Stevioside Counteracts Beta-Cell Lipotoxicity without Affecting Acetyl CoA Carboxylase

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■ Abstract

Chronic exposure to high levels of free fatty acids impairs beta-cell function (lipotoxicity). Then basal insulin secretion (BIS) is increased and glucose-stimulated insulin secretion (GSIS) is inhibited. Acetyl CoA carboxylase (ACC) acts as the sensor for insulin secretion in pancreatic beta-cells in response to glucose and other nutrients. Stevioside (SVS), a diterpene glycoside, has recently been shown to prevent glucotoxic effect by regulating ACC activity. The aim of this study was to investigate whether SVS can alleviate impaired beta-cell function by regulating ACC activity. We exposed isolated rat islets and the clonal beta-cell line, INS-1E, to palmitate concentrations of 1.0 or 0.6 mM, respectively, for a period of 24 h to 120 h. The results showed that lipotoxicity occurred in rat islets after 72 h exposure to 1.0 mM

palmitate. The lipotoxicity was counteracted by 10^{-6} M SVS ($n = 8$, $p < 0.001$). Similar results were obtained in INS-1E cells. Neither SVS nor palmitate had any effect on the gene expression of ACC, insulin 2, and glucose transporter 2 in INS-1E cells. In contrast, palmitate significantly increased the gene expression of carnitine palmitoyl transporter 1 ($n = 6$, $p = 0.003$). However, the addition of SVS to palmitate did not counteract this effect ($n = 6$, $p = 1.0$). During lipotoxicity, SVS did not alter levels of ACC protein, phosphorylated-ACC, ACC activity or glucose uptake. Our results showed that SVS counteracts the impaired insulin secretion during lipotoxicity in rat islets as well as in INS-1E cells without affecting ACC activity.

Keywords: rat islets · INS-1E · CPT1 · insulin secretion · type 2 diabetes mellitus

Introduction

Type 2 diabetes mellitus (T2DM) is associated with abnormal beta-cell function and insulin resistance with hyperinsulinemia, atherogenic dyslipidemia, hypertension, abdominal obesity, and impaired homeostasis [1, 2]. Fatty acids are currently the focus of much research in relation to the etiology and treatment of T2DM.

Free fatty acids (FFAs) are now recognized as potent players in signal transduction, vesicular trafficking and insulin secretion. Acetyl CoA carboxylase (ACC) is currently of great interest in relation to the effect of FFAs and insulin secretion [3-5]. It has been found

that ACC functions as the insulin secreting sensor in pancreatic beta-cells in response to glucose and other nutrients [6-9]. According to this theory, cytosolic acetyl CoA is converted to malonyl-CoA by ACC. An abrupt increase in malonyl-CoA concentration during glucose metabolism results in the inhibition of carnitine palmitoyl transporter 1 (CPT1) as well as the transport and oxidation of long chain-CoA (LC-CoA) in mitochondria [6, 10, 11]. This causes elevation of cytosolic LC-CoA and leads to insulin secretion and modulation of other beta-cell functions [7, 12]. Additionally, Itoh *et al.* [13] reported that a G-protein-coupled receptor, GPR40, which is abundantly expressed in the pancreas, functions as a receptor for

long chain FFAs. However, chronic exposure to high levels of FFAs causes derangement of the beta-cell function i.e. increases basal insulin secretion (BIS) and inhibits glucose-stimulated insulin secretion (GSIS) *in vivo* and *in vitro* [2, 3]. Progressive lipotoxicity and peripheral insulin resistance may play key roles in etiology by causing detrimental effects on gene expression and beta-cell function [14-17]. Previously, we have shown that lipotoxicity has a profound impact on the gene expression in beta-cells [18]. Thus chronically elevated palmitate upregulates the genes involved in fatty acid oxidation (LC-CoA dehydrogenase, CPT-1 and Carnitine/acyl-carnitine carrier) and downregulates the genes involved in both lipogenesis (ACC, fatty acid synthetase (FAS)) as well as in glycolysis (L-type pyruvate kinase (LPK), hepatocyte nuclear factor (HNF-4) and phosphofructokinase (PFK)) [18]. Thus, lipotoxicity affects insulin secretion to some degree through the ACC-malonyl-CoA-CPT1-LC-CoA partnership.

Stevioside (SVS), a diterpene glycoside, possesses insulinotropic, glucagonostatic, antihyperglycemic and blood pressure-lowering effects [19, 20]. *In vitro* we have demonstrated a direct insulinotropic effect of SVS in clonal INS-1 cells and in islets from normal mice and GK diabetic rats [19, 20]. SVS does not, like sulfonylureas, close the ATP-sensitive potassium channels in the beta-cells at low glucose levels [19]. Interestingly, it increases the expression of proinsulin gene at both mRNA and protein levels [21]. Exposure for 24 h to SVS can enhance GSIS in mouse islets even when SVS is not present during the subsequent 1 h test period [22, 23]. SVS improves pancreatic beta-cell function during glucotoxicity by regulating ACC activity (unpublished data). ACC links fatty acid and carbohydrate metabolism through the shared intermediate acetyl CoA. SVS apparently affects fatty acid metabolism by suppressing the genes involved in fatty acid oxidation and by inducing changes in those involved in fatty acid synthesis and glycolysis in INS-1 cells [18]. We hypothesize that SVS may also be able to counteract lipotoxicity in insulin secreting cells. The aim of the present study was to test the potential preventive effects of SVS on lipotoxicity and, furthermore, to investigate a possible mechanism of action using isolated rat islets and the clonal beta-cells, INS-1E.

Materials and methods

Experimental animals

Male Wistar rats (Bomholtgaard Breeding and Research Center, Ry, Denmark), aged 15 weeks and weighing 250 to 350g, were used. The animals were

kept on a standard pellet diet with tap water ad libitum and a light/dark cycle of L12:D12.

This study was carried out in accordance with the guidelines of the Danish Council for Animal Experiments.

Agent preparation

SVS (Wako Pure Chemical Industries, Osaka, Japan) was added to the medium from a stock solution (10^{-3} M) prepared in distilled water. Palmitate (Sigma, St. Louis, Mo) was prepared by dissolving and heating equal molar amounts of NaOH and fatty acids, supplemented with distilled water, to obtain a 500 mM concentration. It was further diluted with 5% BSA (fatty acid free, Sigma) to 50 mM fatty acid. The stock solution was filter sterilized and stored at -20°C . Each fatty acid solution was freshly prepared before each experiment.

Islet isolation

Islets were isolated by the collagenase digestion technique [24, 25]. The islets were then incubated overnight at 37°C and 95% normal atmosphere to 5% CO_2 in 10 ml 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 $\mu\text{g}/\text{ml}$ streptomycin (all from GIBCO BRL, Paisley, UK). Islets for the incubation studies were obtained from 3 to 5 rats to compensate for inter-individual differences.

Islet incubation and insulin secretion studies

This part of the study aimed to determine whether palmitate can cause lipotoxicity and SVS potentially prevent it. After overnight incubation, the rat islets were pre-incubated for 24 h to 120 h in the above mentioned RPMI-1640, although 5.5 mM glucose was used instead of 11.1 mM glucose and 0.5% BSA (fatty acid free, Sigma) was added. The RPMI was supplemented with either 1.0 mM palmitate or 10^{-6} M SVS or both. After pre-incubation, the islets were rinsed once with modified Krebs-Ringer buffer (KRB) [23] and then supplemented with 3.3 mM glucose and 0.1% BSA (fatty acid free, Sigma). After 15 min of pre-incubation in normal atmosphere at 37°C , single islets were hand-picked and incubated in 100 μl KRB at glucose concentrations of 3.3 or 16.7 mM. After 1 h incubation in a normal atmosphere at 37°C , 50 μl of the medium was 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 rat islets could be reproduced in the INS-1E cell line. The day before the experiment, the INS-1E cells were placed in 24-well plates (NUNC Brand Products, NUNC A/S, Roskilde, Denmark) at 0.2×10^6 cell/well with PRMI. Then the cells were cultured in RPMI with 11.1 mM glucose and 0.5% BAS (fatty acid free, Sigma) in the presence or absence of either 0.6 mM palmitate, 10^{-6} M SVS, or both for 72 h in a humidified atmosphere (5% CO₂, 95% air at 37°C). These culture conditions were used in all subsequent studies with the INS-1E cell line except when specifically mentioned. The cells were subsequently divided into three groups for use in insulin secretion, insulin output, and insulin content studies. Insulin secretion was defined as insulin concentration in the medium after 1 h stimulation, whereas insulin output referred to the insulin concentration obtained after 72 h incubation. In the insulin output study, insulin output was measured in 100 µl incubation medium. Insulin content was obtained as previously described [18]. For the insulin secretion study, the cells were incubated with 1 ml KRB containing 3.3 mM or 16.7 mM glucose for 1 hour after pre-incubation in 1 ml KRB supplemented with 3.3 mM glucose for 15 min. Subsequently, 300 µl of the 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 using a Bio-Rad detergent compatible protein kit (Bio-Rad laboratories, Hercules, CA, USA) for adjustment of insulin secretion. A passage number between 67 and 80 was used in all the studies on INS-1E cells.

Insulin assay

Insulin was analyzed by radioimmunoassay using guinea pig anti-porcine insulin antibody (Novo Nordisk, Bagsvaerd, Denmark) and mono-¹²⁵I-(Tyr A14)-labeled human insulin (Novo Nordisk) as tracer and rat insulin as standard (Novo Nordisk). Bound and free radioactivity were separated by ethanol [26]. The inter- and intra-assay variation coefficients were both less than 10%. SVS and palmitate did not interfere with the insulin assay at the concentrations tested.

Isolation of RNA

The cells were cultured as described above in the section entitled "INS-1E cell incubation and insulin secretion studies" except that the 6-well plate was used

(NUNC Brand Products, NUNC A/S, Roskilde, Denmark) in order to obtain sufficient cells for RNA extraction. The cells were washed once with cold PBS. Subsequently, 1 ml TriZol reagent (Gibco BRL, Life Technologies, Roskilde, Denmark) was added to extract total RNA according with 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, CPT1, insulin II (INS2), and glucose transporter isoform 2 (GLUT2) using real-time RT-PCR. cDNA was synthesized using IScript (BioRad, Hercules, CA, USA). The real-time RT-PCR assay was performed using the ABI 7500 FAST machine (ABI, Foster City, CA). 10 µl Real-time RT-PCR reactions consist of 5 µl 2x TaqMan® FAST Universal Master Mix (P/N 43660783; ABI, Foster City, CA), 0.5 µl 20xTaqMan® Assay/probe (AAC (Rn00573474_m1), CPT1 (Rn00580702_m1), INS2 (Rn01774648_g1), or GLUT2 (Rn00563565_m1), (ABI, Foster City, CA) and INS-1E cDNA equivalent to 50 ng of total RNA in 4.5 µl H₂O. Reactions were set up in triplicate for each sample, and the gene expressions were normalized to eukaryotic 18S rRNA expression (Hs99999901_s1; ABI, Foster City, CA). All assays were carried out in 96-well format plates covered with optical adhesive cover (P/N 4346906 and P/N 4311971; ABI, Foster City, CA). We used the 2- $\Delta\Delta C_T$ method to calculate the relative gene expression (as described in User Bulletin 2, 1997, from Perkin-Elmer Corp. in relation to 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 72 h in an 80 cm² flask 1075 (NUNC Brand Products, NUNC A/S, Roskilde, Denmark) with 15 ml culture medium as described above. The medium was removed and the cells washed once with 15 ml PBS at room temperature and trypsinized and centrifuged to obtain cell pellets. After washing with 10 ml cold PBS, the pellets were lysed for 30 min on ice in 500 µl cold RIPA lysis buffer (sc-24948; Santa Cruz, CA, USA) and whole cell lysate was collected after centrifugation. Protein concentration was determined using the Bio-

Rad detergent compatible protein kit (Bio-Rad laboratories, Hercules, CA, USA). 50 µg total protein samples were resolved by PAGE (Criterion™ 4-20% Tris-HCl; Bio-Rad laboratories, Hercules, CA, USA). Protein samples were denatured by boiling for 5 min before loading. Proteins were blotted to PVDF membrane (Amersham International, UK), and the blot was then incubated in blocking buffer (5% non-fat milk in 10 mM Tris-HCl, 1.15 M NaCl and 0.1% Tween-20) for 1 h at room temperature. Immunoblotting was performed as follows. Primary antibody incubation at 4°C was carried out overnight with antibody for ACC (1:500 dilution; #3662; Cell Signaling Technology, MA, USA), phospho-ACC (1:500 dilution; #3661; Cell Signaling Technology, MA, USA), or actin (1:5000 dilution; A5441; Sigma), followed by incubation with the appropriate horse-radish peroxidase-conjugated secondary antibodies (sc-2370 and sc-2375; all Santa Cruz, CA, USA). The labeling was visualized using an enhanced chemiluminescence system (ECL, Amersham International, UK). In each experiment, band densities were normalized against actin, and the results were expressed as protein level to control.

ACC activity determination

The 6-well plate was used (NUNC Brand Products, NUNC A/S, Roskilde, Denmark) to culture cells for determination of ACC activity. The cells were washed once with cold PBS. Subsequently, 0.5 ml cold lysis buffer was added to each well. Lysis buffer contained 5 mM HEPES, 230 mM mannitol, 70 mM sucrose, 1 mM EGTA- Na_2 , 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 1.5 ml Eppendorf tubes where they were sonicated twice at 0°C for 14 s (Branson Sonifier 250, Danbury, CT, USA). After centrifugation for 10 min at 13,000 rpm, the supernatant was collected for ACC determination.

ACC activity was measured using the radioactive CO_2 fixation method described by Inoue and Lowenstein [27]. ACC activity is expressed as the fold change in relation to the control [28].

Triglyceride (TG) content assay

Cells were cultured as described in the section above entitled “INS-1E cell incubation and insulin secretion studies” except that 24-well Black Visiplate™ TC (Wallac Oy, Turku, Finland) plates were used. After 72 h, the cells were washed once with 1 ml PBS (GIBCO) at room temperature, and the number of cells was estimated by nuclear staining using SYTO 24 reagent (Roche; 20 µl/well), and measured by FLU-Ostar Galaxy (BMG, Ramcon, Denmark). The medium was removed and the cells frozen for 1 h at -80°C. Subsequently, the cells were incubated for 20 min with triglyceride (TG) reagents (Roche; 250

Figure 1. Rat islets were pretreated with 5.5 mM glucose supplemented with or without 1.0 mM palmitate for 24 h, 72 h, or 120 h, respectively. Subsequently single rat islets were incubated at 16.7 mM glucose for 1 h (**A**). **B**: rat islets were pretreated with 5.5 mM glucose supplemented with or without 1.0 mM palmitate and 1.0 mM palmitate plus 10^{-6} M SVS for 72 h. Then single islets were incubated at 3.3 mM or 16.7 mM glucose for 1 h. Subsequently, aliquots were collected for insulin measurement. Each bar represents the mean $\pm$ SEM from 8 incubations of single islets. * $p < 0.05$ and ** $p < 0.001$ denote significant difference from that without palmitate. # denotes significant difference from that without SVS ($p < 0.001$).

$\mu\text{l/well}$). TG content was determined by a TG GPO-PAP kit (Roche) and was normalized to cell number. TG recovery was about 90%.

2-Deoxyglucose (2-DOG) uptake assay

After 72 h culture, the cells were washed twice with 1 ml PBS at room temperature. The cells were incubated in 300 $\mu\text{l/well}$ UB buffer containing 0.025 mM 2-deoxyglucose (5 $\mu\text{Ci/ml}$ 2-deoxy-[^3H]glucose, 1.5 $\mu\text{Ci/well}$ tracer) (PerkinElmer, MA, USA) supplemented with 10 $\mu\text{g/ml}$ insulin, for 15 min at 37°C. The cells were washed twice with 1 ml UB buffer supplemented with 50 mM glucose and were lysed in 0.5 ml 0.1 N NaOH. 400 μl of cell lysates were subjected to 6 ml scintillating liquid and counted using the Wallac 1409 Liquid Scintillation Counter (Wallac Oy, Turku, Finland). 50 μl of cell lysates were applied to determine protein concentration using the Bio-Rad detergent compatible protein kit (Bio-Rad laboratories, Hercules, CA, USA). Data are presented in terms of fold change in relation to the control after protein adjustment.

Statistical analysis

We performed the statistical analysis using the one-way ANOVA or Student's unpaired *t* test. Each treatment condition was compared with controls. We considered differences between two groups to be significant at $p < 0.05$. Data are expressed as mean $\pm$ SEM.

Figure 2. After 72 h pretreatment with 11.1 mM glucose supplemented with either 0.6 mM palmitate or 10^{-6} M SVS or both, INS-1 cells were incubated at 3.3 or 16.7 mM glucose for 1 h. Subsequently, aliquots were collected for insulin measurements (A). After the above-mentioned pretreatment, parts of cells underwent insulin output and insulin content determination. Pretreatment medium was taken to measure insulin output (C). Subsequently, the cells were sonicated to release insulin for measurement of insulin content (B). Each bar represents the mean $\pm$ SEM for 12 experiments of insulin secretion, and 18 experiments of insulin content and output. Data in insulin secretion are expressed relative to BIS of control (fold change), whereas data in insulin content and output are expressed relative to that of control (fold change). * $p < 0.001$ denotes significant difference from that of 11.1 mM glucose. # $p < 0.05$ denotes significant difference from that of 0.6 mM palmitate.

Figure 3. ACC, CPT1, INS2, and GLUT2 gene expression were performed by RT-PCR after INS-1 cells were pretreated with 11.1 mM glucose supplemented with or without 0.6 mM palmitate, 10^{-6} M SVS or in combination for 72 h. Each bar represents the mean $\pm$ SEM from 3 to 6 experiments. All data are expressed relative to control (control = 1.00). * denotes significant difference from 11.1 mM glucose ($p < 0.05$).

Results

The time course of lipotoxicity and the counteractive effects of SVS in rat islets

As can be seen in Figure 1A, GSIS (16.7 mM glucose) was unchanged after 24 h pretreatment with 1.0 mM palmitate in the presence of 5.5 mM glucose. However, GSIS decreased after 72 h and 120 h ($n = 8$, $p = 0.001$ and $n = 8$, $p = 0.05$, respectively). As shown in Figure 1B, BIS was increased after 72 h pretreatment with palmitate (1.0 mM) (3.3 mM glucose, $n = 8$, $p < 0.001$), whereas GSIS decreased ($n = 8$, $p < 0.001$). The suppression of GSIS was counteracted by 10^{-6} M SVS ($n = 8$, $p < 0.001$), while SVS did not change BIS during palmitate exposure ($n = 8$, $p = 0.720$).

The effects of palmitate and SVS in INS-1E cells

In the INS-1E cell line, we chose a 11.1 mM instead of a 5.5 mM glucose concentration and 0.6 mM instead of 1.0 mM palmitate. The cells were pretreated with either SVS (10^{-6} M) or palmitate (0.6 mM) or both for 72 h in the presence of 11.1 mM glucose. Insulin secretion (Figure 2A) was expressed as a fold change in BIS of 11.1 mM glucose only. The results in INS-1E cells and in isolated rat islets were similar. BIS was enhanced by palmitate (0.6 mM) pretreatment in the presence of 3.3 mM glucose ($n = 12$, $p < 0.001$), whereas GSIS decreased ($n = 12$, $p < 0.001$) in the INS-1E cells. The suppressed GSIS was counteracted by the addition of SVS (10^{-6} M) ($n = 12$, $p = 0.001$), while SVS caused no change in BIS during palmitate exposure ($n = 12$, $p = 0.290$). Insulin content was reduced by palmitate (0.6 mM) pretreatment

($n = 18$, $p < 0.001$) and further decreased after the addition of SVS (10^{-6} M) ($n = 18$, $p = 0.002$) (Figure 2B). Insulin output was increased by pretreatment with palmitate (0.6 mM) ($n = 18$, $p < 0.001$), while SVS had no impact on insulin output in response to palmitate ($n = 18$, $p = 1.0$) (Figure 2C).

The effect of SVS, palmitate, or a combination of SVS and palmitate on ACC, CPT1, INS2, and GLUT2 gene expression

As seen in Figure 3, SVS, palmitate or the combination of SVS and palmitate had no effect on the ACC, INS2, and GLUT2 gene expressions. Palmitate pretreatment significantly increased CPT1 relative mRNA level (1.03 ± 0.07 vs. 1.67 ± 0.10 , $n = 6$, $p = 0.003$), while the addition of SVS to palmitate did not change the expression of CPT-1 (1.67 ± 0.10 vs. 1.68 ± 0.06 , $n = 6$, $p = 1.0$).

The effect of SVS, palmitate, or a combination of SVS and palmitate on ACC protein levels and phosphorylated ACC

Palmitate did not change the ACC protein level (1.00 ± 0.20 vs. 1.19 ± 0.25 , $n = 3$, $p = 0.5$) (Figure 4A), or the phosphorylated ACC level (1.00 ± 0.15 vs. 0.84 ± 0.16 , $n = 3$, $p = 0.420$) (Figure 4B). The addition of SVS changed neither ACC (1.19 ± 0.25 vs. 0.81 ± 0.21 , $n = 3$, $p = 0.27$) nor phosphorylated ACC (0.84 ± 0.16 vs. 0.77 ± 0.32 , $n = 3$, $p = 1.0$) in the presence of palmitate.

The effect of SVS, palmitate, or a combination of SVS and palmitate on ACC activity

Palmitate did not change ACC activity (1.00 ± 0.08 vs. 0.95 ± 0.04 , $n = 10$, $p = 0.560$) (Figure 5) and neither did any change occur in ACC activity in response to SVS during exposure to palmitate (0.95 ± 0.04 vs. 0.99 ± 0.05 , $n = 10$, $p = 0.63$).

Figure 4. ACC (A) and phosphorylated ACC (B) proteins were measured by Western blotting after INS-1 cells were pretreated with 11.1 mM glucose supplemented with either 0.6 mM palmitate or 10^{-6} M SVS or with both in combination for 72 h. Each bar represents the mean $\pm$ SEM from 3 experiments. All data are expressed relative to control (fold change).

TG content

As illustrated at Figure 6, we found that palmitate increased the TG content in the INS-1E beta-cells (1.00 ± 0.06 vs. 1.55 ± 0.14 , $n = 10$, $p = 0.003$), while the addition of SVS to palmitate had no impact on the TG content (1.55 ± 0.14 vs. 1.78 ± 0.08 , $n = 10$, $p = 0.17$).

Figure 5. ACC activity was determined using the radioactive CO_2 fixation method after INS-1 cells pretreated with 11.1 mM glucose and supplemented with either 0.6 mM palmitate or 10^{-6} M SVS or with both in combination for 72 h. Each bar represents the mean $\pm$ SEM from 10 experiments. All data are expressed in relation to control (fold change).

Glucose uptake

As shown in Figure 7, palmitate did not change glucose uptake in the beta-cells (1.00 ± 0.02 vs. 1.02 ± 0.02 , $n = 6$, $p = 1.0$). The addition of SVS had no effect on glucose uptake in response to palmitate (1.02 ± 0.02 vs. 0.97 ± 0.02 , $n = 6$, $p = 1.0$).

Figure 6. Impact of SVS, palmitate and the both on TG content. TG content was determined after INS-1E cells pretreatment with 11.1 mM glucose and 16.7 mM glucose supplemented with or without 0.6 mM palmitate, 10^{-6} M SVS or the both for 72 h. Each bar represents the mean $\pm$ SEM from 10 experiments. All data are expressed relative to control. * denotes significant difference from 11.1 mM glucose ($p < 0.05$).

Discussion

In the present study, we found that lipotoxicity is a time-dependent process in rat islets. Lipotoxicity occurred after 72 h exposure of rat islets to 1.0 mM palmitate and was characterized by decreased GSIS and elevated BIS. SVS counteracted the decreased GSIS but had no impact on BIS. Similar results were obtained in the clonal pancreatic beta-cell line INS-1E. This implies that the properties of SVS that prevent lipotoxicity directly affect the beta-cells. In INS-1E cells, insulin content decreased during lipotoxicity, whereas insulin output increased. SVS reversed the suppression of GSIS during lipotoxicity, which was accompanied by the decreased insulin content. The gene expressions of ACC, INS2, and GLUT2 were unchanged during lipotoxicity, while CPT1 was significantly increased. The quantity of ACC protein present, as well as that of phosphorylated ACC, was unchanged by SVS. Furthermore, neither ACC activity nor glucose uptake was changed. TG content, however, increased significantly during lipotoxicity and was not influenced by the addition of SVS.

In rat islets, we used a palmitate concentration of 1.0 mM in the presence of 5.5 mM glucose, whereas in the INS-1E cell line, palmitate concentration was lower (0.6 mM) while glucose concentration was increased to 11.1 mM. The reason for changing the culture conditions was the fact that INS-1E cells are normally cultured in 11.1 mM glucose, and that cell growth depends on the glucose concentration. The physiological levels of FFAs in plasma are usually 0.2-1.7 mM, however 99% or more of these FFAs are tightly bound to serum albumin i.e. the concentration of unbound FFAs is in the range of 0.001-10 μM [29]. If we do not consider the 10% fetal calf serum in the culture media, 0.6 mM palmitate in 0.5% BSA (fatty acid free) will yield a level of more than 20 μM of free palmitate [12]. We have used a palmitate concentration higher than the physiological

level, and also slightly higher than the abnormal levels observed in diabetes. Unger *et al.* suggested that increased lipid availability (FFAs levels < 1.5 mM) induces hyperinsulinemia to alleviate insulin resistance, thereby maintaining normoglycemia [30]. A further increase in FFAs levels, however, impairs beta-cell compensation for insulin resistance and hyperglycemia appears.

Figure 7. Glucose uptake was measured after INS-1E cells pretreated with 11.1 mM glucose supplemented with either 0.6 mM palmitate or 10^{-6} M SVS or with both in combination for 72 h. Each bar represents the mean $\pm$ SEM from 6 experiments. All data are expressed relative to control (fold change).

The mechanisms involved in lipotoxicity are not fully understood. Eitel *et al.* observed that apoptosis induced by high palmitate was accompanied by a rapid nuclear translocation of protein kinase C (PKC)-delta [31]. This translocation was impaired by the phospholipase C inhibitor U-73122, which substantially reduced apoptosis. However, Welters *et al.* reported that PKC delta is not required for palmitate-induced cytotoxicity in BRIN-BD11 beta-cells [32].

The FFA concentrations most commonly used to produce the lipotoxicity model were between 0.25 to 2.0 mM for a duration of 48 to 96 h [33-37]. Our results in rat islets as well as in the INS-1E cell line are consistent with the literature. In the INS-1E cell line, palmitate pretreatment decreased GSIS and insulin content, but increased insulin output. This implies an exhaustion of the cells. FFAs are freely taken up by many types of cells [38], including pancreatic beta-cells [39]. If not oxidized, they are stored as TG in many cell types. Elevated FFAs are thus likely to produce elevated TG. Cnop *et al.* reported that an inverse correlation was observed between the percentage of dead beta-cells on day 8 and their cellular TG content on day 2 in rat islets [40]. For equimolar concentrations of the FFAs tested, oleate caused the lowest beta-cell toxicity and the highest cytoplasmic TG accumulation.

On the other hand, oleate exerted the highest toxicity in non-beta islet cells, where no FFAs-induced TG accumulation was detected. It is concluded that FFAs can cause death of normal rat islet cells through an NO-independent mechanism [40]. The ability of normal beta-cells to form and accumulate cytoplasmic TG may serve as a cytoprotective mechanism against FFAs-induced apoptosis by preventing a cellular rise in toxic free fatty acyl moieties [40]. However, contrasting results have been reported [41, 42]. Increased CPT1 mRNA implied an enhanced FFAs oxidation that could, therefore, play an important role for the inhibitory effects of FFAs observed *in vitro*. It is not surprising to observe that palmitate pretreatment increased both CPT1 gene expression as well as TG content in INS-1E cells. Our study showed that TG content increased and GSIS decreased with palmitate pretreatment.

Previously, we showed that SVS enhances ACC gene expression and activity, which in turn inhibits CPT1, increases LC-CoA, and thus reverses decreased GSIS during glucotoxicity (unpublished data). In the present study, we did not see any changes in ACC mRNA, ACC protein or ACC activity. The glucose concentration in the culture media may play a key role. Thus we have previously shown that the influence of SVS on GSIS is critically glucose-dependent in mouse islets [19]. We did not see any effect of SVS on CPT1 during lipotoxicity. The possibility, however, exists that the CPT1 inhibitory effect of SVS may be overwhelmed by the effect of palmitate itself. Further investigations are needed to establish this. TG had a tendency to increase in parallel with SVS as compared to palmitate alone. Interestingly, Zhou *et al.* showed that etomoxir, a CPT1 inhibitor, restored the decreased GSIS to palmitate, a phenomenon that was lost when TG was normalized [42]. Although the expression of INS2 and GLUT2 were unchanged during lipotoxicity, the increased insulin output and decreased insulin content imply beta-cell exhaustion during lipotoxicity.

Prentki *et al.* reported that palmitate downregulates ACC resulting in exaggerated fatty acid oxidation, providing a possible mechanism to explain how fatty acids cause beta cell glucose insensitivity [43]. We did not observe that palmitate induced changes in the expression of ACC mRNA in INS-1 cells incubated for 72 h in 11.1 mM glucose.

Decreased expression of GLUT2 occurs simultaneously with the loss of GSIS in numerous animal models of T2DM [44]. The decreased expression of the transporter is probably secondary to other metabolic alterations in diabetes [45], in particular to increases in plasma FFAs [46]. However, in the present lipotoxicity model using the INS-1E cell line, we did not observe any changes in expression of GLUT2 during lipotoxicity. Furthermore, the preventive effect of SVS on lipotoxicity did not influence GLUT2 expression.

Boden *et al.* found that FFAs caused a dose-dependent inhibition of insulin-stimulated glucose uptake in humans *in vivo* [47, 48]. Our results showed that glucose uptake was unchanged in INS-1E cells exposed to palmitate for 72 h.

Previously, our group has shown that rats fed with SVS demonstrated antihyperglycemic and blood pressure-reducing effects [21]. Recently, Geuns *et al.* reported that, following oral consumption of SVS, only its metabolite, steviol, is absorbed and excreted into the urine [49]. We are now performing further experiments on steviol in the context of studies of this mechanism.

In conclusion, SVS counteracted the functional changes occurring during lipotoxicity i.e. the impaired

GSIS in both isolated rat islets and INS-1E cells. ACC gene expression, ACC protein and ACC activity were unchanged, whereas CPT1 expression and TG content increased during lipotoxicity, irrespective of the presence of SVS. Further long-term and *in vivo* experiments are needed to clarify the possible mechanisms behind these findings. Stevioside may have beneficial potential as a glucose sensitizer for beta-cells in T2DM.

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