●) stevioside (n = 6).

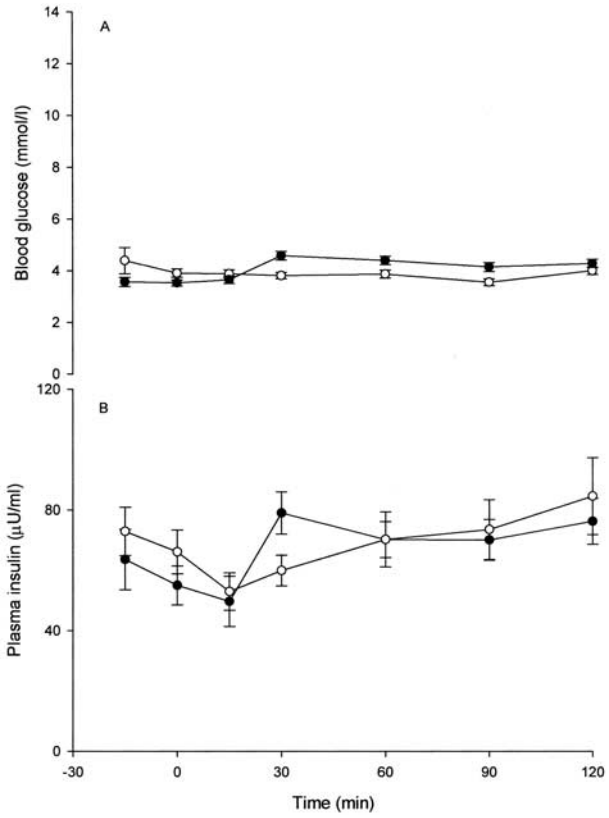

Fig 4. Blood glucose and plasma insulin levels after bolus injection of stevioside (0.2 g · kg^{-1}) in the awake, normal Wistar rat after a 12-hour fast. (○) 0.9% saline alone (n = 12) and (●) stevioside (n = 10). Data given as mean ± SEM.

SEM) 153 ± 5/83 ± 1 mm Hg in the control group versus 135 ± 2/74 ± 1 mm Hg in the stevioside group ($P < .001$). Stevioside caused a progressive lowering of blood pressure from week 1 and onward (Fig 2A). Stevioside had no significant effect on body weight (mean ± SEM).

Effects of a Bolus Infusion of Stevioside on Blood Glucose and Insulin Levels in Conscious Fasting Diabetic GK Rats

As shown in Fig 3, a bolus injection of stevioside (0.2 g · kg^{-1}) in 6 conscious fasting diabetic GK rats did not cause significant changes in blood glucose (TAUC: 1,669 ± 65 [stevioside] *v* 1,439 ± 157 [control] mmol/L glucose × 240 minutes; $P < .20$) or plasma insulin responses (TAUC: 13,995 ± 952 [stevioside] *v* 13,003 ± 705 μU insulin × 240 minutes [control]; $P < .42$) (Fig 3). As expected, the fasting blood glucose level of these animals was lower than that of the GK rats participating in the long-term stevioside treatment because they were 5 weeks older on the day of the IAGTT (Fig 1).

Effects of a Bolus Infusion of Stevioside on Blood Glucose and Insulin Levels in Conscious Fasting Wistar Rats

Figure 4 shows that a bolus injection of stevioside (0.2 g · kg^{-1}; n = 10) in the fasting state in conscious Wistar rats and saline in the control group (n = 12) did not affect the area under the blood glucose (TAUC: 465 ± 15 *v* 505 ± 54 mmol/L × 120 minutes) or plasma insulin response curves (TAUC: 8,245 ± 459 *v* 8,069 ± 904 μU/mL × 120 minutes). Despite similar TAUCs, stevioside induced small increases in

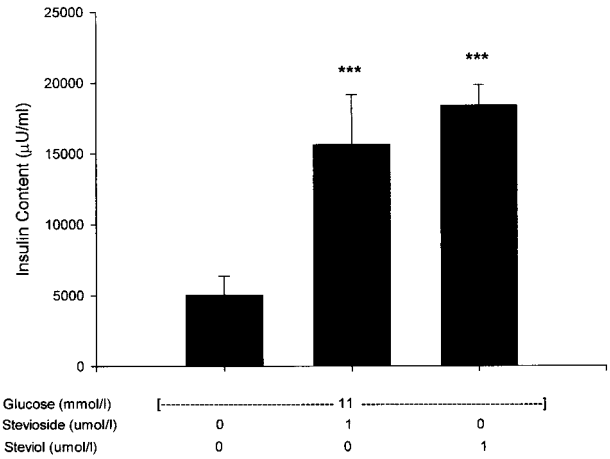

Fig 5. Effects of stevioside and steviol (1 μmol/L) on total insulin content in clonal pancreatic β-cell line, INS-1, incubated overnight. Each bar represents mean ± SEM from 6 incubations of 0.2 × 10^6 cells each.

Table 1. Expression of Gene Profile in INS-1 Cells After 24 Hours incubation With 1 μmol/L Stevioside and the Aglucon of Stevioside, Steviol (1 μmol/L)

GB Acc. No.	Name	Function	Stevid FC	Stevioside FC
L05489	Heparin-binding BGF-like growth factor	Cell growth	10.1	5.5
U21101	Cyclic GMP-stimulated phosphodiesterase (PDE2A2)	cAMP degradation	8.6	7.3
U43534	Double-stranded RNA-specific editase (RED1)	Double-strand RNA synthesis	8.6	7.8
L32591	GADD45	GABA synthesis	8.2	8.3
AB001452	Sck	Signal transduction	7.4	4.3
M91466	A2b-adenosine receptor	Adenyl cyclase activation	7.3	9.3
X66022	Neuro-D4 protein	Unknown	5.8	3.6
AB004096	Lanosterol 14-demethylase	Microsomal cholesterol synthesis	5.4	7.1
X13527	Fatty acid synthetase	Fatty acid synthesis	5.4	4
AF014503	p8	Pancreatitis-associated protein	5.3	4.5
U72741	36 Kd beta-galactoside binding lectin	Unknown	4.9	3.1
X63281	N-myc gene	Oncogene	4.4	4.5
L32591	GADD45	GABA synthesis	4.2	3.6
J03808	Acetyl-coenzyme A carboxylase	Malonyl CoA synthesis	3.7	3.2
J04792	Omithine decarboxylase (CDC)	Polyamine metabolism	3.7	5.4
X05684	L-type pyruvate kinase	Glycolysis	3.3	3.3
J04791	Omithine decarboxylase (CDC)	Polyamine metabolism	3.2	4.2
U35099	Complexin II	Unknown	−3	−5.2
U53486	Corticotropin-releasing factor receptor	Unknown in islets	−3	−3.2
D86039	ATP-sensitive invardly rectifying K^+ channel, BIR (Kir62)	K^+ channel	−3.1	−3.2
M31322	Spermmembrane protein (YWK-II)	Unknown	−3.1	−3.1
U89744	Putative cell surface antigen	Unknown	−3.2	−3.5
L48060	Lactogen receptor	Cell growth	−3.9	−4.9
U44948	Smooth muscle cell LIM protein (SmLIM)	Unknown	−4.1	−3
X05341	3-oxoacyl-CoA thiolase	Fatty acid oxidation	−4.1	−3.7
D86089	ATP-sensitive invardly rectifying K^+ channel, BIR (Kir62)	K^+ channel	−4.6	−8.1
J05029	long-chainacyl-CoA dehydrogenase (LCAD)	Fatty acid oxidation	−4.6	−6.4
M37828	Cytochrome P450	Mitochondrial function	−4.8	−4.1
L07736	Camitine palmitoyltransferase I	Fatty acid oxidation	−4.9	−6.6
M58364	GTP cyclohydrolase I	Tetrahydrobiopterin (BH4) synthesis	−5.6	−3
D28560	Phosphodiesterase I	cAMP degradation	−5.8	−5.1
M23601	Pronoamine oxidase B (Maobf3)	Excytosis	−6.2	−5.2
M85214	trk precursor	Unknown	−6.8	−6.2
D28560	Phosphodiesterase I	cAMP degradation	−7	−5.4
X01785	Cell surface protein (MRC OX-2)	Unknown	−13.6	−4.8

Abbreviation: FC, fold of change. The (−) sign before number indicates decrease, otherwise increase for FC.

blood glucose at time points 15, 30, and 45 minutes and in insulin at time point 30 minutes ($P < .05$).

Insulin Content in INS-1 Cells Treated With Stevioside

The INS-1 cells were incubated for 24 hours with 11 mmol/L glucose with or without stevioside and steviol, respectively. Stevioside and steviol (1 μmol/L) significantly ($P < .05$) increased the insulin content in the clonal β-cell line, INS-1 (Fig 5).

The Effect of Stevioside on the Gene Expression Profile in INS-1 Cells

Thirty-five genes and 19 ESTs (not shown) changed expression more than 3 times in response to stevioside (Table 1). Among them, the proinsulin gene was upregulated 3.8 times. Interestingly, the liver type pyruvate gene, acetyl-coenzyme A carboxylase, and the fatty acid synthetase gene were upregulated. In contrast, genes involved in fatty acid oxidation, ie, the carnitine palmitoyltransferase I (CPT-1) gene, the long-chain acyl-CoA dehydrogenase gene, the 3-oxoacyl-CoA thiolase gene, and the cytosolic epoxide hydrolase gene were all downregulated. Interestingly, phosphodiesterase I (PDE 1) was also downregulated in the INS-1 cells. The aglucon of stevioside, steviol (1 μmol/L), induced similar changes on gene expression as stevioside.

DISCUSSION

We tested the hypothesis that the combined antihyperglycemic and blood pressure–lowering effects were harbored in the diterpene glycoside stevioside, a main component of the dry matter of leaves of SrB. This study is the first to demonstrate that long-term oral stevioside treatment has antihyperglycemic and blood pressure–lowering effects in the diabetic GK rat where it stimulated the first phase insulin secretion and suppressed the glucagon levels in vivo. Stevioside induced the β-cell genes involved in the glycolysis and nutrient-sensing

mechanisms. In the fasting state, no acute hypoglycemic action was discovered after a bolus injection of stevioside in the normal Wistar or in the diabetic GK rat. Stevioside consists of a complex of 3 glucose molecules and 1 molecule of the steviol aglucone, a diterpenic carboxylic alcohol.[17,18]

In a previous pilot study, a single bolus injection of stevioside caused steadily increasing insulin concentrations and a suppression of glucagon during an intravenous (IV) glucose tolerance test (IVGTT) in anesthetized GK rats.[8] In this previous study, stevioside did not, as in the present study, enhance the first-phase insulin response in the anesthetized GK rats.[8] However, data obtained in anesthetized animals should be interpreted with caution as anesthesia may significantly affect glucose and insulin levels, as well as other hormones.

Type 2 diabetes is a chronic metabolic disorder that not only results from insulin resistance and defect in first phase insulin secretion, but is also characterized by a relative glucagon excess and a pancreatic α-cell dysfunction.[19] The suppression of circulating glucagon concentrations reduces blood glucose levels[20] supporting that agents inhibiting the glucagon secretion or action are beneficial for patients with diabetes. In experimental diabetes, an abnormal α-cell function is characterized by an impaired response to glucose and certain glucose metabolites that probably results from a specific defect in glucose recognition.[21] The abnormal α-cell function seems not to be ascribed to an insulin deficiency per se, but rather to an abnormal metabolic state secondary to insulin deficiency.[22] Interestingly, plasma glucagon decreased initially during the IAGTT in the stevioside-treated GK group compared with the control group. The glucagonostatic effect of stevioside treatment is therefore presumably caused either via a direct inhibitory action on the glucagon-producing α cells or an indirect action via either an improved glucose recognition or an insulin-induced suppression of glucagon. We addressed the question whether stevioside in the fasting state may cause hypoglycemia like sulphonylureas and hence become a threat to the diabetic subject by conducting the experiments in fasting, conscious GK, and Wistar rats. We demonstrated that a bolus injection of stevioside in the fasting state did not cause hypoglycemia in the normal Wistar or the diabetic GK rat. This is supported by our previous demonstrations in vitro that the effects of stevioside fade at glucose concentrations comparable with normoglycemia.[6,7]

Interestingly, the long-term administration in vivo in GK rats had no effect on fasting blood glucose, insulin, and glucagon, while it markedly improved the glucose tolerance. The reason could be that the GK rats only display a rather mild fasting hyperglycemia. We have tried to gain insight into the mechanisms of action of stevioside using the INS-1 cells. We are aware that it is not possible to make far-reaching extrapolations from the in vitro effects to the in vivo situation. Chronic exposure of INS-1 cells to stevioside increased the content of proinsulin mRNA and insulin, which may have contributed to the increased glucose-stimulated insulin secretion.[6,7] Previously, we have demonstrated that stevioside does not affect the adenosine triphosphate (ATP)-sensitive potassium channels in the β cells.[7] It is well known that malonyl-CoA plays a key role in the nutrient-sensing mechanism of β cells. Glucose activation of acetyl-CoA carboxylase triggers a rapid production of malonyl-CoA, which inhibits CPT-1 and the importation of fatty acyl-CoA into the mitochondria for oxidation. The increase in cytosolic long-chain fatty acyl-CoA leads to the exocytosis of insulin.[23] Interestingly, stevioside upregulated the expression of the liver type of pyruvate kinase (L-PK) and acetyl-CoA carboxylase, whereas it downregulated the expressions of CPT-1, long-chain acyl-CoA dehydrogenase, cytosolic epioxide hydrolase, and 3-oxoacyl-CoA thiolase. These modifications favor the augmentation of the long-chain fatty acyl-CoA during glucose challenge. Therefore, the improved insulin secretion by stevioside may attribute to the induction of genes involved in glycolysis and the improvement of nutrient-sensing mechanisms. It should be kept in mind that because there is a low expression of acetyl CoA-carboxylase and fatty acid synthetase in islets, the de novo fatty acid synthesis may not result in fat accumulation during long-term treatment. Moreover, the stevioside-induced counteraction of the abnormal insulin and glucagon secretion might inhibit lipolysis in adipose tissue and reduce the accumulation of fat in nonadipose tissue. Further experiments are needed to clarify this question. The downregulation of PDE1 may contribute to the improvement of insulin secretion secondary to increased intracellular cyclic adenosine monophosphate (cAMP) levels, an important physiologic amplifier of the glucose-induced insulin secretion. Pancreatic islets from rats, mice, and humans express functional PDE1, PDE3, PDE4, and PDE5, as well as an IBMX-resistant cAMP PDE8.[24] Finally, the present study also revealed an increase in the A2b-adenosine receptor, which is involved in the adenyl cyclase activation increasing the synthesis of cAMP.[25] Stevioside increases the expression of glucose responsive genes. The mode of action is not clear because the aglucon, steviol, has equal effects as stevioside. It is unlikely that the action is mediated via the glucose residues, but may be ascribed to the carboxylic alcohol and/or the bicyclo [3,2,1] octane C/D ring.

The long-term prognosis of type 2 diabetes relies not only on the treatment of hyperglycemia, but also on the coexistence of hypertension, dyslipidemia, obesity, sedentariness, and smoking as delineated in the United Kingdom Prospective Diabetes Study (UKPDS).[1,2,26,27] Consequently, the pharmacologic treatment of type 2 diabetes should also aim to lower blood pressure and lipid concentrations. The present study demonstrated that long-term stevioside treatment had a significant blood pressure–lowering effect from week 1 and onwards. Our results support the previously demonstrated blood pressure reduction in nondiabetic rats.[28] Recently, a blood pressure-lowering effect has also been reported in nondiabetic subjects with hypertension being treated for 1 year with stevioside.[14] No side effects or beneficial effects on lipid or glucose were reported. We were not surprised to learn that stevioside had no effect on blood glucose because the subjects were normoglycemic, nondiabetic subjects, but it would be interesting to learn whether daily administration of stevioside to patients suffering from the metabolic syndrome would improve their lipid levels secondary to better glycemic control. Although the mechanism underlying the antihypertensive effect of stevioside is not clear, it has been demonstrated that the hypotensive response to

stevioside appears to occur through a calcium antagonist mechanism similar to that of verapamil.[29,30]

In conclusion, long-term oral stevioside treatment improves first-phase insulin response, suppresses glucagon levels, and has antihyperglycemic effects in the diabetic GK rat. Bolus injections of stevioside do not elicit hypoglycemia in conscious normal Wistar or diabetic GK rats. The modification of gene expression involving insulin synthesis, glucose, and fatty acid metabolism may contribute to the beneficial metabolic effect in diabetic animals. Additionally, stevioside accomplishes a pronounced suppression of blood pressure in the GK rat. Whether stevioside may serve as a "new" medicament for the treatment or prevention of the metabolic syndrome with actions on 2 or more of the clinical features remains to be elucidated.

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