EFFECT OF ORAL SEBACIC ACID ON POSTPRANDIAL GLYCEMIA, INSULINEMIA AND GLUCOSE RATE OF APPEARANCE IN TYPE 2 DIABETES

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Submitted 9 April 2010 and accepted 6 August 2010.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes Care. The American Diabetes Association, publisher of Diabetes Care, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes Care in print and online at http://care.diabetesjournals.org.
Objective  Dicarboxylic acids (DAs) are natural products with the potential of being an alternate dietary source of energy. We aimed at evaluating the effect of sebacic acid (a 10 carbons DA; C10) ingestion on postprandial glycemia and glucose rate of appearance (Ra) in healthy and type 2 diabetic (T2D) subjects. Furthermore, the effect of C10 on insulin-mediated glucose uptake and on GLUT-4 expression was assessed in L6 muscle cells in vitro.

Research, Design and Methods Subjects ingested a mixed meal (50% carbohydrates, 15% proteins and 35% lipids) containing 0g (Control), 10g of C10 (10g C10) in addition to the meal or 23g of C10 in substitution of fats (23g C10).

Results  In T2D subjects, the incremental glucose area-under-the-curve (AUC) decreased by 42% (P<0.05) and 70% (P<0.05) in the 10g C10 and 23g C10 groups, respectively. At the largest amounts used, C10 reduced the glucose AUC also in healthy volunteers. When fats were substituted with 23 g of C10, AUC of Ra was significantly reduced in the order of 18% (P<0.05) in both healthy and diabetic subjects. The insulin-dependent glucose uptake by L6 cells was increased in presence of C10 (38.7 ± 10.3 vs. 11.4 ± 5.4 %, p=0.026). This increase was associated with a 1.7 fold raise of GLUT4.

Conclusions  Sebacic acid significantly reduced hyperglycemia after a meal in T2D subjects. This beneficial effect was associated with a reduction of glucose Ra, probably due to a lowered hepatic glucose output and an increased peripheral glucose disposal.

The World Health Report launched in 2002 by the World Health Organization advised that more than one billion adults worldwide are overweight and at least 300 million are clinically obese. Type 2 diabetes (T2D) can be considered a threatening obesity-related disease since hyperglycemia causes relevant complications such as micro- and macro-angiopathy. Patients with T2D exhibit increased hepatic glucose output, which is identified as the main cause of fasting hyperglycemia and is associated with an impaired plasma glucose clearance (1), and a reduced hepatic synthesis of glycogen, of about 25-45% as compared with non-diabetic subjects (2). Increased hepatic gluconeogenesis has been considered responsible for elevated hepatic glucose output in type 2 diabetes (3). When glycogen is available in adequate amount there is an auto-limitation of the hepatic glucose production. In diabetes, a breakdown of this auto-regulation may occur if glycogenolysis is limited by glycogen depletion (4).

Jenkins et al. (5) have shown that spreading the nutrient load over a longer period of time by increased meal frequency, the so-called nibbling diet, is beneficial in terms of reduction of circulating levels of glucose, insulin and free fatty acids in type 2 diabetes. Thus, the availability of snacks poor in fat and that do not induce hyperglycemia and/or over-stimulate insulin secretion would be a good tool in the diet of insulin-resistant, type 2 diabetic subjects.

Dicarboxylic acids (DAs) are natural occurring substances produced by both higher plants and animals via ω-oxidation of fatty acids (6,7). In plants, long-chain DAs are components of natural protective polymers, cutin and suberin, support biopolymesters involved in waterproofing the leaves and fruits, regulating the flow of nutrients among various plant cells and organs, and
minimizing the deleterious impact of pathogens (7). In animals and humans, medium chain DAs, which include prevalently sebacic (C10) and dodecanedioic (C12) acids, derive from the β-oxidation of longer chain DAs (8). Long chain DAs, in turn, are formed from the correspondent fatty acids by ω-oxidation in the microsomial membranes (9) or are taken in with a diet rich in vegetables (7).

We have previously shown that medium chain DAs represent a suitable alternate energy substrate to glucose in clinical conditions with marked insulin resistance and/or impaired aerobic glycolysis (10). Interestingly, in T2D, medium chain DAs are rapidly oxidized, do not stimulate insulin secretion and reduce muscle fatigue (11). Nevertheless, the effect of C10 or C12, not as a substitute but in addition to available carbohydrates, on glucose homeostasis has never been studied. On this ground, we aimed at investigating the effect of oral administration of C10 on postprandial glycemia, insulinemia and glucose rate of appearance (Ra) in T2D subjects as compared with healthy volunteers.

To further elucidate the mechanism of action of sebacic acid in diabetes, insulin mediated glucose uptake and GLUT4 protein expression were assessed in L6 cells in vitro.

SUBJECTS AND METHODS

Inclusion Criteria. From October 2006 to September 2007, 10 obese T2D subjects and 10 healthy volunteers were enrolled. Women in fertile age were asked to engage themselves in avoiding pregnancy during the study protocol. In any case, before starting each experimental session a pregnancy test was performed and pregnant women excluded from the investigation. All women were studied in the follicular phase of their menstrual cycle. None of the subjects have suffered or were suffering major endocrinological, renal, cardiac, respiratory, liver or gastro-intestinal disorders.

All subjects underwent 75-g oral glucose-tolerance test to measure Matsuda Index, as an index of insulin resistance (12). None of the diabetic subjects were under oral hypoglycemic agents, were diagnosed with type 2 diabetes 2-5 years before the study and their glycated hemoglobin was 5.5-7.5%.

The protocol was approved by the Ethical Committee of the Catholic University, School of Medicine in Rome, Italy. All the subjects signed a written informed consent prior to starting the study.

Chemicals. Sebacic acid was purchased from Sigma (St Louis, MO, USA) and purified by Real s.r.l., Como, Italy. It was pyrogen-free and contaminant-free with a degree of purification (gas-liquid chromatography/mass spectrometry) of 99.5%. 6,6-D-glucose was purchased from Mass Trace (Woburn, MA).

Experimental protocol. The subjects were studied on three separate occasions in a randomized way at a distance of 1 month. After the overnight fast, during which subjects were instructed not to drink, the study was initiated at 8:00 A.M. An indwelling catheter was placed into an antecubital vein for isotope infusion. A second catheter was inserted retrogradely into a wrist vein of the ipsilateral hand, and the hand placed into a heated box (60°C) to achieve arterialization of venous blood. Basal rates of glucose turnover were assessed after 2 h of an adjusted primed (22 µmol/kg), continuous (0.22 µmol kg⁻¹ min⁻¹) infusion of 6,6-D₂-glucose (Cambridge Isotope Lab, Boston, MA, USA) (13).

Two hours after the beginning of the session, the subjects ingested a mixed meal. (See Table 1 for meal composition).

Blood samples for the determination of 6,6D-glucose enrichment, plasma C-peptide, and plasma insulin concentrations were obtained before starting tracer infusion and every 10 min during the last 20 min of the 2 hours preceding the meal ingestion (equilibration period). Starting from the meal ingestion
time, plasma samples were obtained every 20 min for 5 hours. Therefore, overall the study duration was 7 hours or 420 minutes. Each subject voided his/her bladder before starting the experimental protocol and the subsequent 24-h urines were collected in a container with 0.1% sodium azide to prevent bacterial growth.

**Body composition.** After voiding and weighing, body composition was assessed by Dual-X-ray Absorptiometry (DXA) (Lunar Prodigy, GE Lunar Corp., Madison, USA) to measure fat-free mass (FFM), fat mass (FM) and percentage of FM.

**Analytical methods.** Plasma insulin and C-peptide were measured in duplicate by double-antibody RIAs (Linco Research, St. Charles, MO). Intra-assay variation was 4.9%, and 2.4%, and inter-assay variation was 5.9%, and 7.1%, respectively. Plasma glucose was monitored immediately after blood withdrawal with an Analox GM9 Glucose-Analyzer (Beckman Instruments, Fullerton, CA).

Plasma glucose enrichment due to 6,6-D-glucose was determined by gas chromatography–mass spectrometry (GCMS) on a Hewlett Packard GC 5890/MS 5972 (Hewlett Packard, Palo Alto, CA) equipped with a 30-m capillary column, as described by Wolfe (13). Briefly, 100 µl of plasma were deproteinized using 2 ml cold methanol, and the supernatant was dried and derivatized using acetic anhydride:pyridine (1:1) to form penta-acetate glucose. The sample was then dried again and dissolved with ethylacetate for injection into the GCMS. The fragments 200, 201, and 202 were monitored, and enrichment calculated as the ratio of 202:200. C10 analysis was performed as previously described (14).

**Calculations.** A physiological and isotopic steady-state was achieved during the last 20 min of the basal period and of the meal period, therefore the glucose rates of appearance ($R_a$) which is equivalent to endogenous glucose production (EGP) and disappearance ($R_d$) were calculated as the tracer infusion rate divided by the tracer-to-tracer ratio. During the meal glucose non-steady conditions prevail and thus glucose rate of appearance and disappearance were calculated using the Steele equation (15). Glucose flux rates were expressed per kg of body weight. Glucose clearance was calculated as Rd divided by plasma glucose concentration. Areas under the curve (AUC) of glucose and insulin concentrations and of glucose rate of appearance and clearance were calculating using the trapezoidal method. AUC of meal glucose clearance was normalized to AUC of insulin to account for different insulin concentrations observed during the three tests. Insulin Secretion Rate (ISR) was computed by C-peptide deconvolution (16).

**Cell culture and Glucose uptake.** L6 myoblasts were grown and maintained in α-MEM containing 2% FBS and 1% antibiotic-antimycotic mixture in an atmosphere of 5% CO$_2$ at 37°C, as described elsewhere (17,18). The cells were cultivated overnight in 12-well plates with or without 0.2 mM albumin bound C10 and serum starved for 4 h before being incubated for 20 min with 10 nM insulin. Subsequently, myoblasts were washed twice and glucose transport was assayed in Heps-buffered saline solution (140 mM NaCl, 20 mM Heps-Na, 2.5 mM MgSO$_4$, 1 mM CaCl$_2$, 5 mM KCl, pH 7.4) containing 10 µM 2-deoxy-D-glucose (0.5 µCi ml$^{-1}$ 2-deoxy-D-[${}^3$H]glucose). The incubation medium was aspirated, the cells were washed with ice-cold saline, and 1 ml of NaOH (0.05 M) was added to each well. Cell lysates were transferred to scintillation vials for radioactivity counting. Non-specific uptake was determined in the presence of cytochalasin B (10 µM) and was subtracted from all values.

**Immunoprecipitation and Western blot.** To measure the total glucose transporter content, cell lysates were subjected to 10% SDS-PAGE and Western blotting (19). Filters were
incubated with GLUT4 antibodies (Abnova, Prodotti Gianni, Milano, Italy) for 14 h at 4°C and then with peroxidated anti-antibodies (Amerham Biosciences, Cologno Monzese, Italy) for 1 h at room temperature. GLUT4 was finally revealed by detection of chemioluminescence by autoradiography.

Statistical analysis. Data are presented as means ± SD, unless otherwise specified. For the clinical study differences between the 3 sessions were analyzed by non-parametric (Wilcoxon signed-rank test) tests for paired samples corrected for Bonferroni inference. Analyses were performed using SPSS for Windows version 13.0 (SPSS, Chicago, IL, USA). Significance was accepted at \( p < 0.05 \).

For in vitro study Student's \( t \)-test was used to evaluate the significance of the effect of sebacic acid and insulin on glucose transport. Results are expressed as means ± SD of five different experiments, each performed in triplicate; SE is reported for percent values.

RESULTS

Type 2 diabetic patients (5 women/5 men) and controls (4 women/6 men) were matched for gender distribution, age (52.1±6.98 vs. 47.2±6.03 years), and body mass index (27.98±4.08 vs. 26.63±3.03 kg/m\(^2\)). Diabetic subjects were insulin resistant with a Matsuda Index of 5.65±1.41 as compared with 15.29±2.63 in controls (\( p<0.01 \)).

All subjects ingested a mixed meal (see Methods) containing either 0 g (Control), additional 10 g of C10 (10g C10) or 23 g of C10 as a substitute of dietary fats (23g C10).

The time courses of plasma glucose and insulin in healthy controls are shown in Figure 1. The ingestion of C10 together with the meal reduced to some extent the glycemic peak, but the glucose incremental area under the curve (AUC) was significantly reduced only after 23 g of C10 (Figure 2). Insulin peak level was clearly reduced in both C10 groups, attaining a value of -39% in the 10g group and -71% in the 23g C10 group (Figure 2; both \( p<0.01 \)).

The effect of C10 on plasma glucose and insulin time courses in type 2 diabetic subjects is depicted Figure 1. A reduction in the incremental glucose AUCs of 42% and 70% was observed in the 10g C10 (\( p=0.037 \)) and 23g C10 (\( p=0.045 \)) groups, respectively (Figure 2). Similarly to what observed in control subjects, the incremental AUCs of insulin were decreased by 39% after 10g C10 intake and by 64% after 23g C10 intake, respectively (Figure 2 panel C, \( p<0.05 \)).

The insulin secretion rate (ISR) was similar across the three studies in both T2D and healthy volunteers (Figure 2).

Glucose Ra AUC was decreased similarly in control and in diabetic subjects after C10 supplement (by ~18% after 23g C10, \( p<0.05 \), Figure 2 panel B) while glucose clearance, which is an index of peripheral insulin sensitivity, tended to increase after C10 supplementation reaching, however, statistical significance only in controls after 23g C10 (Figure 2 Panel D).

As shown in Figure 1, the concentration of plasma C10 in diabetic patients (Panel F) tended to be higher than in controls (Panel E), without reaching however a statistical significance, and peaked at the same times. Furthermore, in diabetics the two curves (10 g vs. 23 g) were overlapped up to 320 minutes after the meal, and then the concentration of C10 declined slower in the 23g C10 fed patients compared with 10 g of C10.

The in vitro experiments were conducted by using a concentration of 0.2 mM, on the basis of the average plasma concentration of C10 reached in humans after C10 ingestion. In fact, a level of 40 µg/ml of plasma C10, whose molecular weight is 202, corresponds to a concentration of 0.198 mM.

The insulin dependent glucose uptake from L6 cells increased more in presence of C10 (1.83±0.40 vs. 1.37±0.28 pmol/mg·10 min; \( p=0.01 \) vs. 1.73±0.19 vs. 1.57±0.14
pmol/mg·10 min; p=0.019), corresponding to a percent increase of 38.7 ± 10.3 (SE)% in presence of C10 vs. 11.4 ± 5.4% in its absence (p=0.026). This increase was associated with a 1.74 ± 0.27 fold increase of glucose transporters GLUT4 (Figure 3), in agreement with the slight increase in glucose clearance observed after C10 (Figure 2, panel D).

DISCUSSION
The major finding of the present study is that sebacic acid significantly reduces post-meal glucose circulating levels as well as the glucose rate of appearance in both healthy and insulin resistant, type 2 diabetic subjects. This occurs when C10 is either added to a mixed meal or substituted to lipids in an equivalent caloric manner. The above effect is less pronounced in healthy controls. In L6 cells C10 significantly increases insulin mediated glucose uptake and GLUT4 expression.

There is general agreement that post-prandial hyperglycemia in type 2 diabetic subjects mainly depends on a defect in the endogenous glucose output (EGO) suppression. The defect in suppressing EGO after a meal has been estimated to account for 35–50% of the excess circulating glucose in this state (20), the rest being attributed to impaired postprandial insulin stimulation of peripheral glucose uptake. The major contributor to the lack of EGO suppression and, thus, to the exaggerated postprandial hyperglycemia in diabetes seems to be an increase in gluconeogenesis (21).

Our study does not allow discriminating among systemic rate of appearance of ingested glucose, postprandial endogenous glucose production and peripheral tissue uptake. In fact, to measure the rate of appearance of ingested glucose we should have used a double tracer, one infused, as we did, and the other labeling the carbohydrate content in the meal. However, also in this case the rate of appearance of meal-derived glucose would have been influenced by a variety of factors, such as the type of complex carbohydrates, the rate of gastric emptying and oro-cecal transit time, and the fraction extracted by the liver, the latter being largely dependent on the prevailing glucose and, to a lesser extent, insulin concentrations. At this regard, it is of note that when the meal is composed of complex carbohydrates the glucose tracer can give a wide variability of the estimates of initial splanchnic glucose uptake as well as of the pattern of endogenous glucose production, which has been reported to be rapidly suppressed in some studies while in others it showed an initial paradoxical rise followed by a subsequent fall (22).

Therefore, with all the precautions of incomplete information, we can remark that sebacic acid induces a significant reduction in the glucose rate of appearance, which can be ascribed to a reduced hepatic glucose output and/or to reduced glucose absorption.

A possible mechanism through which sebacic acid might reduce EGO after a meal could be related to its mitochondrial β-oxidation with production of succinic acid (10), which enters the mitochondrial tricarboxylic acid cycle. Reduced in vivo mitochondrial function in skeletal muscle under resting conditions as well as during post-exercise recovery has been observed in type 2 diabetic subjects (23). This impaired mitochondrial oxidative capacity seems to be attributable to a reduced mitochondrial TCA cycle flux (24). C10 might overcome this defective mitochondrial TCA function in diabetic skeletal muscles by providing succinic acid and thus increasing whole body glucose clearance, being skeletal muscle tissue the major contributor to glucose uptake.

At this regard, we have observed that in L6 cells, 0.2 mM C10 significantly increased glucose uptake of about 30% as compared with insulin alone and increased 1.7 fold GLUT4 protein expression, likely improving the insulin signaling and the docking of insulin.
GLUT4 to the cell membrane. This in vitro effect of C10 might partially explain the marked decrease of plasma glucose concentrations since it is well known that the skeletal muscle mass represents the most important consumer of glucose.

We note that a true dose-response effect of C10 on glucose disposal was not present in our investigation. At this regard, it is necessary to point out that, as previously (25) demonstrated, the whole body rate of C10 tissue uptake in male healthy volunteers was $180.9 \pm 4.5 \mu\text{mole/min}$, which equals to about $0.5 \text{mg/kg bw/min}$ or to $2.1 \text{g/h}$ in a subject weighing 70 kg. Therefore, in the 5 hours following C10 ingestion with the mixed meal 10.5 g would be metabolized, thus explaining why the effects obtained with 10 or 23 g were similar.

In conclusion, although more detailed studies are required to elucidate the mechanism of action of sebacic acid in T2D, this study provides relevant clues about its effect in reducing glucose circulating levels when added to standard meals.

**Contributions.** GM and KM: participated in the conception and design of the study; AI, DG, and AF: participated in the study coordination, participant enrolment, medical oversight of participants and data collection; AG: participated in the analysis of stable isotopes; CC: participated in the in vitro studies; GM: participated in the data analysis and interpretation and wrote the article; and KM, CB and AG provided critical revisions.

**ACKNOWLEDGEMENTS**

We would like to thank Mrs. Anna Caprodossi, Catholic University, Department of Medicine in Rome, for her precious technical assistance.

The clinical study was sponsored by Nestec Ltd.

**REFERENCES**

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Table 1. Mixed Meal Composition

<table>
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<tr>
<th></th>
<th>Carbohydrates (kcal%)</th>
<th>Proteins (kcal%)</th>
<th>Lipids (kcal%)</th>
<th>Sebacic acid (kcal%)</th>
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<td>35</td>
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<td>13</td>
<td>31</td>
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<td>23g C10 (450 kcal)</td>
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FIGURE LEGENDS

**Figure 1**
Time course of plasma glucose (mmol/l), insulin (pmol/l) and sebacic acid concentrations (µg/ml) in healthy volunteers (Left Panels) and in type 2 diabetic subjects (Right Panels). Black squares indicate the control study (0 g C10), white circles the 10g C10 study and grey squares the 23 g C10 study. Data are mean ± SE. As indicated by an arrow, the meal was ingested after 120 minutes of 6,6-D$_2$-glucose primed-constant infusion to assess basal glucose Ra.

**Figure 2**
Left panels: Incremental AUC of glucose (Panel A) and insulin (Panel C) concentrations after the meal, and ratio between ∆AUC of insulin and glucose (Panel E). Right panels: AUC of glucose rate of appearance (Ra, Panel B), AUC of glucose clearance rate normalized by AUC insulin concentrations (Panel D) and insulin secretion rate (Panel F). Black bars indicate the control study (0 g C10), white bars the 10g C10 study and stripe bars the 23 g C10 study. Data are mean ± SE. * indicates statistical significance (P<0.05) between the study with and without C10.

**Figure 3**
Left panel: Effect of sebacic acid on insulin-stimulated 2-deoxyglucose (2-DG) uptake by L6 cells. Serum-starved L6 myotubes were exposed to 0.2 mM concentration of sebacic acid overnight followed by exposure to 10 nM insulin for 20 min and then incubated with 2-[H]$^3$DG for 10 min at 37°C. Results are means ± SE of 5 experiments performed in duplicate. Results are expressed as pmol/mg proteins during 10 minutes. P < 0.05 vs. insulin alone.

Right panel: Western blot analysis of GLUT-4 expression in total protein extracts from L6 myoblasts treated with 0.2 mM sebacic acid overnight. Expression of tubulin was determined to ensure similar protein loading. Y-axis represents arbitrary units. Results are means±SD.
Figure 1

**A**

Glucose Concentration (mmol/l)

**B**

MEAL

**C**

Insulin Concentration (pmol/l)

**D**

MEAL

**E**

Plasma Sebacic Acid (mg/ml)

**F**

MEAL

Time (minutes)