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Metabolism Clinical and Experimental 54 (2005) 1181–1188

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Preventive effects of a soy-based diet supplemented with stevioside on the development of the metabolic syndrome and type 2 diabetes in Zucker diabetic fatty rats

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Received 15 December 2004; accepted 14 March 2005

Abstract

The world witnesses an explosive increase in diabetes, demanding intensified prevention and treatment not least for the low-income population. The plant, *Stevia rebaudiana* Bertoni, has been used for the treatment of diabetes in traditional medicine. We have previously demonstrated that stevioside, a diterpene glycoside isolated from the plant *Stevia rebaudiana* Bertoni, possesses insulinotropic, glucagonostatic, antihyperglycemic, and blood pressure–lowering effects in animal studies. We have also found that a dietary supplement, Abalon, of soy protein, isoflavones, and cotyledon fiber has beneficial effects on cardiovascular risk markers in type 2 diabetes. The aim of this study was to investigate if the combination of stevioside and a dietary supplement of soy protein possesses beneficial qualities in the treatment of type 2 diabetes and the metabolic syndrome. We randomized male Zucker diabetic fatty rats into 4 groups and fed them the different test diets for 10 weeks: (A) standard carbohydrate-rich laboratory diet (chow), (B) chow + stevioside (0.03 g/kg body weight [BW] per day), (C) 50% soy (Abalon) + 50% chow (adjusted for vitamins and minerals), and (D) 50% soy (Abalon) + 50% chow + stevioside 0.03 g/kg BW per day. We measured plasma glucose, blood pressure, weight, and food intake once weekly. The animals were equipped with an intra-arterial catheter, and at week 10, the conscious rats underwent an intra-arterial glucose tolerance test (2.0 g/kg BW). Stevioside exerts beneficial effects in type 2 diabetic Zucker diabetic fatty rats, that is, lowers blood glucose (area under the glucose curve [AUC_{30min}]: group A vs B, a 19% reduction; and group C vs D, a 12% reduction; $P < .001$). We did not detect any effect on insulin or glucagon responses. After 2 weeks of treatment, a decrease in the systolic blood pressure was observed in the stevioside-treated groups ($P < .01$). Abalon had beneficial effects on cardiovascular risk markers, that is, (1) lowers total cholesterol ($P < .01$), (2) reduces triglycerides ($P = .01$), and (3) reduces free fatty acids ($P < .001$). The combination of stevioside and soy supplementation appears to possess the potential as effective treatment of a number of the characteristic features of the metabolic syndrome, that is, hyperglycemia, hypertension, and dyslipidemia. A long-term human study of the concept in type 2 diabetic subjects is needed to verify these promising results in animal diabetes.

1. Background

Type 2 diabetes is a global epidemic that currently affects more than 150 million people, a number that is expected to rise to 300 million by 2025 [1]. The cluster of hypertension, central adiposity, dyslipidemia, and diabetes, that is, the metabolic syndrome [2,3], is tightly associated with coronary artery disease.

According to several large intervention studies, it seems possible to improve insulin sensitivity and to prevent the development of type 2 diabetes by lifestyle changes, primarily weight reduction, dietary interventions, and exercise [4-8]. Moreover, pharmacological intervention with metformin [8], troglitazone [9], or acarbose [10] has been shown to delay the development of type 2 diabetes from a prediabetic state. The fact that type 2 diabetes has a 4- to 6-fold increase in cardiovascular mortality [11] makes prevention of type 2 diabetes a key issue. Interestingly, diets low in fat and high in carbohydrates from grains, fruits, and vegetables are associated with a lower risk of cardiovascular

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doi:10.1016/j.metabol.2005.03.026

disease [12]. Furthermore, the major components of soybean flour (ie, soy protein, soy cotyledon fiber, and isoflavones) appear to independently decrease serum cholesterol [13]. A recent meta-analysis of 38 controlled clinical trials indicates that soy protein is effective in lowering plasma cholesterol, low-density lipoprotein cholesterol, and triglyceride concentrations [14]. In addition, the Food and Drug Administration states that 25 g of soy protein a day may reduce the risk of heart disease [15].

Next to hyperglycemia and dyslipidemia, blood pressure has been identified as an important factor in determining the risk of macrovascular and microvascular complications in type 2 diabetes [16]. Both the Heart Outcomes Prevention Evaluation [17] study and the UK Prospective Diabetes Study Group–Hypertension in Diabetes Study [16] provide convincing evidence on the beneficial effects of blood pressure lowering on microvascular complications of type 2 diabetes.

Extracts of leaves of the plant *Stevia rebaudiana* Bertoni have been used for many years in traditional medicine in South America for the treatment of diabetes [18]. The glycoside stevioside constitutes about 10% of the dry matter of *Stevia rebaudiana* Bertoni. Our group has previously shown that stevioside in vitro exerts a direct insulinotropic action in both isolated mouse islets and the clonal beta cell line, INS-1 [19,20]. We have shown that long-term treatment with stevioside in the nonobese animal model of type 2 diabetes, the Goto-Kakizaki (GK) rat [21,22], lowers blood glucose and blood pressure [23].

Furthermore, a double-blind placebo-controlled human study in nondiabetic hypertensive subjects showed that 250 mg stevioside thrice daily for 2 years had a clear-cut blood pressure–lowering effect [24].

Thus, stevioside seems to have a dual beneficial quality in type 2 diabetes, with an antihyperglycemic, and a blood pressure–lowering effect. The beneficial effect has, however, only been demonstrated in the nonobese mild type 2 diabetic GK rat. We wanted to extend our investigation to another diabetes model, that is, the obese type 2 diabetic Zucker diabetic fatty (ZDF) rat. These animals progress through several stages in the development of diabetes in a relatively predictable age-dependent fashion, when maintained under standard conditions [25]. The ZDF rats rapidly progress from a hyperinsulinemic-euglycemic (insulin-resistant) state to a hyperglycemic insulin-deficient state when approximately 12 weeks of age [25].

Consequently, we wanted to explore the impact of the combination of stevioside and soy supplement on the glycemic control and the blood pressure in the obese male ZDF rats.

2. Materials and methods

2.1. Animals

Forty-eight 8-week-old male obese ZDF rats (Charles River Genetic Models, Indianapolis, Ind, USA) were randomized to 4 groups (12 animals in each group) and fed different diets (A, B, and C, D). Diet A consisted of a standard chow laboratory animal diet (Altromin 1324, Altromin, Lage, Germany). Diet B consisted of chow and a supplement of stevioside (0.03 g/kg body weight [BW] per day) put into the drinking water once daily (8 mL). Group C received a diet consisting of 50% soy protein (Abalon, Nutri Pharma ASA, Oslo, Norway) + 50% chow, adjusted for vitamins and minerals, and finally, group D was given the same soy protein–enriched diet but with a supplement of stevioside (0.03 g/kg BW per day) put into the drinking water (8 mL) once daily. The animals had free access to tap water.

Before entering the experiment, all the animals were fed the standard chow diet for laboratory rats (Altromin). We performed the experiments in accordance with the Danish Council on Animal Care.

2.2. Stevioside

Stevitafarm Industrial S/A (Maringa, Parana, Brazil) supplied the stevioside supplement. It consisted of 91% pure stevioside, 4% rebaudioside A, and 5% other glycosides. The plants used for the production originate from Paraguay and Brazil (Amambay mountain area).

2.3. Measurements during the study period

We monitored the weight and food consumption throughout the study period. Blood glucose was measured once weekly after an overnight fast, with OneTouch (Johnson&Johnson, Milpitas, Calif, USA) using blood collected after transection of the tip of the tail. Systolic blood pressure was monitored weekly with the automatic system 209002 (TSE GmbH, Bad Homburg, Germany) with animals placed in a restrainer under calm conditions. Before the experiment, animals were trained in the use of the restrainer.

2.4. Intra-arterial glucose tolerance test

After 9 weeks of dietary treatment, the animals were equipped with an intravascular catheter for blood sampling and infusion as described elsewhere [23]. In brief, the animals were anesthetized by SC injection of 1 mL/kg BW of a mixture of 0.08 mg/mL fentanyl citrate + 2.5 mg/mL fluanisone (Janssen Pharmaceutica, Beerse, Belgium) and 1.25 mg/mL midazolam (Dumex-Alpharma A/S, Oslo, Norway). We inserted the catheter (Tygon Microbore Tubing, Norton Performance Plastics, Akron, OH; internal diameter 0.40 mm, outer diameter 0.78 mm) into the right carotid artery and exteriorized it at the neck of the animal. The catheter was filled with 0.9% saline containing 10 U/mL heparin (Løvens Kemiske Fabrik, Ballerup, Denmark). Naloxone (DuPont Pharmaceuticals Ltd, Hertfordshire, UK) 0.08 mg was injected IM after surgery. The animals caged individually continued on their respective diets. We secured patency of the catheter by daily flushing with 0.2 mL of the saline/heparin solution. After 5 days of recovery, the animals underwent an intra-arterial glucose tolerance test (IAGTT).

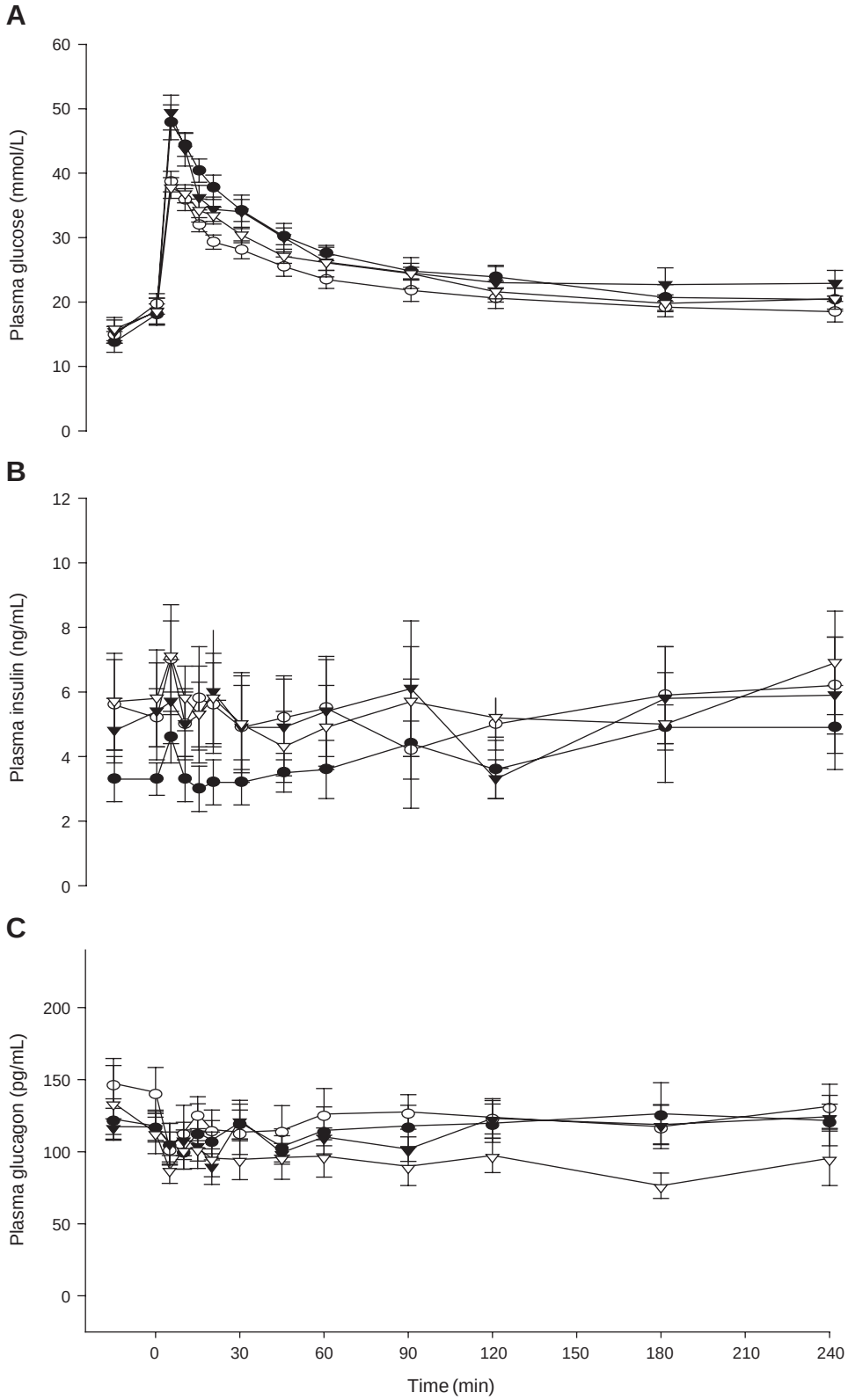

Fig. 1. Effect of stevioside and a soy-rich diet on plasma glucose levels (A), plasma insulin responses (B), and plasma glucagon (C) during IAGTT in conscious ZDF rats after 10 weeks' intervention. (●) Standard chow diet, (○) standard chow diet + stevioside, (▼) soy-rich diet, and (▽) soy-rich diet + stevioside. Data are shown as mean ± SEM (n = 11-12 in each group).

Animals were fasted 14 hours (water ad libitum) before the IAGTT, which started at 8:30 AM. The animals were conscious and allowed to move freely in separate plastic cylinders. The catheter connected to a tubing (Tygon Microbore Tubing) allowed blood sampling and infusion of glucose. Glucose (2.0 g D-glucose/kg BW) was prepared as a 30% D-glucose solution in 0.9% saline and infused as a bolus over 20 seconds.

Blood samples were withdrawn at 15 minutes before and immediately before the glucose infusion and hereafter at time points 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 minutes after glucose infusion. Two hundred–microliter blood samples were taken on chilled tubes containing heparin/aprotinin and centrifuged (4000 g, 60 seconds, 4°C), and plasma was frozen for subsequent analysis of insulin, glucose, glucagon, free fatty acids (FFAs), triglycerides, and cholesterol. The blood cells drawn from the animals were resuspended in 0.9% saline (in a volume equal to the plasma drawn from each sample) and reinfused to prevent volume depletion.

2.5. Assays

Blood glucose during the IAGTT was determined using the glucose oxidase method (GOD-PAP, Boehringer Mannheim, Mannheim, Germany). Insulin was determined by radioimmunoassay with a guinea pig antiporcine insulin antibody (PNILGP4, Novo Nordisk, Bagsvaerd, Denmark), and mono-[^{125}I]-(Tyr A14) labeled human insulin (Novo Nordisk) as tracer and rat insulin (Novo Nordisk) as standard. We separated free and bound radioactivity using ethanol [26]. Interassay and intra-assay variation was below 10%. Stevioside did not interfere with the insulin assay at the concentrations studied.

Triglycerides, FFAs, and total cholesterol were determined using colorimetric kits (Boehringer Mannheim).

The animals continued on the same treatments after the IAGTT. One week later, we anesthetized the animals as described previously and conducted bioimpedance measurements to determine the fat mass. Using SEAC Multiple Frequency Bioimpedance meter (Model SFB3, UniQuest Ltd, Queensland, Australia), the distance between the electrodes, and a mathematical equation, it is possible to obtain a measurement of the fat mass in percent of the total BW [27].

2.6. Statistical analysis

We used a 1-way analysis of variance test to assess overall differences between groups. If significant, we used the Newman-Keuls test for multiple differences to compare differences between experimental groups.

If the data did not approximate to the normal distribution, even after log transformation, we used the Kruskal-Wallis test to assess overall differences between groups. If significant, we used the Mann-Whitney *U* test to compare differences between experimental groups. We applied the Bonferroni method to correct for multiple comparisons. We used a 2-way analysis of variance test with soy and stevioside as dependent variables to test for effects of different diets. We applied the trapezoidal method to calculate the area under the curve (AUC). Results are presented as mean ± SEM.

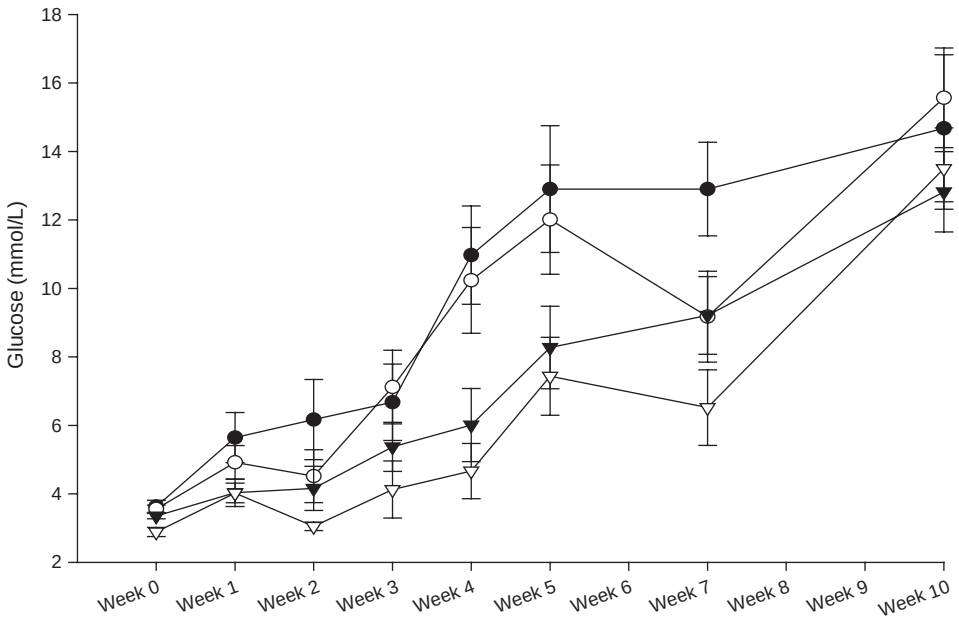

Fig. 2. Effects of stevioside and a soy-rich diet on fasting plasma glucose levels during the 10 weeks' treatment period. The glucose levels were measured using a OneTouch glucose monitoring apparatus after an overnight fast. (●) Standard chow diet, (○) standard chow diet + stevioside, (▼) soy-rich diet, and (▽) soy-rich diet + stevioside. Data are shown as mean ± SEM (n = 11-12 in each group).

3. Results

3.1. Effects of long-term treatment with a soy-rich diet and stevioside on IAGTT in type 2 diabetic obese ZDF rats

Before the glucose infusion, we did not detect any difference in fasting levels of either plasma glucose (A, 18.1 ± 1.5 mmol/L; B, 19.7 ± 1.5 mmol/L; C, 18.6 ± 1.9 mmol/L; D, 18.5 ± 2.0 mmol/L; $P = .9$) (Fig. 1A), insulin (A, 3.7 ± 0.5 ng/mL; B, 5.2 ± 0.9 ng/mL; C, 5.4 ± 1.4 ng/mL; D, 5.7 ± 1.4 ng/mL; $P = .8$) (Fig. 1B), or glucagon (A, 117.8 ± 10.3 pg/mL; B, 141.2 ± 16.3 pg/mL; C, 117.2 ± 8.7 pg/mL; D, 113.2 ± 13.5 pg/mL; $P = .6$) (Fig. 1C) between the 4 groups (n = 8-10 in each group) of overnight fasted ZDF rats. After the glucose injection, there was a smaller increase in plasma glucose in the stevioside-treated groups for both the standard chow ($AUC_{0\text{-}30min}$: A, 943 ± 32 vs B, 1163 ± 41 mmol/L × 30 minutes) and the soy-rich diet ($AUC_{0\text{-}30min}$: C, 992 ± 37 vs D, 1121 ± 58 mmol/L × 30 minutes), respectively ($P < .001$). During the 240-minute period, plasma glucose tended to be lower in the 2 stevioside-treated groups compared with the groups fed a standard chow diet ($AUC_{0\text{-}240min}$: A, 5352 ± 332 vs B, 6165 ± 300 mmol/L × 240 minutes) or soy-rich diet ($AUC_{0\text{-}240min}$: C, 5721 ± 282 vs D, 6235 ± 505 mmol/L × 240 minutes), respectively. However, this did not reach significance ($P = .08$).

First-phase insulin response, defined as 0 to 30 minutes, was similar in the stevioside-treated groups compared with the standard chow and soy-fed animals without stevioside ($AUC_{0\text{-}30min}$: A, 168 ± 35 vs B, 104 ± 19 ng/L ± 30 minutes, and $AUC_{0\text{-}30min}$: C, 174 ± 33 vs D, 163 ± 38 ng/L × 30 minutes), respectively ($P = .2$). Insulin responses was similar during the IAGTT overall for the 2 stevioside-treated groups compared with the 2 groups receiving the same diets without stevioside ($AUC_{0\text{-}240min}$: A, 1296 ± 260 vs B, 995 ± 275 ng/L × 240 minutes, and $AUC_{0\text{-}240min}$: C, 1296 ± 193 vs D, 1252 ± 283 ng/L × 240 minutes) for chow-fed and soy-fed groups, respectively ($P = .6$).

The level of plasma glucagon, measured as AUC, was similar between the groups during the IAGTT, and thus we did not detect any effect of stevioside on the glucagon level.

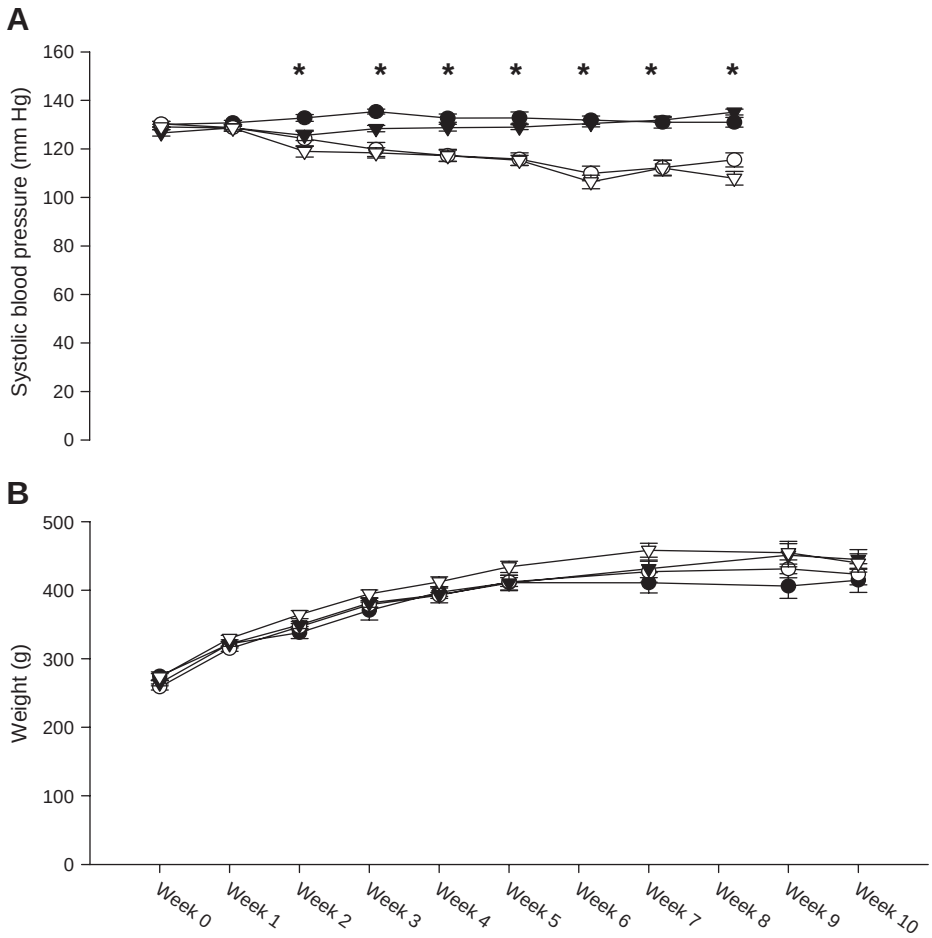

Fig. 3. Effects of stevioside and a soy-rich diet on systolic blood pressure (A) and BW (B). (●) Standard chow diet, (○) standard chow diet + stevioside, (▼) soy-rich diet, and (▽) soy-rich diet + stevioside. Blood pressure was significantly lowered for stevioside-treated groups compared with respective treatment without stevioside (*$P < .01$). We did not detect any significant differences in weight development between the groups at any time point. Data are shown as mean ± SEM (n = 11-12 in each group).

Table 1
Effects of 11 weeks' stevioside and soy-rich diets on blood lipids measured 1 week after IAGTT

	Group A (chow)	Group B (chow + stevioside)	Group C (soy)	Group D (soy + stevioside)	*P* value (soy effect)
FFA (mmol/L)	2.5 ± 0.3	2.3 ± 0.4	1.2 ± 0.2	1.3 ± 0.2	<.001
Cholesterol (mmol/L)	3.7 ± 0.2	3.6 ± 0.2	2.7 ± 0.3	3.3 ± 0.3	<.01
Triglycerides (mmol/L)	3.9 ± 0.8	4.4 ± 0.9	2.4 ± 0.2	3.1 ± 0.5	.01

The *P* values refer to the effect of soy treatment on the measured blood lipids. We did not detect any independent effect of stevioside or any interaction between stevioside and soy treatment. Data are shown as mean ± SEM (n = 9-11).

3.2. Effects of long-term treatment with a soy-rich diet and stevioside on the level of fasting blood glucose in type 2 diabetic obese ZDF rats

As indicated in Fig. 2, we observed a difference in the course of blood glucose development in the soy-treated groups compared with the groups fed a standard chow diet. The soy-fed groups tended to have lower blood glucose levels compared with the chow-fed groups until the end of the study period, where the values did not differ.

3.3. Effects of long-term treatment with a soy-rich diet and stevioside on blood pressure and BW in type 2 diabetic obese ZDF rats

As illustrated in Fig. 3A, initial systolic blood pressures were similar in the 4 groups. After 2 weeks' treatment and for the rest of the study period, a significant difference in the systolic blood pressure was observed in the 2 groups receiving stevioside compared with the 2 groups which received the same diet without stevioside ($P < .01$). The last blood pressure measurement was, for technical reasons, performed after 8 weeks of treatment. At this time point, systolic blood pressure had dropped by 14 ± 3 mm Hg (in group B) and 21 ± 3 mm Hg (in group D) in the 2 groups receiving chow and soy supplemented with stevioside, respectively, compared with the blood pressure before treatment. The soy diet did not have any independent effect on the blood pressure.

Weight gain was similar in all 4 experimental groups throughout the study period (Fig. 3B). Bioimpedance measurements at the end of the study showed similar body composition (average percent fat mass) among the groups (A, 55.7 ± 1.6; B, 51.7 ± 1.9; C, 54.6 ± 1.2; D, 55.2 ± 1.1; $P = .3$) (Fig. 4).

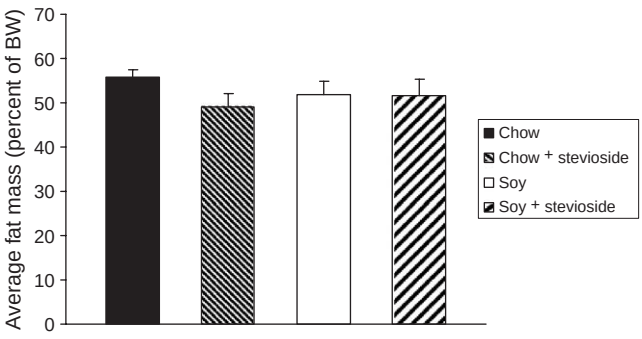

Fig. 4. Effects of stevioside and a soy-rich diet for 10 weeks on body composition displayed as average fat mass in percentage of BW. No significant difference in fat mass in percentage of BW between the groups was observed ($P = .3$). Data are shown as mean ± SEM (n = 8-11 in each group).

3.4. Effects of long-term treatment with a soy-rich diet and stevioside on fasting plasma levels of FFA, triglycerides, and cholesterol in type 2 diabetic obese ZDF rats

The levels of fasting lipids (Table 1) were significantly lower in the soy-treated group compared with the normal chow diet when measured as plasma FFA (in millimole per liter) ($P < .001$), plasma total-cholesterol (in millimole per liter) ($P < .01$), and triglycerides (in millimole per liter) ($P = .01$) at the end of the study. Stevioside treatment did not have any independent effect, and we did not detect any interaction between stevioside and soy treatment, on the measured plasma lipid levels.

4. Discussion

We tested the hypothesis that the diterpene glycoside stevioside exerts both antihyperglycemic and blood pressure–lowering effects and that supplement with the soy supplement, Abalon, exerts lipid-lowering effects after long-term treatment in the obese ZDF rat.

We studied male ZDF rats, which spontaneously develop a type 2 diabetic state with age. The launching of the experiments was postponed due to different external factors (ie, the animals had reached the age of 18 weeks at the end of the study). The advanced age of the rats resulted in relatively high fasting blood glucose levels, compatible with a rather severe diabetic state. In spite of this, we detected a significantly larger reduction in the glucose AUC during the first 30 minutes of the IAGTT in the stevioside-treated rather than in the nontreated groups (by 19% vs 12%, $P < .001$), respectively. Because no difference in insulin responses was detected concomitantly, this indicates that stevioside may improve the insulin sensitivity. It should, however, be kept in mind that the insulin and glucagon levels derive from peripheral blood sampling. Consequently, we cannot exclude that there has been a change in the hormone secretion from the pancreas at the portal vein level, an effect that may have disappeared due to dilution in the periphery.

Throughout the treatment period, we detected lower levels of fasting blood glucose in the soy-treated groups (Fig. 2). This effect faded at the end of the study period. A possible explanation could be that the animals at this point

had developed a severe insulinopenic state. We did not detect any separate effect of stevioside on fasting blood glucose, which corroborates our previous findings in the GK rat [23].

Figs. 1A and 2 show a difference in the fasting glucose levels at the age of 18 weeks between blood collected from the tail and blood taken from the carotid artery before the IAGTT. We believe that this difference at least in part is due to different methods applied to determine the glucose levels. Stevioside seems able to improve the insulin sensitivity during the IAGTT despite the severe diabetic state.

We have previously demonstrated a steadily increasing insulin concentration and suppression of glucagon both during the glucose tolerance test in long-term stevioside-treated GK rats [23] and after a single-bolus injection of stevioside in anesthetized GK rats [28]. The present study corroborates our previous demonstration of a reduced level of blood glucose during a glucose tolerance test after stevioside treatment. However, in the present study, we were not able to detect any effect of stevioside on insulin responses. An explanation for this discrepancy could at least in part be that the animals in the present study had developed severe diabetes with a subdued endogenous insulin secretion.

One week after the IAGTT, we detected improved lipid profiles in the soy groups, compared with the chow groups. Thus, plasma FFA, triglycerides, and total cholesterol were lower in the soy-treated groups, irrespective of addition of stevioside.

Type 2 diabetes is a chronic metabolic disorder that not only results from insulin resistance and reduced first-phase insulin secretion, but is also characterized by a relative glucagon excess and a pancreatic alpha-cell dysfunction [29]. A reduction in the circulating glucagon concentration is accompanied by a fall in the blood glucose [30], supporting that agents inhibiting the glucagon secretion or action are beneficial for patients with type 2 diabetes. In experimental diabetes, an abnormal alpha-cell function is characterized by an impaired response to glucose and certain metabolites, probably secondary to a defect in the glucose recognition [31]. The abnormal alpha-cell function seems not to be ascribed to insulin deficiency per se, but rather to an abnormal metabolic state secondary to insulin deficiency [32]. In the present study, we did not reveal any effect on glucagon levels neither corresponding to the soy-enriched diet, nor the supplementation with stevioside. This is in contrast to our previous finding in GK rats [23] where stevioside suppressed the glucagon level during the first 30 minutes of the glucose tolerance test. Differences in the rat type or the severity of diabetes may explain this controversy.

As can be seen from Fig. 3B, the average weight development did not differ among the groups throughout the study period. Bioimpedance measurements performed at the end of the study showed similar body composition among the groups. Thus, we conclude that the dietary intervention with supplementation with soy and stevioside does not cause any effect on the weight development or body composition in the ZDF rat. This corroborates our previous findings of similar weight developments in stevioside-treated GK rats [23].

The initial systolic blood pressure was similar in the 4 groups (Fig. 3A). Two weeks' treatment with stevioside reduced the systolic blood pressure (groups B and D). This supports our previous finding of a blood pressure–lowering effect of stevioside observed after 2 weeks' treatment in the GK rat [23]. The soy diet did not have any independent effect on the blood pressure.

In conclusion, long-term administration of a combination of stevioside and soy protein possesses beneficial effects on the main features of the metabolic syndrome, that is, it lowers blood glucose, reduces blood pressure, and improves the blood lipid profile.

The expected large increase in diabetes worldwide demands intensified investigations in new approaches for prevention and treatment of diabetes suitable even for low-income populations in developing countries. In this respect, stevioside and other diterpene glycosides from the *Stevia* plant may be a new useful treatment modality. Supplementation with soy protein may further reduce the risk of cardiovascular disease, the main death cause in type 2 diabetes, and the metabolic syndrome.

Acknowledgment

The study was supported by the Danish Medical Research Council; Institute of Experimental Clinical Research, Aarhus University; Aarhus Amtssygehus Forskningsfond; Research Foundation of Aarhus University, The Faculty of Health Science, Aarhus University; The A.P. Møller Foundation for the Advancement of Medical Science; and the Novo Nordisk Foundation.

The authors thank Lene Trudsø, Kirsten Eriksen, Tove Skrumsager, and Dorthe Rasmussen for skillful technical assistance.

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