

## Stevioside improves pancreatic $\beta$ -cell function during glucotoxicity via regulation of acetyl-CoA carboxylase

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**Chen J, Jeppesen PB, Nordentoft I, Hermansen K.** Stevioside improves pancreatic  $\beta$ -cell function during glucotoxicity via regulation of acetyl-CoA carboxylase. *Am J Physiol Endocrinol Metab* 292: E1906–E1916, 2007. First published March 6, 2007; doi:10.1152/ajpendo.00356.2006.—Chronic hyperglycemia is detrimental to pancreatic  $\beta$ -cells, causing impaired insulin secretion and  $\beta$ -cell turnover. The characteristic secretory defects are increased basal insulin secretion (BIS) and a selective loss of glucose-stimulated insulin secretion (GSIS). Several recent studies support the view that the acetyl-CoA carboxylase (ACC) plays a pivotal role for GSIS. We have shown that stevioside (SVS) enhances insulin secretion and ACC gene expression. Whether glucotoxicity influences ACC and whether this action can be counteracted by SVS are not known. To investigate this, we exposed isolated mouse islets as well as clonal INS-1E  $\beta$ -cells for 48 h to 27 or 16.7 mM glucose, respectively. We found that 48-h exposure to high glucose impairs GSIS from mouse islets and INS-1E cells, an effect that is partly counteracted by SVS. The ACC dephosphorylation inhibitor okadaic acid (OKA,  $10^{-8}$  M), and 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR,  $10^{-4}$  M), an activator of 5'-AMP protein kinase that phosphorylates ACC, eliminated the beneficial effect of SVS. 5-Tetradecyloxy-2-furancarboxylic acid (TOFA), the specific ACC inhibitor, blocked the effect of SVS as well. During glucotoxicity, ACC gene expression, ACC protein, and phosphorylated ACC protein were increased in INS-1E  $\beta$ -cells. SVS pretreatment further increased ACC gene expression with strikingly elevated ACC activity and increased glucose uptake accompanied by enhanced GSIS. Our studies show that glucose is a potent stimulator of ACC and that SVS to some extent counteracts glucotoxicity via increased ACC activity. SVS possesses the potential to alleviate negative effects of glucotoxicity in  $\beta$ -cells via a unique mechanism of action.

acetyl-coenzyme A carboxylase; mouse islets; INS-1E  $\beta$ -cell line; carnitine palmitoyltransferase I; insulin secretion; type 2 diabetes mellitus

TYPE 2 DIABETES MELLITUS (T2DM) has become a worldwide epidemic. The disease results from defects in both insulin action and insulin secretion. The secretory defect in  $\beta$ -cells is characterized by increased basal insulin secretion (BIS) and a selective loss of glucose-stimulated insulin secretion (GSIS). Chronic hyperglycemia is also detrimental to the regulation of  $\beta$ -cell turnover (29); the exact mechanisms of action of glucotoxicity are, however, not well defined. Several studies support the view that the long-chain fatty acyl-CoA (LC-CoA) is important for GSIS (9, 13, 16). According to this model, cytosolic acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). A sudden augmentation in the malonyl-CoA

concentration during glucose metabolism results in an inhibition of carnitine palmitoyltransferase I (CPT I) and transport and oxidation of LC-CoA in mitochondria (9, 13, 16). Thus, malonyl-CoA, the rate-limiting step in the biosynthesis of LC-CoA, stimulates insulin secretion and modulates other  $\beta$ -cell functions (41, 42). Interestingly, Zhang et al. (51) have confirmed that ACC plays a key role for GSIS in INS-1 cells. However, the malonyl-CoA hypothesis is currently controversial. Antinozzi et al. (1) and Mulder et al. (34) found that INS-1 cells treated with a recombinant adenovirus containing the cDNA encoding malonyl-CoA decarboxylase, an enzyme that decarboxylates malonyl-CoA to acetyl-CoA, dramatically lowered intracellular malonyl-CoA levels, whereas insulin secretion to glucose was unaltered. A more inclusive model of nutrient-stimulated secretion involves two arms of signal transduction that occur simultaneously (15). One arm is dependent on modulation of the  $K_{ATP}$  channel, evoked by changes in the ATP/ADP ratio. This causes increased cytosolic  $Ca^{2+}$  and, secondary to this, changes in cAMP and phospholipids. The other arm is dependent on anapleotic input into the tricarboxylic acid cycle, generation of excess citrate, and increases in cytosolic malonyl-CoA (44) and subsequently increased LC-CoA. Signaling through both arms is required for normal secretion. LC-CoA esters and products formed from them are potent regulators of enzymes and channels. It is hypothesized that the elevations of LC-CoA directly modulate the activity of enzymes, genes, and various  $\beta$ -cell functions or modify the acylation state of key proteins involved in regulation of ion channels and exocytosis (15).

The diterpene glycoside stevioside (SVS) stimulates insulin secretion both *in vitro* and *in vivo* and lowers blood glucose (11, 12, 19, 22–24). We (24) have demonstrated that SVS increases ACC gene expression in  $\beta$ -cells by use of gene chip analysis. The questions arise 1) whether ACC is changed in response to glucotoxicity with regard to expression and activity and 2) whether ACC could be the target of new forms of therapy. In this study, we wanted to explore whether SVS was able to counteract the glucotoxicity via the regulation of ACC.

### MATERIALS AND METHODS

**Experimental animals.** Adult female NMRI mice (Bomholtgaard Breeding and Research Centre, Ry, Denmark) weighing 20–25 g were used. The animals were kept on a standard pellet diet and tap water *ad libitum* and a 12:12-h light-dark cycle. This study was carried out in accordance with the guidelines of the Danish Council for Animal Experiments.

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**Agent preparation.** SVS (Wako Pure Chemical Industries, Osaka, Japan) was added to the medium from a stock solution ( $10^{-3}$  M) in distilled water. OKA (Sigma Chemical, St. Louis, MO) and 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR; Sigma) were added from stock solutions ( $10^{-5}$  M and  $10^{-2}$  M, respectively) in distilled water. 5-Tetradecyloxy-2-furancarboxylic acid (TOFA; Sigma) was added from stock solution ( $6 \times 10^{-3}$  M) in dimethylsulfoxide (DMSO; Merck, Frankfurt, Germany). The final concentration of DMSO in all media concerning the studies of TOFA was 0.1%.

**Islet isolation.** Islets were isolated by the collagenase digestion technique (28). In brief, the animals were anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg), and a midline laparotomy was performed. The pancreas was retrogradely filled with 3 ml of ice-cold Hanks' balanced salt solution (HBSS; Sigma) supplemented with 0.3 mg/ml collagenase P (Boehringer Mannheim, Mannheim, Germany). HBSS and collagenase P were filter sterilized before use. The pancreas was subsequently removed, incubated for 19 min at 37°C in a water bath, and subsequently rinsed with ice-cold HBSS, and the islets were handpicked under a stereomicroscope. The islets were then incubated overnight at 37°C and 95% normal atmosphere-5% CO<sub>2</sub> in 10 ml of RPMI 1640 containing 11.1 mM glucose supplemented with 10% fetal calf serum, 2.06 mM L-glutamine, 100 IU/ml penicillin G, and 100  $\mu$ g/ml streptomycin (all GIBCO-BRL, Paisley, UK). Islets for the incubation studies were obtained from 6–10 mice to compensate for interindividual differences.

**Islet incubation and insulin secretion studies.** This part of the study aimed at determining the antiglucotoxicity effect of SVS and the possible blocking effect of OKA and AICAR. After overnight incubation, mouse islets were preincubated for 48 h in RPMI with 11.1 or 27 mM glucose in the presence or absence of  $10^{-6}$  M SVS supplemented with or without  $10^{-8}$  M OKA or  $10^{-4}$  M AICAR, respectively. After preincubation, islets were rinsed once with modified Krebs-Ringer buffer (KRB) supplemented with 3.3 mM glucose and 0.1% human serum albumin (Sigma). The KRB contained (in mM) 125 NaCl, 5.9 KCl, 1.2 MgCl<sub>2</sub>, 1.28 CaCl<sub>2</sub>, and 25 HEPES (pH 7.4; all Sigma). After 30 min of preincubation in normal atmosphere at 37°C, single islets were handpicked and incubated in 100  $\mu$ l of KRB with glucose concentrations of 3.3 or 16.7 mM. After 60 min of incubation in normal atmosphere at 37°C, 50  $\mu$ l of the medium were collected and frozen for analysis of insulin.

**INS-1E cell incubation, insulin secretion, insulin output, and insulin content studies.** This part was done to explore whether the effects demonstrated in mouse islets could be reproduced in the INS-1E cell line. Prior to this study, the INS-1E cells were maintained and cultured in the RPMI as described above. The cells were passed weekly. The day before the experiment, the INS-1E cells were plated onto 24-well plates (NUNC Brand Products, Roskilde, Denmark) in a number of  $0.2 \times 10^6$  cells/well with PRMI. Then the cells were cultured in RPMI with 11.1 or 16.7 mM glucose in the presence or absence of  $10^{-6}$  M SVS for 48 h in a humidified atmosphere (5% CO<sub>2</sub>-95% air at 37°C). This culture condition was used in all of the following studies with INS-1E cell line except those especially mentioned. Thereafter, the cells were divided into three parts according to insulin secretion, insulin output, and insulin content study. For the insulin output study, 100  $\mu$ l of incubation medium were taken to measure insulin output. For the insulin content, cells were washed once with 1 ml of cold PBS, and then 1 ml of glycine-bovine serum albumin (BSA) buffer (glycine 100 mM, 0.25% BSA, pH 8.8; Roche Molecular Biochemicals, Mannheim, Germany) was added. Cells were scratched and treated with sonication twice at 0°C for 14 s (Branson Sonifier 250, Danbury, CT). After centrifugation for 30 min at 16,000 rpm, the supernatant was collected and frozen at -20°C for later insulin assay. For the insulin secretion study, the cells were incubated with 1 ml of KRB containing 3.3 or 16.7 mM glucose for 1 h after preincubation in 1 ml of KRB supplemented 3.3 mM glucose for 30 min. Supplementary experiments were made with the ACC inhibitor TOFA. In brief, INS-1E cells (passage 90 or 93) were

cultured in RPMI with 11.1 or 16.7 mM glucose in the presence or absence of  $10^{-6}$  M SVS,  $6 \times 10^{-6}$  M TOFA, or both for 48 h in a humidified atmosphere. The cells were incubated with 1 ml of KRB containing 3.3 or 16.7 mM glucose with or without  $10^{-6}$  M SVS and 30 mM KCl (Sigma) for 1 h after preincubation in 1 ml of KRB supplemented with 3.3 mM glucose for 30 min. Subsequently, 300  $\mu$ l of supernatants were collected, centrifuged, and kept for insulin analysis. After the secretion study, the cells were lysed in 0.1 N NaOH. We measured the total protein with a detergent-compatible protein kit (Bio-Rad Laboratories, Hercules, CA) for adjustment of insulin secretion. A passage number between 67 and 80 was used in all of the studies on INS-1E cells except that specially mentioned.

**Insulin assay.** Insulin was analyzed by radioimmunoassay using a guinea pig anti-porcine insulin antibody (Novo Nordisk, Bagsvaerd, Denmark) and mono-<sup>125</sup>I-(Tyr A14)-labeled human insulin (Novo Nordisk) as tracer and rat insulin as standard (Novo Nordisk). The separation of bound and free radioactivity was performed using ethanol (18). The inter- and intra-assay variation coefficients were both <10%. SVS, OKA, and AICAR did not interfere with the insulin assay at the studied concentrations.

**Isolation of RNA.** The cells were cultured in accordance with section "INS-1E cell incubation and insulin secretion studies" except that the 6-well plate was used (NUNC Brand Products, Roskilde, Denmark) to get enough cells for RNA extraction. The cells were washed once with cold PBS. Subsequently, 1 ml of TRIzol reagent (GIBCO-BRL, Life Technologies, Roskilde, Denmark) was added to extract total RNA according to the manufacturer's instructions. RNA was quantified by measuring absorbency at 260 and 280 nm. The integrity of the RNA was checked by visual inspection of the two ribosomal RNAs 18S and 28S on an agarose gel.

**Real-time RT-PCR.** We investigated the expression of ACC, CPT I, insulin II (INS2), and glucose transporter 2 (GLUT2) by real-time RT-PCR. cDNA was synthesized using IScript (Bio-Rad) in accordance with the manufacturer's instructions. Total RNA of 50 ng/10  $\mu$ l reaction mixture was used for measurement of the target mRNA. The real-time RT-PCR assay was performed using the ABI 7500 FAST machine (ABI, Foster City, CA). Ten-microliter real-time RT-PCR reactions consist of 5  $\mu$ l of 2 $\times$  TaqMan FAST Universal Master Mix (P/N 43660783; ABI), 0.5  $\mu$ l of 20 $\times$  TaqMan Assay/probe [AAC1 (Rn00573474\_m1), CPT I (Rn00580702\_m1), INS2 (Rn01774648\_g1), or GLUT2 (Rn00563565\_m1); ABI], and INS-1E cDNA equivalent to 50 ng of total RNA in 4.5  $\mu$ l of H<sub>2</sub>O. The thermal FAST cycle program was 20 s at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Reactions were set up in triplicate for each sample, and ACC, CPT I, INS2, and GLUT2 expressions were normalized to eukaryotic 18S rRNA expression (Hs99999901\_s1; ABI). All assays were carried out in 96-well format plates covered with optical adhesive cover (P/N 4346906 and P/N 4311971; ABI). We used the 2<sup>- $\Delta\Delta$ C<sub>T</sub></sup> method to calculate the relative gene expression (as described in *User Bulletin 2*, 1997, from PerkinElmer, covering the aspect of relative quantization of gene expression). No template controls (NTC) and no amplification controls (NAC) were included for each gene as negative controls.

**Western blotting.** The cells for Western blotting were cultured for 48 h in 80-cm<sup>2</sup> flask 1075 (NUNC Brand Products) with 15 ml of culture medium as mentioned above. The medium was removed and the cells washed once with 15 ml of room temperature PBS and trypsinized and centrifuged to get cell pellets. After a wash with 10 ml of cold PBS, the pellets were lysed for 30 min on ice in 500  $\mu$ l of cold RIPA lysis buffer (sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA), and whole cell lysate was collected after centrifugation (1 ml of 1 $\times$  RIPA contains 1 $\times$  TBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 10  $\mu$ l of sodium orthovanadate, and 10  $\mu$ l of protease inhibitor cocktail solution). The protein concentration was determined by Bio-Rad detergent-compatible protein kit (Bio-Rad Laboratories). Fifty micrograms of total protein samples were resolved by PAGE (Criterion 4–20% Tris·HCl; Bio-Rad Lab-

oratories). Protein samples were denatured by boiling for 5 min before loading. Proteins were blotted onto PVDF membrane (Amersham International), and the blot was then incubated in blocking buffer (5% nonfat milk in 10 mM Tris·HCl, 1.15 M NaCl, and 0.1% Tween-20) for 1 h at room temperature. Immunoblotting entailed the following: primary antibody incubation at 4°C overnight with antibody for ACC1 (1:500 dilution, no. 3662; Cell Signaling Technology), phosphorylated (p)ACC (1:500 dilution, no. 3661; Cell Signaling Technology), or actin (1:5,000 dilution, A5441; Sigma) followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (sc-2370 and sc-2375, all Santa Cruz Biotechnology). The labeling was visualized using an enhanced chemiluminescence system (ECL; Amersham International). In each experiment, band densities were normalized against actin, and the results are expressed as protein level to control.

**ACC activity determination.** The 6-well plate was used (NUNC Brand Products) for culture cells to determine ACC activity. The cells were washed once with cold PBS. Subsequently, 0.5 ml of cold lysis buffer was added to each well. Lysis buffer contained 5 mM HEPES, 230 mM mannitol, 70 mM sucrose, 1 mM Na<sub>2</sub>-EGTA, 1 mM DTT, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. The cells were kept on ice for 10 min, scraped, and transferred to a 1.5-ml Eppendorf tube where they were sonicated twice at 0°C for 14 s (Branson Sonifier 250). After centrifugation for 10 min at 13,000 rpm, the supernatant was collected for ACC determination.

ACC activity was measured using the radioactive CO<sub>2</sub> fixation method described by Inoue and Lowenstein (21). The principle is based on the reaction: acetyl-CoA + CO<sub>2</sub> + ATP ↔ malonyl-CoA + ADP + P<sub>i</sub>. [<sup>14</sup>C]bicarbonate is converted into the carboxyl group of malonyl-CoA. In brief, the reaction mixture (400 μl total volume) consisted of 10.0 mM MgCl<sub>2</sub>, 10.0 mM sodium citrate, 10 mg/ml BSA (free fatty acid), 100 mM Tris·HCl (pH 7.4), 1 mM DTT, 0.3 mM acetyl-CoA, 4 mM ATP, 20 mM NaHCO<sub>3</sub>, and 200 μl of whole cell lysate. The reaction was initiated by the addition of [<sup>14</sup>C]sodium bicarbonate (0.0075 mCi/ml final; Amersham International); the reaction was carried out at 37°C for 20 min in a shaking water bath. The reaction was terminated by the addition of 5 N HCl. The content of the tube was shaken gently by hand and left on ice for 10 min and then centrifuged (13,000 rpm, 4°C, 10 min). Four hundred microliters of the supernatant were transferred into a scintillation vial and dried with nitrogen. Residue in the vial was dissolved in 400 μl of distilled water and mixed, and 3 ml of scintillating liquid were added and counted for 5 min by Wallac 1409 Liquid Scintillation Counter (Wallac Oy, Turku, Finland). The activity of ACC is expressed as the fold change to the control (27).

**Triglyceride content assay.** Cells were cultured as described in the "INS-1E cell incubation and insulin secretion studies" except that 24-well Black Visiplate TC (Wallac Oy) plates were used. After 48 h, the cells were washed once with 1 ml of PBS at room temperature, and the number of cells was estimated using nuclear staining with SYTO 24 reagent (20 μl/well, Roche) and measured by FLUOstar Galaxy (BMG, Ramcon, Denmark). The medium was removed, and the cells were frozen for 1 h at -80°C. Subsequently, the cells were incubated for 20 min with triglyceride (TG) reagents (250 μl/well, Roche). TG content was determined with a TG GPO-PAP kit (Roche) and was normalized to cell number. The recovery of TG was ~90%.

**2-Deoxyglucose uptake assay.** After 48 h of culture, the cells were washed twice with 1 ml of PBS at room temperature. The cells were incubated in 300 μl/well UB buffer containing 0.025 mM 2-deoxyglucose (5 μCi/ml 2-deoxy-[<sup>3</sup>H]glucose, 1.5 μCi/well tracer; PerkinElmer) supplemented with 10 μg/ml insulin for 15 min at 37°C. The cells were washed twice with 1 ml of UB buffer supplemented with 50 mM glucose and lysed in 0.5 ml of 0.1 N NaOH. Cell lysates (400 μl) were subjected to 6 ml of scintillating liquid and counted with a Wallac 1409 liquid scintillation counter (Wallac Oy). Cell lysates (50 μl) were applied to determine the protein concentration by Bio-Rad detergent-compatible protein kit (Bio-Rad Laboratories).

Data are presented in terms of fold change to the control after adjustment by protein.

**Statistical analysis.** In the islet study, we performed three independent experiments. For each experiment we calculated and applied the mean value from 12–13 islets for each condition, taken from a pool of isolated islets derived from pancreata of 6–10 mice;  $n = 3$  represented the number of experiments. We performed statistical analysis using unpaired two-tailed Student's *t*-test with unequal variances. In gene expression and Western blotting analysis, we also compared variations using one-way ANOVA with Bonferroni's correction. We applied the software INTERCOOLED STATA 8.2 (StataCorp, College Station, TX) to perform statistical analysis. Each treatment condition was compared with controls. We considered differences between two groups significant at  $P$  values < 0.05. Data are expressed as means ± SE.

## RESULTS

**Effect of SVS on insulin secretion in mouse islets and INS-1E cells in the presence or absence of OKA, AICAR, or TOFA.** In the presence of 3.3 mM glucose, BIS was elevated after pretreatment of the islets with 27 mM glucose for 48 h ( $n = 3$ ,  $P = 0.000$ ; Fig. 1A). We found a decrease in GSIS to 16.7 mM glucose after 48-h pretreatment of the islets at 27 mM glucose compared with 48-h pretreatment at 11.1 mM glucose ( $n = 3$ ,  $P = 0.025$ ). SVS ( $10^{-6}$  M) did not alter GSIS from mouse islets pretreated with 11.1 mM glucose for 48 h ( $n = 3$ ,  $P = 0.248$ ). In contrast, SVS increased GSIS from mouse islets pretreated for 48 h with 27 mM glucose ( $n = 3$ ,  $P = 0.007$ ; Fig. 1A). When OKA ( $10^{-8}$  M) and SVS were added to the medium containing 27 mM glucose, the subsequent GSIS was totally blocked ( $n = 3$ ,  $P = 0.000$ ). Similar results of GSIS were obtained after addition of AICAR ( $10^{-4}$  M;  $n = 3$ ,  $P = 0.002$ ).

To establish glucotoxicity, a lower glucose concentration (16.7 mM) was applied in INS-1E cells than in isolated islets (27 mM) (Fig. 1B). Insulin secretion was expressed as fold change in BIS (pretreated for 48 h with 11.1 mM glucose). Comparable results were obtained in INS-1E cells as in isolated mouse islets (Fig. 1B and Fig. 2A). Additionally, glucotoxicity decreased the insulin secretory capacity to 30 mM KCl. Incubation for 1 h with SVS enhanced the GSIS. This was demonstrated at 11.1 mM glucose ( $n = 12$ ,  $P = 0.007$ ), 16.7 mM glucose ( $n = 12$ ,  $P = 0.001$ ), and 16.7 mM glucose supplemented with SVS ( $n = 12$ ,  $P = 0.018$ ). The effects of SVS on GSIS were blocked by TOFA irrespective of the presence of SVS during the 48-h pretreatment ( $n = 12$ ,  $P = 0.076$  and  $n = 12$ ,  $P = 0.056$ , respectively). However, the insulin output was decreased after pretreatment with high glucose (16.7 mM,  $n = 24$ ,  $P = 0.000$ ), whereas SVS ( $10^{-6}$  M) partly reversed the decreased insulin output ( $n = 24$ ,  $P = 0.037$ ; Fig. 2B). High-glucose pretreatment also reduced the insulin content ( $n = 18$ ,  $P = 0.034$ ), which was further reduced by SVS ( $10^{-6}$  M,  $n = 18$ ,  $P = 0.002$ ; Fig. 2C).

**Effect of SVS on ACC, CPT I, INS2, and GLUT2 gene expression.** SVS ( $10^{-6}$  M) did not influence ACC gene expression in INS-1E cells incubated for 48 h in 11.1 mM glucose ( $1.1 ± 0.46$  vs.  $1.1 ± 0.19$ ,  $n = 6$ ,  $P = 1.000$ ; Fig. 3). High glucose (16.7 mM) significantly increased ACC expression ( $1.1 ± 0.46$  vs.  $1.9 ± 0.54$ ,  $n = 6$ ,  $P = 0.020$ ). Interestingly, SVS ( $10^{-6}$  M) markedly increased ACC gene expression in the presence of 16.7 mM glucose ( $1.9 ± 0.54$  vs.  $3.2 ± 1.10$ ,  $n = 6$ ,  $P = 0.026$ ). In contrast, CPT I gene expression was signifi-



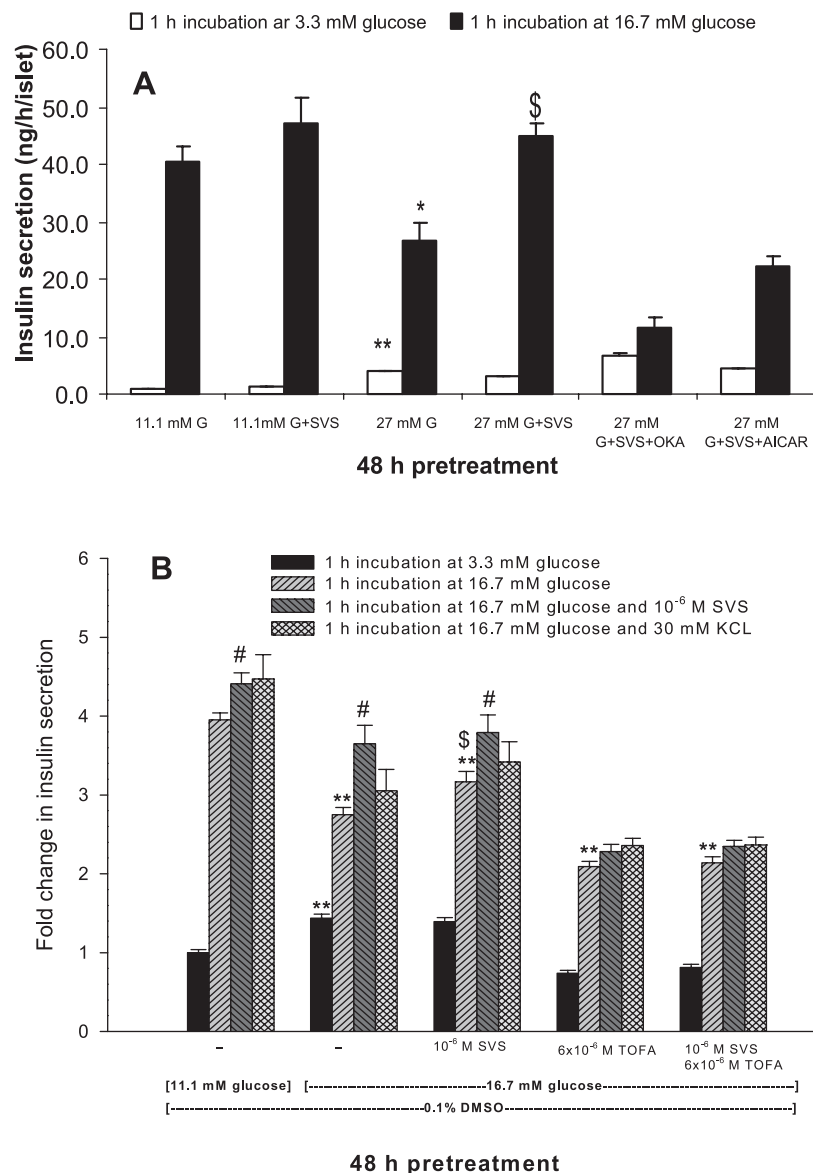


Fig. 1. Antiglucotoxicity effect of stevioside (SVS) was blocked by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), okadaic acid (OKA), and 5-tetradecyloxy-2-furancarboxylic acid (TOFA). A: after 48-h pretreatment of mouse islets with 11.1 or 27 mM glucose supplemented with or without 10<sup>-6</sup> M SVS and 10<sup>-8</sup> M OKA or 10<sup>-4</sup> M AICAR, we incubated single mouse islets at 3.3 or 16.7 mM glucose for 1 h. B: INS-1E cells were pretreated for 48 h with 11.1 mM glucose, 16.7 mM glucose supplemented with or without 10<sup>-6</sup> M SVS, 6 × 10<sup>-6</sup> M TOFA, or both. Subsequently cells were incubated at 3.3 mM glucose, 16.7 mM glucose supplemented with or without 10<sup>-6</sup> M SVS, or 30 mM KCl for 1 h. We collected aliquots for insulin assay. Bars represent means ± SE. We performed 3 independent experiments. We performed 12 experiments with INS-1E cells from 2 separate experiments (days) with insulin level expressed as fold change of basal insulin stimulation (BIS) of the control. Unpaired Student's *t*-test was used for statistical analysis. \**P* < 0.05 and \*\**P* < 0.001 denote significant difference from the corresponding control of 48-h pretreatment with 11.1 mM glucose. #Significant difference from corresponding control without SVS from the same pretreatment group (*P* < 0.05); \$significant difference from the corresponding control in the group of high glucose pretreatment alone (*P* < 0.001).

cantly reduced in cells incubated at high glucose with or without 10<sup>-6</sup> M SVS (1.0 ± 0.21 vs. 0.45 ± 0.20, *n* = 6, *P* = 0.001 or 1.0 ± 0.21 vs. 0.37 ± 0.21, *n* = 6, *P* = 0.000, respectively). SVS (10<sup>-6</sup> M) alone did not change the CPT I gene expression. High glucose (16.7 mM) reduced INS2 gene expression (1.0 ± 0.07 vs. 0.55 ± 0.08, *n* = 3, *P* = 0.002), which was not counteracted by SVS (10<sup>-6</sup> M, 0.55 ± 0.08 vs. 0.54 ± 0.06, *n* = 3, *P* = 0.750). GLUT2 gene expression was not changed during glucotoxicity with or without SVS.

**Effect of SVS on protein levels of ACC and pACC.** Compared with 11.1 mM glucose, 16.7 mM glucose significantly increased the protein level of ACC in the presence and absence of 10<sup>-6</sup> M SVS (1.0 ± 0.25 vs. 2.2 ± 0.30, *n* = 3, *P* = 0.020 with SVS or 1.0 ± 0.25 vs. 2.4 ± 0.05, *n* = 3, *P* = 0.003 without SVS, respectively; Fig. 4A). Glucose at 16.7 mM significantly enhanced the protein levels of pACC (1.0 ± 0.25 vs. 2.12 ± 0.28, *n* = 3, *P* = 0.041; Fig. 4B) and the protein ratio of ACC-pACC to pACC (1.00 ± 0.27 vs. 1.89 ± 0.19,

*n* = 3, *P* = 0.029; Fig. 4C). SVS did not change pACC (2.12 ± 0.28 vs. 1.82 ± 0.38, *n* = 3, *P* = 0.510; Fig. 4B) or the (ACC-pACC)/pACC protein ratio (1.89 ± 0.19 vs. 1.25 ± 0.27, *n* = 3, *P* = 0.077; Fig. 4C) in the presence of 16.7 mM glucose.

**Effect of SVS on ACC activity.** At 11.1 mM glucose, 10<sup>-6</sup> M SVS did not change the ACC activity (1.0 ± 0.08 vs. 1.24 ± 0.26, *n* = 6, *P* = 0.097). High glucose (16.7 mM) significantly increased ACC activity (1.0 ± 0.08 vs. 1.97 ± 0.15, *n* = 6, *P* = 0.002). In the presence of high glucose (16.7 mM glucose), SVS (10<sup>-6</sup> M) further increased the ACC activity (1.97 ± 0.15 vs. 3.03 ± 0.14, *n* = 6, *P* = 0.000; Fig. 5).

**TG content.** As illustrated in Fig. 6, we found that SVS (10<sup>-6</sup> M) at high glucose (16.7 mM) increased the TG content in β-cells (*n* = 16, *P* = 0.020).

**Glucose uptake.** As seen in Fig. 7, high glucose did not change glucose uptake (1.0 ± 0.10 vs. 1.1 ± 0.08, *n* = 12, *P* = 0.690). SVS had no impact on glucose uptake when glucose was 11.1 mM (1.0 ± 0.10 vs. 1.0 ± 0.09, *n* = 12, *P* = 0.823),

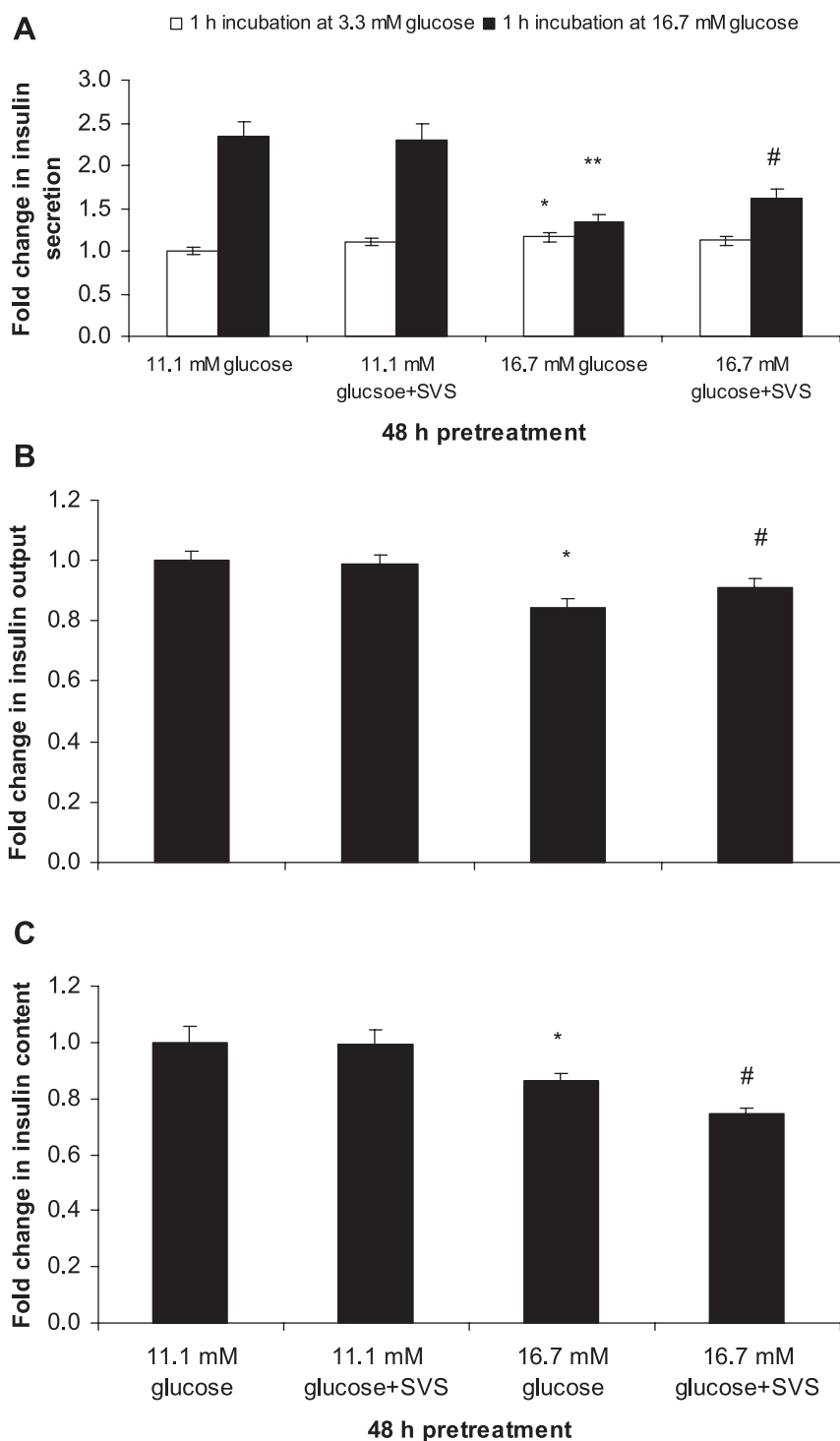


Fig. 2. Antiglucotoxicity effects of SVS in INS-1E cell line. **A:** after 48-h pretreatment with 11.1 or 16.7 mM glucose supplemented with or without  $10^{-6}$  M SVS, INS-1E cells were incubated at 3.3 or 16.7 mM glucose for 1 h. Subsequently, we collected aliquots for insulin secretion assay. **B:** after pretreatment, parts of cells underwent insulin output and insulin content determination. Pretreatment medium was taken to measure insulin output. **C:** subsequently, cells were sonicated to release insulin for measurement of insulin content. Bars represent means  $\pm$  SE. We performed 24 experiments with INS-1E cells, measuring insulin secretion and output and 18 experiments measuring insulin content obtained from 3 separate experiments (days). Unpaired Student's *t*-test was used for statistical analysis. Data on insulin secretion are expressed relative to BIS of control, whereas data in insulin content and output are expressed relative to that of control. \*\* $P < 0.001$  and \* $P < 0.05$  denote significant difference from that of 11.1 mM glucose. # $P < 0.05$  denotes significant difference from that of 16.7 mM glucose.

whereas it enhanced glucose uptake when glucose was 16.7 mM ( $1.1 \pm 0.09$  vs.  $1.6 \pm 0.12$ ,  $n = 12$ ,  $P = 0.002$ ).

#### DISCUSSION

In this study, we have demonstrated that pretreatment with high glucose for 48 h enhanced BIS and decreased GSIS from both isolated mouse islets and pancreatic  $\beta$ -cells. Glucotoxicity increased the ACC gene expression, depressed CPT I expression, and augmented the protein content of ACC and pACC as

well as ACC activity. SVS partly counteracted the impaired GSIS but not the increased BIS. As illustrated in Fig. 8, SVS increased ACC gene expression, ACC activity, TG content, and glucose uptake concomitantly with SVS-induced improvement of GSIS.

Glucose is known to directly stimulate insulin release as well as modulate insulin response to many nonglucose secretagogues. However, persistent high glucose can cause glucotoxicity (5, 30, 46) and as such is a target for primary prevention.

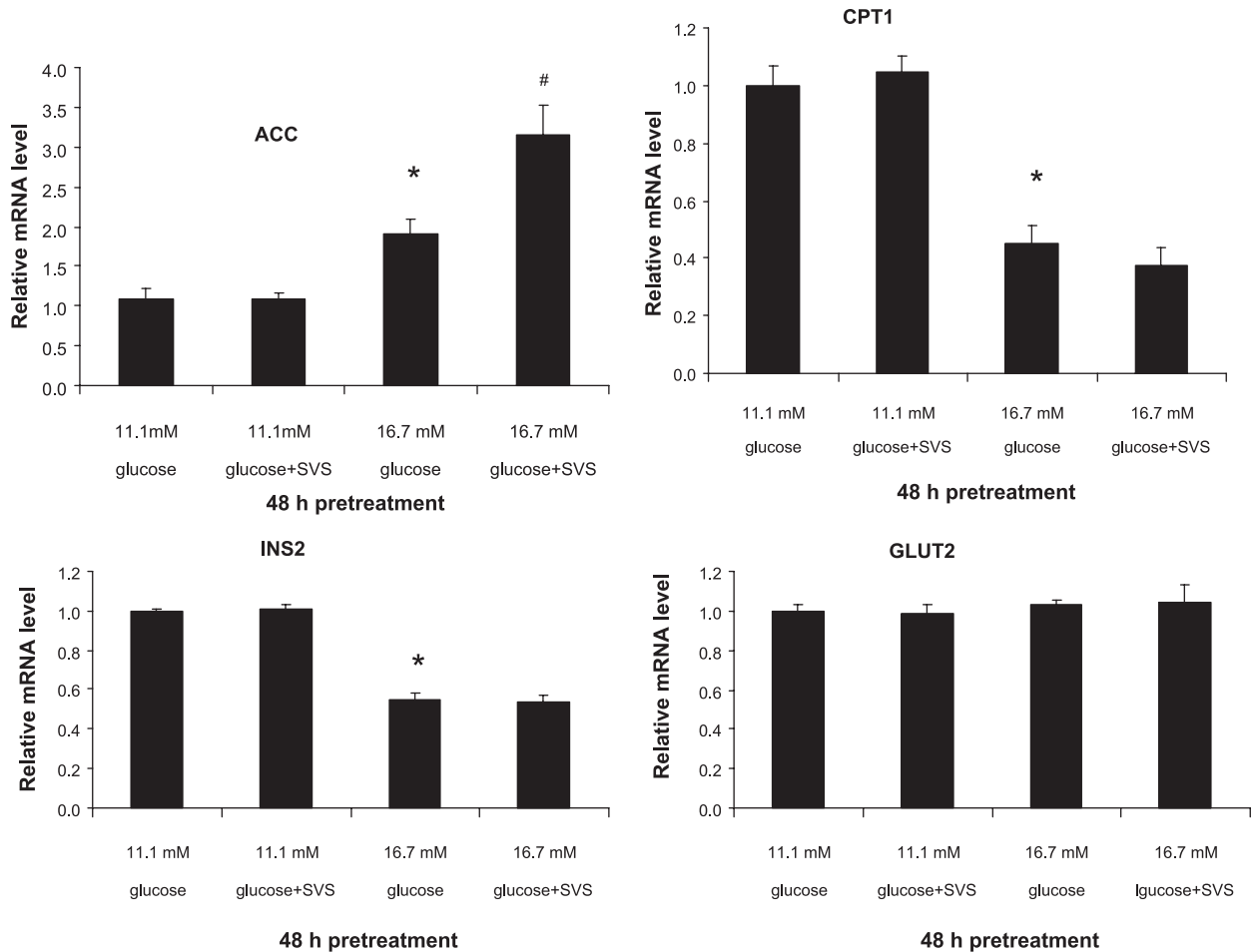


Fig. 3. Effects of SVS on acetyl-CoA carboxylase (ACC), carnitine palmitoyltransferase I (CPT I), INS2, and GLUT2 gene expressions, which were measured by RT-PCR in INS-1E cells pretreated with 11.1 mM glucose or 16.7 mM glucose supplemented with or without  $10^{-6}$  M SVS. Bars represent means  $\pm$  SE from 3–6 experiments. Statistical analysis was carried out with 1-way ANOVA with Bonferroni's correction. All data are expressed relative to control. \*Significant difference from 11.1 mM glucose ( $P < 0.05$ ); #significant difference from the corresponding one without SVS ( $P < 0.05$ ).

The Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) have shown that tight blood glucose control is beneficial in both type 1 diabetes mellitus (T1DM) and T2DM (16a, 47a, 47b). A substantial amount of data has been generated in animal models to support the concept that chronic hyperglycemia causes the loss of glucose recognition, the so-called glucotoxicity hypothesis (29).

Attempts to identify specific defects have been complicated by the lack of suitable in vitro models. Isolated islets from hyperglycemic rodent models have been a major disappointment because they failed to exhibit the same profound lack of glucose-induced insulin secretion that is found in vivo. Another limitation with islet studies is that  $\sim 20\%$  of cells in normal islets are non- $\beta$ -cells, a fact that complicates the localization of a  $\beta$ -cell defect. However, an alternative approach with prolonged culture of normal islets at high glucose concentrations has been found to cause profound glucose unresponsiveness (17, 52). Similar findings have been obtained in the clonal  $\beta$ -cell line INS-1 (5).

We used intact mouse islets to test insulin secretion after 48-h incubation at 11.1 or 27 mM glucose, according to preliminary results in a pilot study showing that glucotoxicity

took place after 48 h. We chose a glucose concentration of 11.1 mM as control because the ability of insulin secretion was best kept at this concentration. As expected, islets treated for 48 h with high glucose experienced elevated BIS and decreased GSIS, which could be partly counteracted by SVS. Incubation time and the glucose concentration played important roles for the effect of SVS. A 24-h pretreatment with SVS at 11.1 mM glucose increases GSIS in isolated islets (11), whereas 48-h pretreatment does not. As reported previously, SVS has no effect on BIS (11).

We compared the results in islets with those obtained in INS-1E cells. The INS-1E cell line derives from cells isolated from an X-ray-induced transplantable rat insulinoma (2). It has morphological characteristics typical for native  $\beta$ -cells that remained unchanged over a period of 2 yr. The cells express insulin 1 and INS2 as well as GLUT2, similar to rat  $\beta$ -cells (48). Since we have experienced higher glucose sensitivity in INS-1E cells compared with islets, we chose the lower glucose concentration of 16.7 mM to induce glucotoxicity. At 16.7 mM glucose, we demonstrated that SVS counteracted the glucotoxicity. This implies that the antiglucotoxic effect of SVS seems to be mediated via a direct effect on the pancreatic  $\beta$ -cells. In the experiment that

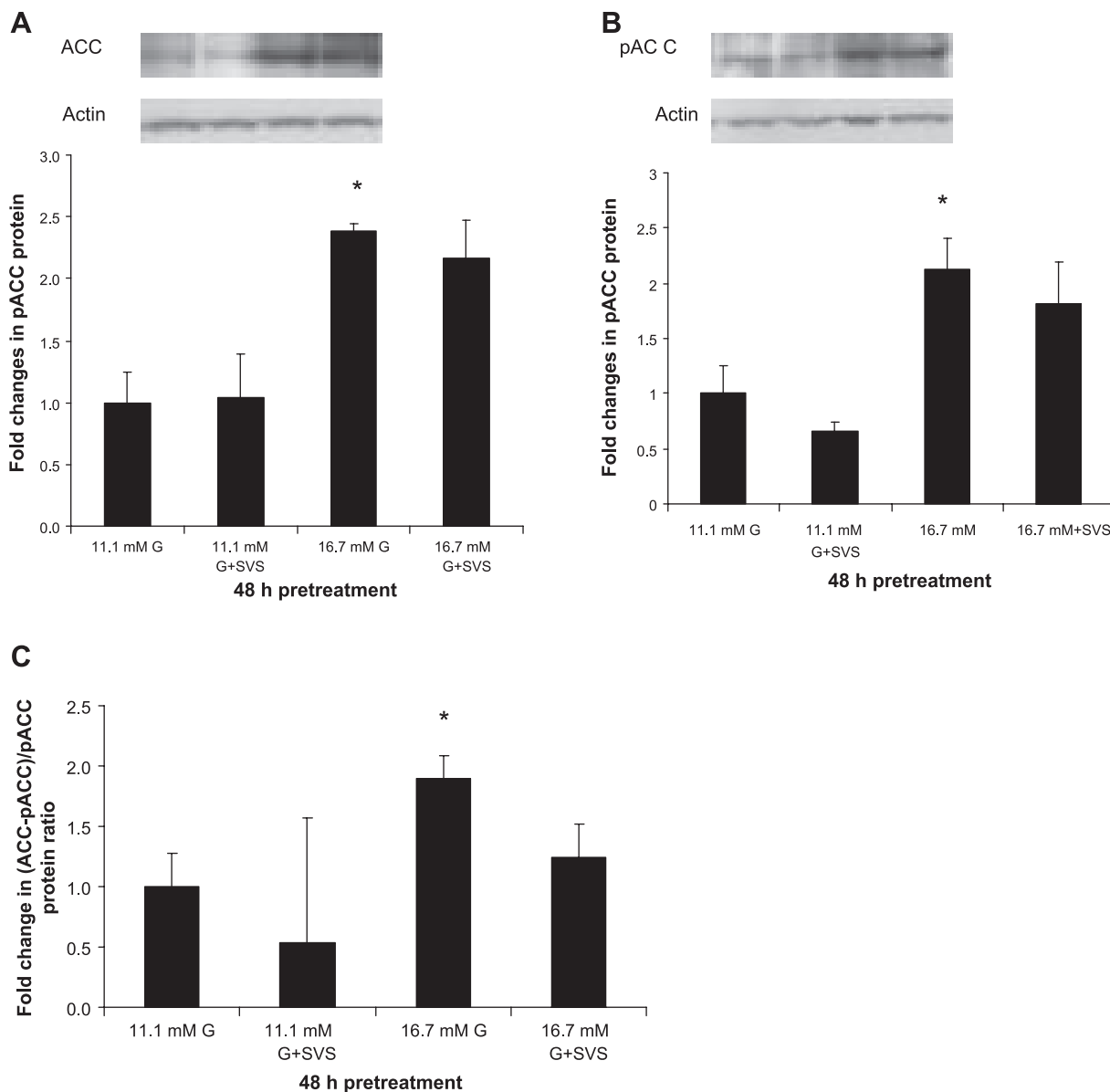


Fig. 4. Effects of SVS on ACC and phosphorylated (p)ACC protein levels. ACC (A) and pACC (B) proteins were measured by Western blotting in INS-1E cells pretreated with 11.1 mM glucose or 16.7 mM glucose supplemented with or without  $10^{-6}$  M SVS. C: fold change in (ACC-pACC)/pACC protein ratio. Bars represent means  $\pm$  SE from 3 experiments. Statistical analysis was carried out with 1-way ANOVA with Bonferroni's correction. All data are expressed relative to control. \*Significant difference from 11.1 mM glucose ( $P < 0.05$ ).

included TOFA, we found a relatively greater fold increase in GSIS, which might have been due to the different passage number of the INS-1E cells.

The increased BIS in glucotoxicity (25) is considered to be the result of enhanced activity of hexokinase, as a threefold increase in the rate of glucose usage has been observed (33). SVS did not change this elevated BIS, indicating that SVS did not inhibit the activity of hexokinase. Concerning the decreased GSIS, several hypotheses that are being investigated involve glucose transport, storage, metabolism, oxidation, and the second messengers (29). Recently, it was found that ACC functions as the sensor in pancreatic  $\beta$ -cells and responds to glucose and other nutrients for insulin secretion (16, 41, 45, 51). ACC exists in different isoforms, of which ACC1 (also known as ACC $\alpha$ ) is a cytosolic enzyme catalyzing acetyl-CoA

to form malonyl-CoA, whereas ACC2 (also known as ACC $\beta$ ) regulates CPT I for LC-CoA translocation from cytosol to mitochondria. Pancreatic  $\beta$ -cells contain primarily ACC1 and a barely detectable amount of ACC2 (50). ACC is regulated through a complex array of control mechanisms: regulation of both transcription and translation; regulation by covalent modification; and allosteric regulation of the enzyme by cellular metabolites (26).

Incubation for 48 h at high glucose increased ACC gene expression and total content of ACC as well as pACC in INS-1E cells, which was accompanied by increased ACC activity. During glucotoxicity, SVS increased ACC gene expression, activity, TG content, and glucose uptake in the cells followed by improved GSIS. These findings fit well with Randle's cycle (43). However, Brun et al. (10) found that

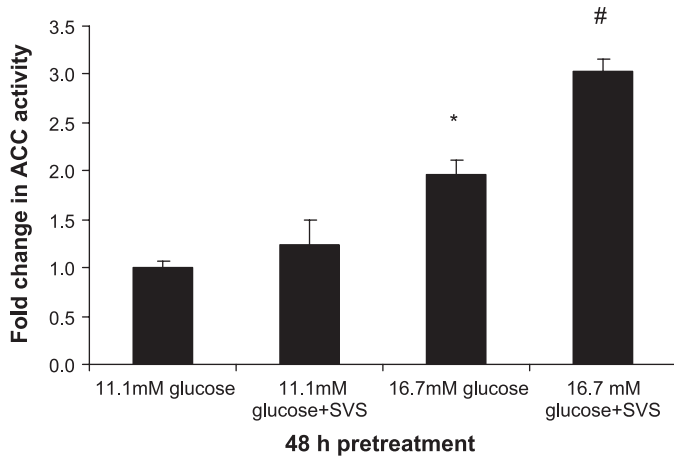


Fig. 5. Regulation of ACC activity with SVS. ACC activity was determined using the radioactive CO<sub>2</sub> fixation method in INS-1E cells pretreated with 11.1 mM glucose or 16.7 mM glucose supplemented with or without 10<sup>-6</sup> M SVS. Bars represent means ± SE from 6 experiments. Unpaired Student's *t*-test with unequal variances was applied for statistical analysis. All data are expressed relative to control. \*Significant difference from 11.1 mM glucose (*P* < 0.001); #significant difference from the corresponding one without SVS (*P* < 0.001).

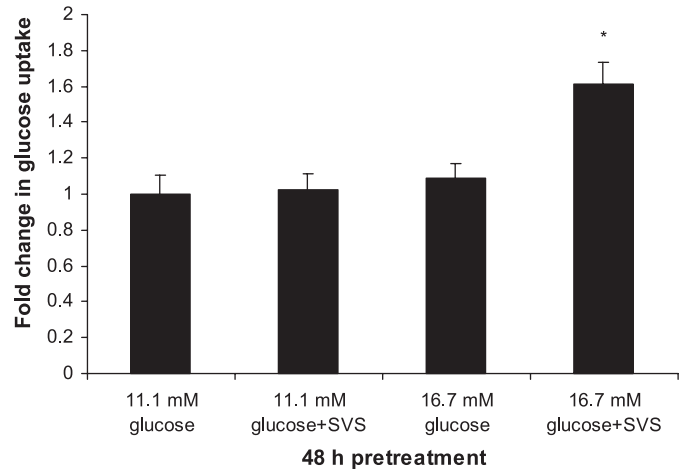


Fig. 7. Effect of SVS on glucose uptake. Glucose uptake was measured in INS-1E cells pretreated with 11.1 mM glucose or 16.7 mM glucose supplemented with or without 10<sup>-6</sup> M SVS. Bars represent means ± SE from 12 experiments. Unpaired Student's *t*-test with unequal variances was applied for statistical analysis. All data are expressed relative to control. \*Significant difference from 16.7 mM glucose (*P* < 0.05).

glucose increased ACC mRNA, ACC protein, and insulin secretion in INS-1 cells preincubated for 24 h. In the present study, we demonstrated that 48-h preincubation of INS-1E cell at high glucose increased ACC mRNA as well as ACC and pACC and ACC activity but decreased GSIS. Interestingly, acutely increased ACC activity enhances malonyl-CoA and LC-CoA that may increase insulin exocytosis directly or indirectly via the increase of diacylglycerol (DAG) (31, 32, 35, 40). However, chronically elevated LC-CoA caused decreased GSIS (40). Our observations corroborate this theory. Those authors proposed a “dual” action of LC-CoA to explain the different effects of LC-CoA between the acute and the chronic conditions (40). Another explanation could be that there is a “set point” of LC-CoA for GSIS that can be changed and switch the impact on insulin secretion. Accordingly, GSIS may be determined by the set point and the LC-CoA concentration. In the acute situation, elevated ACC activity and LC-CoA

concentration overwhelm the set point for GSIS, leading to increased GSIS. In the chronic situation, the set point for GSIS was changed eliciting decreased GSIS. In the chronic situation, SVS increased further LC-CoA concentration and enhanced GSIS. Unfortunately, we did not have the technology to measure the cytosolic LC-CoA. However, Oakes et al. (36) previously showed that the total LC-CoA concentration tends to parallel that of TG. Further experiments are needed to test this hypothesis.

Phosphorylation and dephosphorylation of ACC cause the enzyme's inactivation and activation, respectively, and serve as the enzyme's short-term regulatory mechanism (26). ACC

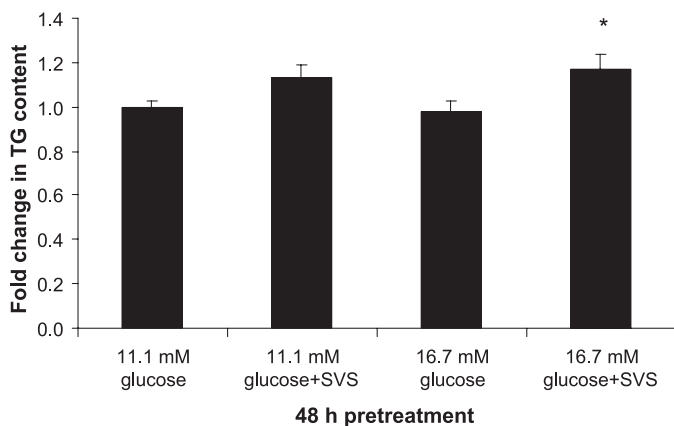


Fig. 6. Impact of SVS on triglyceride (TG) content. TG content was determined in INS-1E cells pretreated with 11.1 mM glucose or 16.7 mM glucose supplemented with or without 10<sup>-6</sup> M SVS. Bars represent means ± SE from 16 experiments. Unpaired Student's *t*-test with unequal variances was applied for statistical analysis. All data are expressed relative to control. \*Significant difference from 11.1 mM glucose (*P* < 0.05).

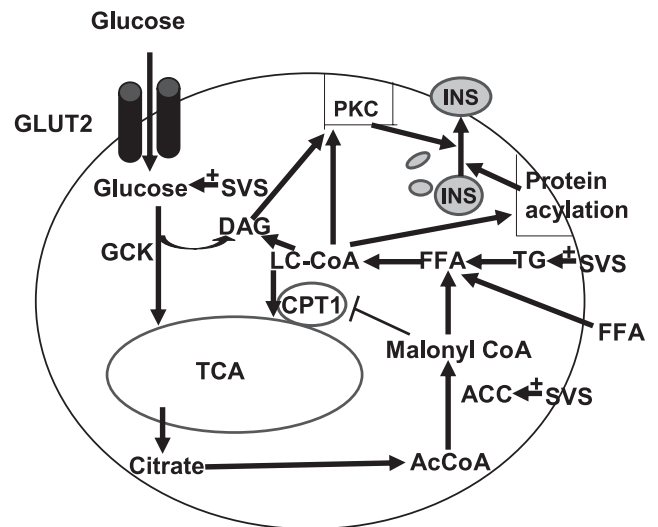


Fig. 8. Schema illustrating the mechanism of SVS enhancing insulin secretion from pancreatic β-cells. Our schema is based on the model of Prentki et al. (42) and shows that SVS increases glucose uptake, ACC gene expression and activity, and TG content. By this, SVS may increase long-chain acyl-CoA (LC-CoA) that may provide substrate for protein acylation or may modulate PKC enzyme activity with resulting insulin release. GSK3, glucokinase; TCA, tricarboxylic acid cycle; AcCoA, acetyl-CoA; FFA, free fatty acids; DAG, diacylglycerol; INS, insulin granulae; +, enhancement; -, inhibition.



colocalizes with CPT I at the "contact sites" of the mitochondrial membranes and regulates mitochondrial fatty acid oxidation as well as inhibiting CPT I by its product malonyl-CoA. ACC antibody detects endogenous level of ACC protein. These protein changes represent long-term regulation. pACC (Ser<sup>79</sup>) antibody detects endogenous levels of ACC only when phosphorylated at Ser<sup>79</sup>. Glucotoxicity increased the protein ratio of (ACC-pACC)/pACC, whereas SVS did not change this ratio, according to the Western blotting experiments.

The ACC activity can be turned off by phosphorylation via AMP-activated protein kinase and turned on by dephosphorylation via protein phosphatase A2. OKA is a powerful inhibitor of phosphatase A2. Mutational analysis showed that Ser<sup>79</sup> was critical for inactivation of ACC by 5'-AMP-dependent protein kinase, which can be activated by AICAR (26). Interestingly, the counteractive effect of SVS to glucotoxicity was totally blocked by OKA as well as by AICAR, which gave further evidence that counteractive effects played an important role via the active form of ACC. Since AICAR and OKA may not be that specific as ACC inhibitors, we included experiments with the ACC specific inhibitor TOFA (53). The results with TOFA demonstrated a similar influence on GSIS as AICAR and OKA. Additionally, we demonstrated that 1-h incubation with SVS induced a clear-cut increase in GSIS, which was blocked by TOFA. The 30 mM KCl-stimulated insulin secretion was also decreased during glucotoxicity. Tsuboi et al. (47) showed that 50 mM KCl-stimulated insulin secretion was not affected by glucotoxicity (30 mM glucose) after 2 days of incubation of dispersed rat islet cell. The different cell type and different dose of KCl may explain this difference.

Increased ACC gene expression and protein level are not always accompanied by increased ACC activity. Therefore, we also determined the ACC activity. High glucose increased ACC activity, which was further increased by SVS pretreatment. SVS pretreatment also increased the TG content. The impact of SVS was glucose dependent, i.e., at 16.7 mM glucose but not at 11.1 mM glucose. This corroborates our previous demonstration of glucose dependency of SVS on GSIS where SVS did not alter insulin release at 11.1 mM but did so at 16.7 mM (23). The mechanism of the glucose dependency is not known. Interestingly, Brachet et al. (3) reported that SVS inhibited the fluxes of palmitate across the cell membrane in the intact rat liver. Furthermore, SVS also suppressed both ketogenesis and <sup>14</sup>CO<sub>2</sub> production from [<sup>14</sup>C]palmitate in perfused rat liver (14). Our data are consistent with these results.

Previously, we reported that the INS2 gene expression decreased in INS-1 cells after 4-day incubation in 16.7 mM glucose (5). In the present study, we observed similar changes; however, SVS could not counteract the suppressed INS2 gene expression during glucotoxicity. GLUT2 gene expression has been reported to decrease in many animal models, e.g., the diabetic Zucker *fafa* rat, the GK rat, and the BB rat (37–39). However, exposure of pancreatic islets to high glucose in vitro leads to an increase in GLUT2 mRNA and protein levels (20, 48, 49). Our data did not reveal any change in GLUT2 during glucotoxicity with or without SVS. This implies that GLUT2 gene expression does not play an important role, at least in this model, of glucotoxicity in INS-1 cells. Taken together, during glucotoxicity, insulin secretion, insulin content, and INS2 gene expression decreased. SVS increased insulin secretion but not

insulin content nor INS2 gene expression, implying that the effect of SVS may take place via the enhancement of insulin exocytosis rather than by increasing insulin synthesis.

Studies trying to disentangle the mechanism of action of the insulinotropic effect of SVS have revealed that 1) SVS does not close ATP-sensitive potassium channels or affect the cAMP system in the  $\beta$ -cells at normal glucose levels (23); 2) SVS increases ACC gene expression as well as ACC activity; 3) SVS increases TG content; 4) OKA, AICAR, and TOFA block the effects of SVS; and 5) SVS inhibits palmitate oxidation (14). We (12) previously proposed that SVS probably increases insulin secretion via activation of phospholipases and PKC. According to Bratanova-Tochkova et al. (4), there are no cAMP changes involved in this signal transduction. This pathway increases phosphoinositide turnover, resulting in mobilization of stored calcium to increase intracellular Ca<sup>2+</sup> and increase production of DAG, which activates PKC isoforms. The present study shows that SVS increases ACC activity and inhibits CPT I, which may lead to inhibition of fatty acid oxidation, increased de novo lipid synthesis, an expansion of the LC-CoA pool (31), and a rise in DAG content (16). The finding of raised TG content in  $\beta$ -cells also supports this view, since the LC-CoA concentration tends to parallel that of TG (36). This implies that SVS stimulates insulin secretion via the regulation of ACC activity.

In conclusion, glucose is a strong stimulator of ACC, but during glucotoxicity the upregulated set point of LC-CoA reduces GSIS, which is enhanced by SVS via an increased ACC activity to overwhelm the raised set point. SVS has the potential to counteract the negative effects of glucotoxicity in the  $\beta$ -cells and may serve as a new antihyperglycemic agent in T2DM with unique mechanisms of action.

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