

Stevioside counteracts the α -cell hypersecretion caused by long-term palmitate exposure

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Hong, J., L. Chen, P. B. Jeppesen, I. Nordentoft, and K. Hermansen. Stevioside counteracts the α -cell hypersecretion caused by long-term palmitate exposure. *Am J Physiol Endocrinol Metab* 290: E416–E422, 2006. First published October 10, 2005; doi:10.1152/ajpendo.00331.2005.—Long-term exposure to fatty acids impairs β -cell function in type 2 diabetes, but little is known about the chronic effects of fatty acids on α -cells. We therefore studied the prolonged impact of palmitate on α -cell function and on the expression of genes related to fuel metabolism. We also investigated whether the antihyperglycemic agent stevioside was able to counteract these effects of palmitate. Clonal α -TC1-6 cells were cultured with palmitate in the presence or absence of stevioside. After 72 h, we evaluated glucagon secretion, glucagon content, triglyceride (TG) content, and changes in gene expression. Glucagon secretion was dose-dependently increased after 72-h culture, with palmitate at concentrations ≥ 0.25 mM ($P < 0.05$). Palmitate (0.5 mM) enhanced TG content of α -cells by 73% ($P < 0.01$). Interestingly, stevioside (10^{-8} and 10^{-6} M) reduced palmitate-stimulated glucagon release by 22 and 45%, respectively ($P < 0.01$). There was no significant change in glucagon content after 72-h culture with palmitate and/or stevioside. Palmitate increased carnitine palmitoyltransferase I (CPT I) mRNA level, whereas stevioside enhanced CPT I, peroxisome proliferator-activated receptor- γ , and stearoyl-CoA desaturase gene expressions in the presence of palmitate ($P < 0.05$). In conclusion, long-term exposure to elevated fatty acids leads to a hypersecretion of glucagon and an accumulation of TG content in clonal α -TC1-6 cells. Stevioside was able to counteract the α -cell hypersecretion caused by palmitate and enhanced the expression of genes involved in fatty acid metabolism. This indicates that **stevioside may be a promising antidiabetic agent in treatment of type 2 diabetes.**

fatty acids; glucagon; gene expression; α -TC1-6 cells

THE PATHOGENESIS OF TYPE 2 DIABETES is complex and usually requires defects both in islet cell function and insulin sensitivity. The islet cell dysfunction is characterized by failure of β -cells to compensate for insulin resistance and by a concomitant enhanced glucagon release from α -cells. Diabetic patients are usually obese with ectopic fat deposition in pancreatic islets, liver, heart, skeletal muscle, and blood vessels (5, 21, 22, 24, 27). Growing evidence suggests that fatty acid accumulation in nonadipose tissues may play an essential role in the pathogenesis of diabetes (5, 22, 24, 27). Considerable attention has been paid to the deleterious effect of fatty acids on the pancreatic β -cell. In vitro observations have shown that elevated fatty acids increase basal insulin secretion but inhibit glucose-induced insulin release (4, 21, 31–33). In addition, moderately elevated fatty acids, especially palmitate, a pre-

dominant fatty acid in human plasma, are toxic to β -cells (2, 11, 13, 31, 32).

Recent studies using microarray technology indicate that fatty acids change the expression of hundreds of β -cell genes (2, 31). Palmitate modifies a series of gene expressions in β -cells, depending on the concentration and exposure time of palmitate (31, 32). Disruption of the lipid metabolism is implicated as a key contributor to fatty acid accumulation (5, 22, 24, 27, 6). Chronically elevated levels of fatty acids dramatically influence β -cell lipid metabolism via changes in the activity and expression of enzymes that are involved in glucose and lipid metabolism as well as in the expression level of key transcription factors (2, 4, 21, 23, 24, 26, 31).

Thirty years ago, Unger and Orci (28) proposed the bihormonal-abnormality hypothesis, which highlighted that both deficient insulin secretion and excessive glucagon level contribute to the hyperglycemic state. Although considerable studies on β -cell function have been carried out, much less attention has been focused on the influence of fatty acids on the α -cell. Recently, we have demonstrated that acute exposure to fatty acids directly stimulates glucagon release from pancreatic α -cells (7). The stimulatory action on glucagon of individual fatty acids is influenced by chain length, spatial configuration, and degree of unsaturation of fatty acids (7). However, the long-term effect of fatty acids on α -cell function remains to be clarified. To our knowledge, there is no report focused on the chronic effect of fatty acids on α -cells.

The present study was designed to elucidate the chronic effects of palmitate on glucagon release, glucagon content, and triglyceride (TG) content of α -tumor cell 1 clone 6 (α -TC1-6) cells as well as on the expression of genes involved in fuel metabolism. In addition, we wanted to evaluate the potential modifying impact of stevioside, an antihyperglycemic, insulinotropic, and glucagonostatic substance (8, 10), on palmitate-induced effect on α -cells.

MATERIALS AND METHODS

α -TC1-6 cell and culture conditions. The α -TC1 line was derived from an adenoma in transgenic mice expressing the SV40 large T-antigen oncogene. The α -TC1-6 cells were cloned by a limiting dilution method and secrete only glucagon without detectable insulin (6). α -TC1-6 cells (passage 26–49) were cultured in DMEM (GIBCO-BRL, Paisley, UK) containing 18 mM glucose and 10% FCS (GIBCO-BRL) under an atmosphere of 95% humidified air-5% CO₂ at 37°C. Cells were passaged once a week after trypsinization and replaced with new medium twice weekly. They were seeded in wells

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Table 1. PCR primers for α -TC1-6 cells

Name	Sense Primer (5'-3')	Antisense Primer (5'-3')	Base Pairs
ACC1	AGATCGGCACCTTCTTCTACTGG	CCTTCACTGCGCCTTCAACTTCTA	154
CPT I	CAAAATGAGCCGCAGCACCAGAAT	AGGCAGGGCGGCAGAGACG	103
GG	CAGGGCACATTCACGAGCGACTAC	TGTTCCGGTTCCTCTTGGTGTCA	97
PPAR γ	GTGCCCTTGCTGTGGGGATGTCTCA	CCCTCGCCTTGGCTTTGGTCAG	197
SCD	GGCGCCACCAAGTTCTCAG	GGCGGGGTTGTAGTACCTCCTT	193
SREBP-1c	CTGGATTTGGCCCGGGAGATTTT	TGAGCAGGTGGCGGATGAGGTTT	140
GAPDH	CAAGTTCATCCATGACAACCTTG	GGCCATCCACAGTCTTCTGG	69

ACC, acetyl-CoA carboxylase-1; CPT I, carnitine palmitoyltransferase 1; GG, glucagon gene; PPAR γ , peroxisome proliferator-activated receptor- γ ; SCD, stearoyl-CoA desaturase; SREBP-1c, sterol regulatory element-binding protein-1c; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

at a density of 0.25×10^6 cells per culture well (24-well plate) and 0.75×10^6 cells per culture well (6-well plate) 24 h before use in the following experiments.

Glucagon secretion. α -TC1-6 cells were incubated for 72 h at 37°C in DMEM with 18 mM glucose in the presence of 0.25–1.0 mM palmitate (Sigma Chemical, St. Louis, MO), 0.5% BSA (fatty acid free; Roche, Mannheim, Germany) with or without 10^{-8} to 10^{-6} M stevioside (Sigma). The solution of palmitate was prepared as previously described (7, 31). Briefly, palmitate was prepared by dissolving and heating to equal molar amounts of NaOH and palmitate supplemented with distilled water to a concentration of 500 mmol/l. They were further diluted with 5% BSA (fatty acid free) to 50 mmol/l palmitate. The stock solution was filter sterilized and stored at -20°C , and palmitate solution was freshly prepared before each experiment.

After 72 h, the cells were preincubated for 30 min in Krebs-Ringer buffer (KRB) containing 3.3 mM glucose and 0.5% BSA (pH 7.4) and

subsequently incubated for 2 h with KRB containing 18 mM glucose and 0.5% BSA, with or without 0.25–1.0 mM palmitate.

After incubation, supernatants were collected and kept frozen at -20°C for glucagon by radioimmunoassay kit (Linco Research, St Charles, MO).

Glucagon content. Cells were cultured in six-well plates for 72 h using the same incubation protocol as described above. Then the medium was replaced by glycine-BSA (glycine 100 mM, 0.25% BSA, pH 8.8; both Sigma). α -Cells were disrupted by sonication twice (Branson Sonifier 250, Danbury, CT) and kept on ice for 15 s. After centrifugation at 16,000 rpm for 30 min, the supernatants were collected and stored at -20°C for assay.

Gene expression. After 72 h of culture, total RNA was extracted from α -TC1-6 cells using TRIzol (GIBCO). To eliminate possible contamination of genomic DNA, the RNA was treated with DNase I (Invitrogen, Taastup, Denmark). The cDNA was synthesized from 1

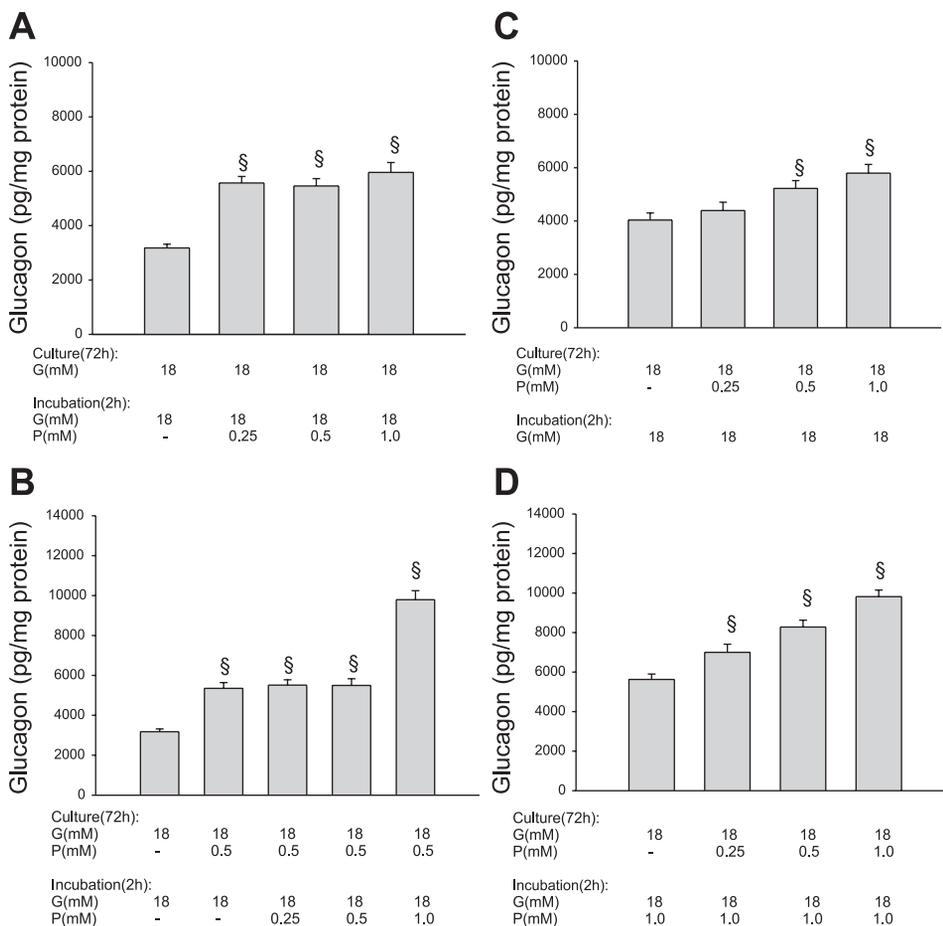


Fig. 1. Dose dependence of palmitate-induced glucagon secretion. α -TC1-6 cells were cultured with palmitate (P) (0–1.0 mM; C and D) or 0.5 mM palmitate + 18 mM glucose (G; A and B). After 72-h culture, cells were incubated for 2 h with KRB containing palmitate (0–1.0 mM; A and B) or 1.0 mM palmitate (A and B; $n = 18$). Data are given as means \pm SE. §Significant difference ($P < 0.05$) compared with control.

μ g of total RNA by means of iScript reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

The primers for selected genes were designed using the Primer Select program of the Lasergene software package (DNA-STAR, Madison, WI; Table 1).

Before routine use, the optimal annealing temperature and predicted size of the PCR product for each gene were verified by gradient tests and electrophoresis. Samples were analyzed by real-time RT-PCR on an i-Cycler PCR system (Bio-Rad) using SYBR green detection. The thermal cycling reactions included an initiating hot start at 95°C for 3 min, 45–50 cycles at 95°C for 15 s, 55–65°C for 15 s, and 72°C for 25 s followed by an elongation step at 72°C for 5 min. A single fluorescence measurement was taken after each annealing step. After amplification, the melting curves of the amplicon were determined to examine the specificity of amplification. The relative quantitative expression was normalized to GAPDH mRNA level.

TG assay. Cells were exposed to 18 mM glucose with or without 0.5 mM palmitate as well as stevioside (10^{-7} or 10^{-6} M) in 24-well plates. After 72 h, the cells were washed once with 1.2 ml of PBS (GIBCO), and the number of cells was estimated using nuclear staining with SYTO 24 reagents (Roche; 24 μ l/well) and measured by FLUOstar Galaxy (BMG, Ramcon, Denmark). The medium was removed, and the cells were frozen for 1 h at -80°C . Subsequently, cells were incubated for 20 min with TG reagents (Roche; 270 μ l/well). TG content was determined by a TG GPO-PAP kit (Roche) and was normalized to cell number. The recovery of TG content was $\sim 90\%$.

Protein assay. After the secretion study, the medium was removed and cells were lysed in 0.1 M NaOH (Sigma). Intracellular protein was measured by Bio-Rad detergent compatible protein assay kit. The glucagon levels were adjusted to protein concentration.

Statistics. One-way ANOVA was used for the statistical analysis. Significant differences were considered for P values < 0.05 . Data are expressed as means \pm SE.

RESULTS

Effects of 72-h exposure to palmitate on glucagon secretion. Figure 1 shows the impact of palmitate (0.25–1.0 mM) on glucagon secretion from α -TC1-6 cells in the presence of high glucose (18 mM glucose) with or without palmitate (0.25–1.0 mM).

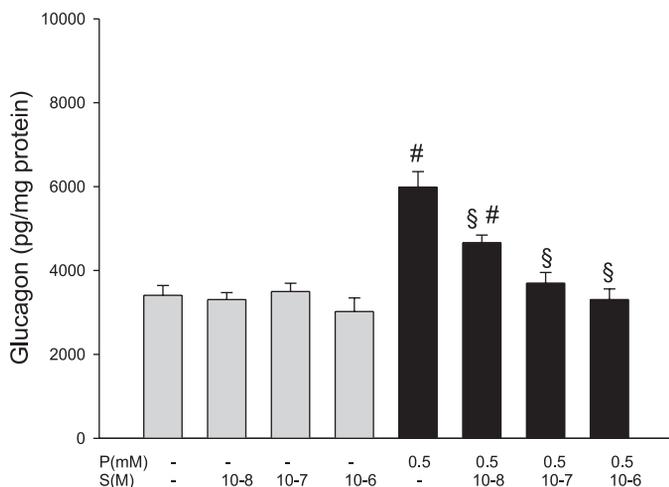


Fig. 2. Effect of stevioside on glucagon secretion from α -TC1-6 cells exposed to 0.5 mM palmitate (P) (gray bars, without palmitate; filled bars, with palmitate) in the presence or absence of stevioside (S) (10^{-8} to 10^{-6} M) for 72 h ($n = 18$). Values are means \pm SE. #Significant difference ($P < 0.05$) between the group with palmitate and the group without palmitate; §significant difference ($P < 0.05$) between the group with palmitate and the group with palmitate as well as stevioside.

Table 2. Effect of palmitate on glucagon content in α -TC1-6 cells

Treatment	Glucagon Content, ng/ml
Control (18 mM glucose)	297.9 \pm 33.2
18 mM glucose + 0.25 mM palmitate	248.4 \pm 62.2
18 mM glucose + 0.5 mM palmitate	255.2 \pm 16.9
18 mM glucose + 1.0 mM palmitate	227.4 \pm 43.8

Values are means \pm SE. α -TC1-6 cells were cultured for 72 h with palmitate (0–1.0 mM; $n = 6$).

α -TC1-6 cells were exposed to 18 mM glucose for 72 h in the absence (Fig. 1A) or presence (Fig. 1B) of 0.5 mM palmitate, followed by a 2-h incubation with 18 mM glucose and palmitate at concentrations ranging between 0.25 and 1.0 mM. A stimulatory effect on glucagon secretion was found at palmitate concentrations ≥ 0.25 mM ($P < 0.05$; Fig. 1, A and B), being most pronounced at 1.0 mM palmitate ($P < 0.01$; Fig. 1B).

α -TC1-6 cells were also cultured for 72 h with palmitate concentrations ranging from 0 to 1.0 mM in the presence of 18 mM glucose, followed by a 2-h incubation without (Fig. 1C) or with 1.0 mM palmitate (Fig. 1D).

As shown in Fig. 1C, a significant stimulation of glucagon release was observed at palmitate concentrations ≥ 0.5 mM ($P < 0.05$). In the presence of 0.5 and 1.0 mM palmitate for 72 h, glucagon secretion was increased by 29 and 44% ($P < 0.05$), respectively.

Figure 1D shows that palmitate (0.25–1.0 mM) induces a subsequent stimulation to 1.0 mM palmitate ($P < 0.05$). The percentage increases to 0.25, 0.5, and 1.0 mM palmitate were 24, 47, and 75% ($P < 0.05$), respectively.

Effects of stevioside on palmitate-induced changes of glucagon secretion. Figure 2 indicates the effect of 72-h culture with stevioside on glucagon secretion in the presence of 18 mM glucose and without (left) and with (right) 0.5 mM palmitate. Stevioside per se (10^{-8} to 10^{-6} M) had no effect on glucagon secretion. However, in the presence of 0.5 mM palmitate, stevioside caused a dose-dependent inhibition of glucagon release ($P < 0.01$). Stevioside at concentrations of 10^{-8} , 10^{-7} , and 10^{-6} M reduced palmitate-induced glucagon release by 22, 38, and 45% ($P < 0.01$), respectively.

Influence of palmitate and stevioside on glucagon content. Although we found a tendency toward reduced glucagon content after 72 h of culture with palmitate (0.25–1.0 mM), this did not attain statistical significance (Table 2). Stevioside had no

Table 3. Effect of stevioside on glucagon content in α -TC1-6 cells

Treatment	Glucagon Content, ng/ml
Control (18 mM glucose)	304.3 \pm 37.5
18 mM glucose + 10^{-7} M stevioside	280.8 \pm 29.8
18 mM glucose + 10^{-6} M stevioside	266.4 \pm 25.4
18 mM glucose + 0.5 mM palmitate	236.1 \pm 6.5
18 mM glucose + 0.5 mM palmitate + 10^{-7} M stevioside	229.5 \pm 16.7
18 mM glucose + 0.5 mM palmitate + 10^{-6} M stevioside	235.1 \pm 16.1

Values are means \pm SE. Cells were cultured for 72 h with palmitate (0.5 mM) and/or stevioside (10^{-7} to 10^{-6} M; $n = 8$).

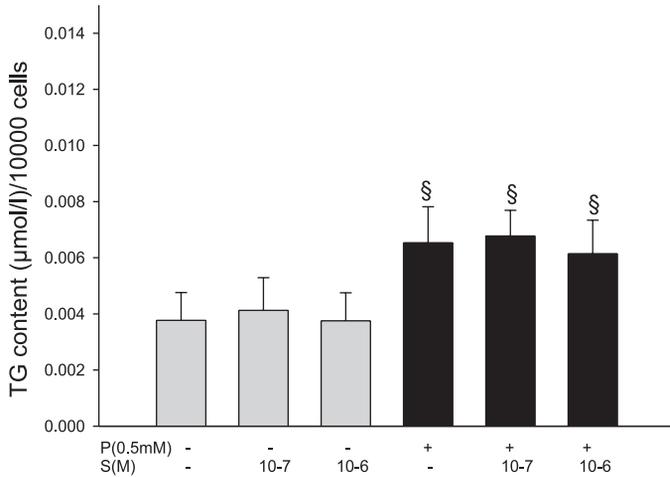


Fig. 3. Impact of palmitate and stevioside on TG content in α -TC1-6 cells cultured with (filled bars) or without (gray bars) 0.5 mM palmitate (P) as well as stevioside (S) (10^{-7} or 10^{-6} M) for 72 h ($n = 16$). Values are means \pm SE. §Significant difference ($P < 0.05$) compared with control.

impact on glucagon content after 72-h culture with 0.5 mM palmitate (Table 3).

Impact of palmitate and stevioside on TG content. As seen in Fig. 3, the TG content of α -cells exposed to palmitate (0.5

mM) for 72 h was increased by 73% ($P < 0.01$). Stevioside (72 h) did not influence the TG content ($P > 0.05$) and was not able to alter the increased TG content seen in the presence of palmitate.

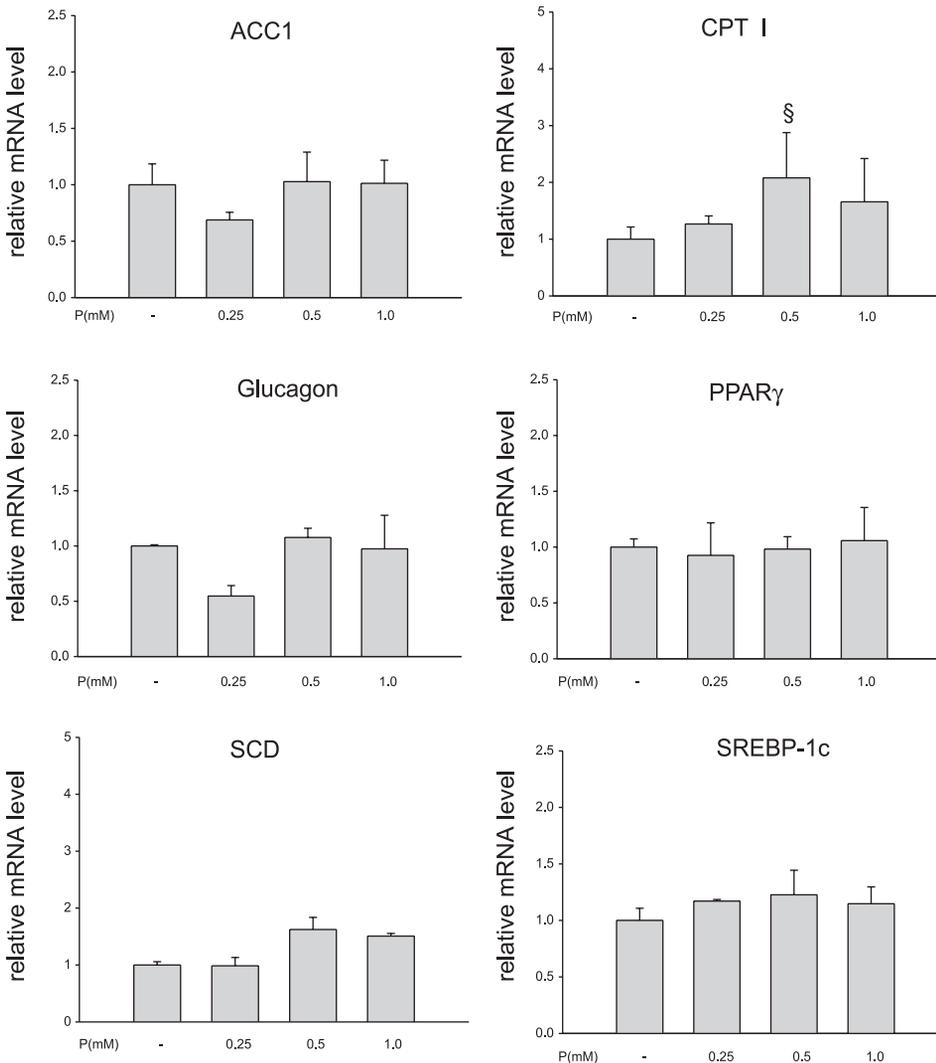


Fig. 4. Gene expression (ratios compared with GAPDH) in α -TC1-6 cells culture for 72 h with palmitate (P) (0–1.0 mM) ($n = 3$). All data are expressed relative to control. §Significant difference ($P < 0.05$) compared with control.

Gene expression. Figure 4 shows the effect of palmitate on the gene expression profile of acetyl-CoA carboxylase-1 (ACC1), carnitine palmitoyltransferase I (CPT I), glucagon, peroxisome proliferator-activated receptor- γ (PPAR γ), stearoyl-CoA desaturase (SCD), and sterol regulatory element-binding protein-1c (SREBP-1c) in α -TC1-6 cells. Compared with control, only 0.5 mM palmitate increased CPT I mRNA level 2.1-fold ($P < 0.05$), whereas palmitate did not affect the expressions of the other genes.

As seen in Fig. 5, stevioside per se did not cause any major change in the expression of the selected genes. However, in the presence of palmitate (0.5 mM), stevioside (10^{-7} and 10^{-6} M) caused an upregulation of the CPT I expression by 150 and 170%, respectively. The expressions of PPAR γ and SCD were also enhanced by the higher stevioside concentration (10^{-6} M) in the presence of palmitate, the ratios being increased 2.7- and 1.7-fold, respectively ($P < 0.05$).

DISCUSSION

We used clonal α -TC1-6 cells to study the impact of palmitate and stevioside on glucagon release and changes in the

expression of selected genes. These cells possess the advantage over primary islets of being a homogeneous cellular population and have been previously used to study glucagon secretion (7, 12, 15) and gene expressions (15, 16, 29).

In this study, we demonstrated that long-term exposure of α -cells to elevated fatty acids caused glucagon hypersecretion and TG accumulation. Palmitate enhanced glucagon secretion after both 72-h culture and the subsequent 2-h incubation in the presence of high glucose to mimic a diabetes-like condition. Whether the hypersecretion of α -cell reflects "lipotoxicity" or a normal response is unclear. Because palmitate increases glucagon secretion and the net cellular glucagon content appears stable, this indicates that glucagon biosynthesis is enhanced. Data presented on a per-cell basis rather than normalized to protein content may be a more accurate method.

Lipid overflow causes excessive storage of TG in nonadipocyte cells, leading to impairment of cellular metabolism (4, 21, 22, 24, 34). Little is known about the effect of fatty acids on α -cells. Here, palmitate caused a marked increase in TG content in α -cells and enhanced the expression of CPT I, which plays a central role in the partitioning of fatty acids in mito-

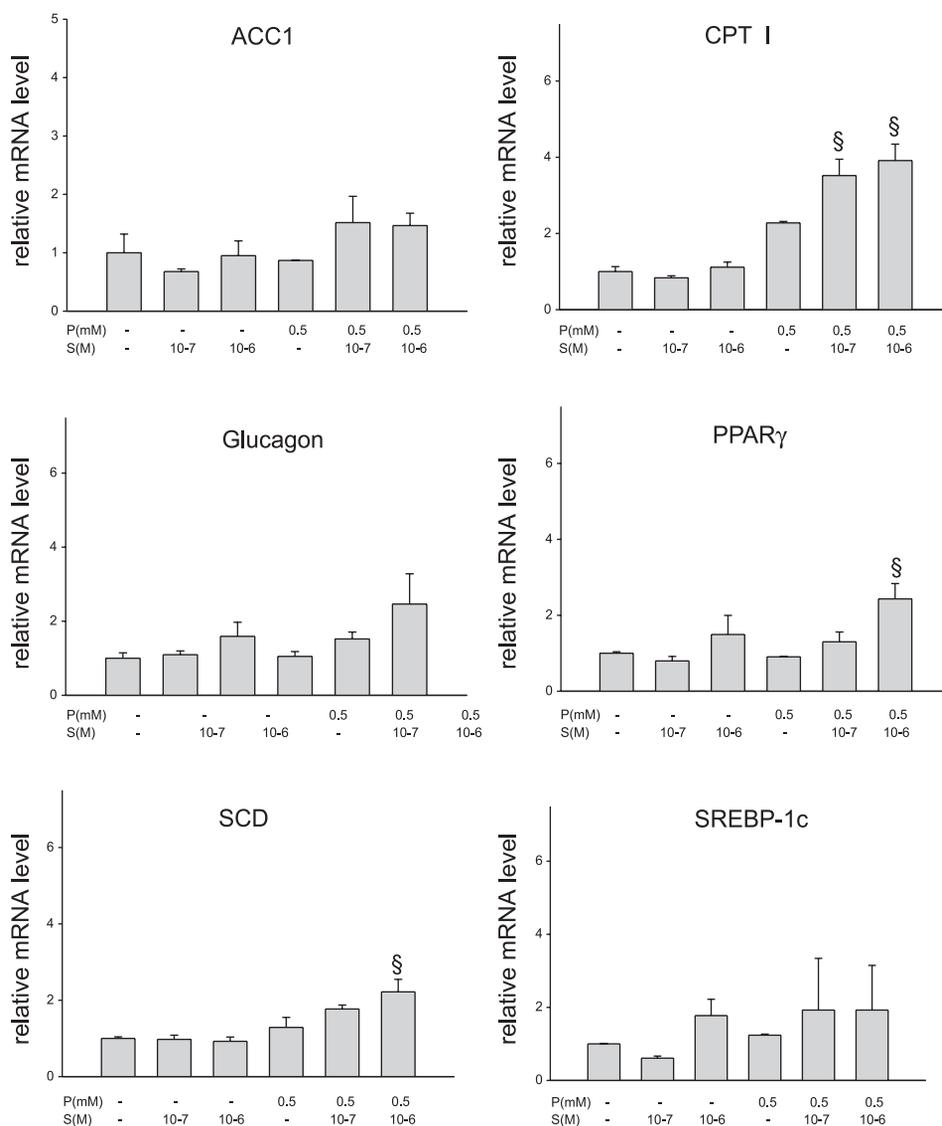


Fig. 5. Gene expression (ratios compared with GAPDH) in α -TC1-6 cells culture for 72 h with 0.5 mM palmitate (P) as well as stevioside (S) (10^{-7} to 10^{-6} M) ($n = 3$). All data are expressed relative to control. §Significant difference ($P < 0.05$) compared with control.

chondrial oxidation (14). The reports of Unger (27) and Zhou et al. (34) indicate that fatty acids raise TG content, upregulate enzymes of oxidation (acyl-CoA oxidase and CPT I) in normal nonadipocytes such as islets, and direct fatty acids into oxidative rather than lipogenic pathways. This leads to a disposal of unwanted fatty acids and prevents their accumulation as TG (25). Enhancing the expression of CPT I by fatty acids with a possible reduction in malonyl-CoA formation could result in elevated fatty acid oxidation (1, 23, 27). Thus we propose that an increase in β -oxidation via upregulation of the expression of CPT I gene in α -cells protects against fatty acid-induced α -cell hypersecretion. Causative relationships between these alterations in gene expression and secretory function will need to be substantiated in future experiments.

In the diabetic rat, stevioside significantly lowers blood glucose, increases insulin release, and suppresses glucagon levels (8, 10). A stimulation of insulin secretion and insulin content was observed in a clonal β -cell line (9, 10). In rat liver, stevioside also slows down gluconeogenesis by decreasing phosphoenolpyruvate carboxykinase gene expression (3). The direct role of stevioside on α -cells is still not clarified. Interestingly, we demonstrated that stevioside is able to reduce the exaggerated glucagon secretion caused by fatty acids and enhance expressions of CPT I, PPAR γ , and SCD in the presence of palmitate. Stevioside per se did not cause any changes in glucagon levels or in the expression of selected genes. This indicates that stevioside may not influence α -cell metabolism under normal metabolic conditions. CPT I gene is an early-response gene induced by fatty acids in β -cells (1). Upregulation of CPT I can improve the insulin response and is protective against fatty acid-induced insulin resistance (20). This may also be the case for α -cells. Parton et al. (19) showed that enhanced activation of PPAR γ in islet leads to the stimulation of multiple metabolic pathways that favor the disposal of fatty acids (mitochondrial and peroxisomal oxidation, cellular export, and incorporation into diglycerides) rather than the synthesis of fatty acids. In the presence of palmitate, stevioside also enhanced the gene expression of SCD, a central enzyme catalyzing the synthesis of monounsaturated fatty acids, mainly oleate (C18:1) and palmitoleate (C16:1) (18). This conversion of palmitate to palmitoleate would represent a beneficial adaptation to elevated palmitate because palmitoleate protects against β -cell apoptosis induced by saturated fatty acids (30). The transcription factor SREBP-1c was associated with control to the regulation of enzymes implicated in fatty acid synthesis (17, 26).

Taken together, the beneficial effects of stevioside on α -cell function are likely to relate to enhanced expression of genes involved in fatty acid oxidation (CPT I), fatty acid disposal (PPAR γ), and fatty acid conversion (SCD). The genes encoding lipogenesis were unchanged, including ACC1 and SREBP-1c. Therefore, stevioside seems not to be associated with genes that regulate fatty acid synthesis. Our work analyzed only a few selected genes related to fuel metabolism; this does not exclude other pathways being involved simultaneously. In future studies, it will be important to clarify possible changes of enzyme activities in lipid metabolism and elucidate the complex gene-regulatory networks to understand the precise mechanisms of the action of stevioside.

In conclusion, we have demonstrated that long-term exposure to palmitate causes a hypersecretion of glucagon and an

accumulation of TG in clonal α -TC1-6 cells similar to the diabetic state. Stevioside was able to counteract the α -cell hypersecretion to palmitate and enhanced the expression of genes involved in fatty acid metabolism but did not reduce the TG content. This indicates that stevioside may be a promising antidiabetic agent in treatment of type 2 diabetes via counteraction of α -cell dysfunction.

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