Comparative Effects of Stevia rebaudiana Leaves and Stevioside on Glycaemia and Hepatic Gluconeogenesis

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Comparative Effects of *Stevia rebaudiana* Leaves and Stevioside on Glycaemia and Hepatic Gluconeogenesis

Abstract

The purpose of the present study was to compare the effect the oral treatment (gavage) with *Stevia rebaudiana* (Bert.) Bertoni (SRB) and stevioside (STV) on glycaemia and gluconeogenesis of 15-h fasted rats. For this purpose, the rats received SRB (20 mg/kg × day), STV (5.5 mg/kg × day) or an equal volume of water (controls) during 15 days. To measure hepatic gluconeogenesis, liver perfusion and isolated hepatocytes were used. Glycaemia and gluconeogenesis from L-alanine (5 mM), L-glutamine (5 mM) and L-lactate (2 mM) were decreased ($P < 0.05$) after pre-treatment with SRB. However, the treatment with STV did not influence glycaemia and gluconeogenesis. Moreover, to get further information about the mechanism by which SRB leaves inhibit gluconeogenesis their potential role as a PPARγ agonist was investigated. The data showed absence of activation of PPARγ receptors. In summary, our results showed that the reduction of glycaemia promoted by the treatment with SRB leaves was mediated, at least in part, by an inhibition of hepatic gluconeogenesis. However, this effect did not involve stevioside and the activation of PPARγ receptors.

Key words
Stevia rebaudiana (Bert) Bertoni · stevioside · gluconeogenesis · glycaemia · PPARγ agonist

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Introduction

Native to north-eastern Paraguay and southern Brazil, the leaves of *Stevia rebaudiana* (Bert) Bertoni (SRB) have been used by the Guarani Indians to treat diabetes and this popular knowledge have been passed on by oral tradition for many centuries.

This empirical knowledge led researchers to study the antidiabetic properties of this plant. The reports about the influence of SRB upon diabetes started with Oviedo et al. [1], who first demonstrated the hypoglycaemic effect of SRB leaves. From this study, several publications, including our previous investigations in rats [2] and human [3] confirmed that the treatment with SRB leaves could reduce fasting glycaemia.

Several reports [4], [5], [6], [7], [8], [9] suggest that the effect of SRB leaves on glycaemia could be mediated, at least in part, by the main sweet component, i.e., stevioside (STV). However, the hypoglycaemic effect of oral STV is still controversial, because STV is degraded by the intestinal microflora of rats [10], pigs [11] and human [10] to the diterpenoid aglycone steviol. However, the presence of steviol in the blood after oral ingestion of stevioside was not confirmed [12].

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Therefore, considering that the effect of SRB leaves on fasting glycaemia mediated by STV was not well established, the present study was undertaken to investigate this possibility. Moreover, the role of liver gluconeogenesis, that is crucial for the maintenance of fasting glycaemia [13], and the participation of peroxisome proliferator-activated receptors gamma (PPARγ), a nuclear mediator of insulin sensitivity [14], [15] were examined. For this purpose, the experiments were executed by using 15-h fasted rats, which represents a favourable system for gluconeogenesis [13] and reporter gene transcription assays modulated by PPARγ in mammalian cells [16].

Materials and Methods

Plant material: SRB leaves and stevioside

Dried powdered leaves manufactured under the highest quality control were obtained from Steviafarma (Maringá, Brazil). From these dried powdered leaves standardised stevioside was obtained by a method that produces a mixture of stevioside and rebaudioside [17].

Animals and treatment protocol

Male Wistar rats (Maringá, Brazil) weighing about 220 g were employed. The manipulation of the animals followed the Brazilian Law on the protection of animals.

Experimental groups received 20 mg/kg × day SRB (SRB group) or 5.5 mg/kg × day STV (STV group) dissolved in water and administered (gavage) at 5 : 00 p. m. during 15 days. Control groups received water (gavage). During the treatment food and water were freely available.

Determination of glycaemia

After 15 days of treatment food was withdrawn and the rats were killed by decapitation 15 h later. Blood was immediately collected, centrifuged and the glycaemia was determined [18].

Liver perfusion experiments

After 15 days of treatment food was withdrawn and the rats were anaesthetised by an *i.p.* injection of sodium pentobarbital (40 mg/kg) and submitted to laparotomy. The livers were perfused *in situ* using Krebs Henseleit bicarbonate buffer (KHB), pH 7.4, saturated with O_2/CO_2 (95/5%). The perfusion fluid was pumped through a temperature controlled (37 °C) membrane oxygenator prior to entering the liver via the portal vein. The perfusion was performed in an open system, without recirculation of the perfusate. A constant flow rate in each experiment was adjusted according to the liver weight (4 mL/g of tissue fresh weight × min).

Fig. **1** illustrates the result of a liver perfusion experiment in which the protocol described above was employed. As shown in Fig. **1** after a pre-perfusion period (10 min), the gluconeogenic substrate was dissolved in the perfusion fluid and infused between the 10th and 30th min of the perfusion period, followed by a post-infusion period (20 min) to allow the return to basal levels.

Samples of the effluent perfusion fluid were collected at 2-min intervals and the glucose concentration was analysed [18]. The differences in the glucose production during (10 – 30 min) and before (0 – 10 min) the infusion of glycerol allowed calculation of the areas under the curves (AUC), expressed as μmol/g. Moreover, similar procedures were performed when L-glutamine (5 mM), L-alanine (5 mM) and L-lactate (2 mM) were used as the gluconeogenic substrates. During the liver perfusion experiments, L-lactate [19], pyruvate [20] and urea [21] production were also measured. Thus, the AUC of Tables 1**4** were obtained from similar experiments shown in Fig. **1**.

Isolation and incubation of isolated hepatocytes

The isolation of hepatocytes was executed by a technique employing collagenase first described by Berry and Friend [22] and modified by Bazotte [23]. For this purpose 15-h fasted rats were anaesthetised and submitted to laparotomy. The liver perfusion started with Ca^{2+}-free KHB. Afterwards, this perfusion fluid was replaced by KHB containing collagenase (0.035%) and Ca^{2+} (2.5 mM). In this phase the perfusion was done with recirculation of the perfusate that returned to the reservoir and recirculated. After 5 – 10 min, the fluid commenced to ooze freely from the surface of the liver. Within 10 – 30 min, the loss of fluid from the liver surface was too great for the perfusion to be maintained. At this point the consistency of the liver was so soft that it disintegrated on pressure unless handled very gently. The liver was then carefully removed, transferred to a Petri dish with 10 – 20 mL of enzyme medium at 4 °C, and broken up with a blunt spatula. After the filtration and centrifugation, we obtained a supernatant with 95 – 98% of isolated hepatocytes that, upon microscopic examination (Newbauer chamber), did not stain with trypan blue. The isolated hepatocytes (2×10^6 cells/mL) obtained by the technique described above were incubated during 60 min with KHB (5 mL) containing L-alanine (5 mM), L-lactate (2 mM), L-glutamine (5 mM), glycerol (2 mM) or no substrate (to get basal values). All flasks were gassed (O_2/CO_2 : 95/5%) and maintained in a shaking water bath at 37 °C during the incubation period. Last-

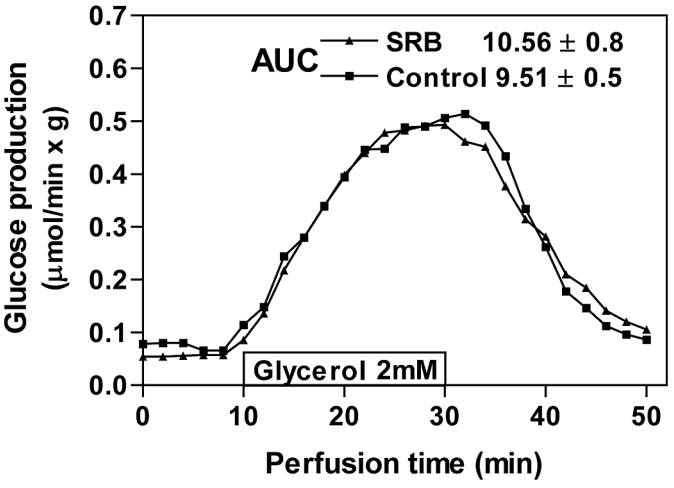

Fig. **1** Demonstrative experiment of glucose production from glycerol in isolated perfused liver from 15-h fasted rats that received during 15 days *Stevia rebaudiana* (Bert.) Bertoni leaves (SRB group) or water (control group) orally administered. The livers were perfused as described in Materials and Methods. The differences in the glucose production during (10 – 30 min) and before (0 – 10 min) the infusion of glycerol allowed calculation of the areas under the curves (AUC), expressed as μmol/g.

ly, the incubation medium was centrifuged and the supernatant was used for glucose [18] determination.

Cell culture, transfection and PPAR reporter transactivation assay

Human promonocyte U937 cells (Cell Culture Facility, University of California; San Francisco, CA, USA) were maintained on 37 °C, CO_2 (5 %) subcultured in RPMI-1640 (GIBCO; Grand Island, NY, USA) medium with foetal calf serum (10 %), glutamine (2 mM), penicillin (50 units/mL) and streptomycin (50 μg/mL). For transfection assays, the cells were collected by centrifugation and resuspended in transfection solution (0.5 mL/1.5 × 10^7 cells) containing PBS (phosphate-buffered saline), Ca^{2+} (100 mM) plus dextrose (0.1 %) and mixed with 1.5 μg of human PPARγ expression vector (provided by J. Magae, Japan) [16] and 3 μg of the luciferase (Luc) reporter (provided by J. Magae, Japan). This reporter plasmid has a synthetic PPARγ response element, containing one copy of DR-1 (5′-agcttcaggtcagaggtcagag-3′) cloned immediately upstream of a minimal thymidine kinase (tk) promoter (–32/+45) linked to luciferase coding sequences. Afterwards, the cells were transferred to a cuvette and electroporated using a Bio-Rad gene pulser at 300 mV and 950 μF. Immediately after electroporation, the cells were transferred to fresh RPMI-1640 medium, plated in a 12-well dish and treated in triplicates with ethanol:DMSO 1 : 1 (vehicle), Pioglitazone (Sigma; St. Louis, MO, USA) at 10^{-5} M (PPARγ agonist) and lyophilised SRB leaves. After 24 h, cells were collected by centrifugation, lysed by the addition of 150 μmL 1 × lysis buffer (Promega; Madison, WI, USA) and assayed for luciferase activity (kit from Promega Corp.). Transfection experiments were performed at least three times. Fold activation was calculated using the ratio of luciferase numbers obtained by the samples treated with ligands with the templates treated with vehicle. Finally, to the transfection assay [24], 1 g of dried and pulverised leaves was heated/boiled for 5 min in 100 mL of water, the decoction was allowed to stand for 30 min and then filtered through a paper filter. Then, the decoction was lyophilised, and this material was kept at –20 °C until use.

Statistical analysis

The GraphPad Prism program (version 2.0) was used to calculate the areas under the curves (AUC). Data were analysed statistically by the unpaired Student t test and ANOVA. A 95 % level of confidence ($P < 0.05$) was accepted for all comparisons. Results were reported as means ± SEM.

Results and Discussion

There are few studies showing the effectiveness of herbal therapy for the management of diabetes [25]. In this context, our data shown quite clearly that SRB leaves, but not stevioside, orally-administered during 15 days decreased ($P < 0.05$) glycaemia (Table **1**).

In contrast to our results, Jeppesen et al. [6] demonstrated favourable effects of stevioside on glycaemia. Since we used approximately the same daily dose, such a discrepancy could be attributed to several possibilities: 1) the experimental model (Wistar rats vs. type 2 Goto-Kakizaki rats); 2) the period of treatment (2 weeks vs. 6 weeks); 3) the parameter evaluated (fasting glycaemia vs. glucose tolerance test) etc. Another possibility not mentioned by Jeppesen et al. [6] could be the composition of the stevioside.

Since hepatic gluconeogenesis is crucial for glucose maintenance during fasting [13] this metabolic pathway was investigated in livers from rats treated with SRB leaves (SRB group) and the results were compared with livers of rats which received an equal volume of water (control group).

The SRB group showed lower liver glucose production ($P < 0.05$) from L-alanine, whereas the catabolism of L-alanine, inferred from the production of urea, pyruvate and L-lactate was not modified (Table **2**). Thus, the possibility of a decreased glucose production from the pyruvate step should be considered. In confor-

Table **2** Glucose, urea, pyruvate and l-lactate production from L-alanine (5 mM) in isolated perfused liver from 15-h fasted rats that received *Stevia rebaudiana* (Bert.) Bertoni leaves (SRB group) or water (control group). SRB (20 mg/kg × day) or water was administered by gavage during 15 days. The values of area under the curves (AUC) were calculated as described in Materials and Methods (Fig. **1**). The data are reported as mean ± SEM of 4 – 6 experiments

Parameters	*Values of AUC (µmol/g)*	
	Control	*SRB*
Glucose	6.08 ± 0.73	3.45 ± 0.39*
Urea	25.94 ± 1.69	27.55 ± 1.90
Pyruvate	3.51 ± 0.52	4.25 ± 0.61
l-Lactate	5.61 ± 0.88	5.99 ± 1.10

* $P < 0.05$ vs. control group.

Table **1** Effect of the treatment with *Stevia rebaudiana* (Bert.) Bertoni leaves (SRB) or stevioside (STV) on glycaemia (mg/dL). Experimental groups received 20 mg/kg × day SBR (SRB group) or 5.5 mg/kg × day STV (STV group) dissolved in water and daily administered (gavage) at 5 : 00 p. m. during 15 days. The control groups received an equal volume of H_2O (gavage). Blood samples were collected from 15-h fasted rats

Parameter	*SRB treatment*		*STV treatment*	
	Control	*SRB*	*Control*	*STV*
Glycaemia	94.10 ± 2.99	67.83 ± 5.70*	86.39 ± 4.64	91.50 ± 6.00

$P < 0.05$ vs. control group.

Table **3** Glucose and pyruvate production from L-lactate (2 mM) in isolated perfused livers from 15-h fasted rats that received *Stevia rebaudiana* (Bert.) Bertoni leaves (SRB group) or water (Control group). SRB (20 mg/kg x day) or water was administered by gavage during 15 days. The values of area under the curves (AUC) were calculated as described in Materials and Methods (Fig. **1**). The data are reported as mean ± SEM of 4 – 6 experiments.

Parameters	*Values of AUC (µmol/g)*	
	Control	*SRB*
Glucose	10.81 ± 1.19	6.50 ± 0.95*
Pyruvate	3.30 ± 0.50	6.75 ± 0.98*

* P < 0.05 vs. control group.

Table **4** Glucose production from L-glutamine (5 mM) and glycerol (2 mM) in isolated perfused livers from 15-h fasted rats that received *Stevia rebaudiana* (Bert.) Bertoni leaves (SRB group) or water (control group). SRB (20 mg/kg x day) or water was administered by gavage during 15 days. The values of area under the curves (AUC) were calculated as described in Materials and Methods (Fig. **1**). The data are reported as mean ± SEM of 4 – 6 experiments

Parameters	*Values of AUC (µmol/g)*	
	Control	*SRB*
Glucose from L-glutamine	24.82 ± 1.0	16.93 ± 1.0*
Glucose from glycerol	9.51 ± 0.5	10.51 ± 0.8

* P < 0.05 vs. control group.

mity with this proposition, livers from SRB group exhibited higher (P < 0.05) pyruvate and lower (P < 0.05) glucose production from L-lactate (Table **3**). These results suggest that gluconeogenesis from the pyruvate step was decreased in the livers from the SRB group. Furthermore, lower glucose production (Pc< 0.05) was also obtained when L-glutamine was used as glucose precursor (Table **4**).

In contrast to L-alanine, L-lactate and L-glutamine the glucose production from glycerol was not affected by the treatment with SRB leaves (Table **4**).

The results obtained from the liver perfusion experiments were summarised in the Fig. **2**. Starting with L-alanine and L-lactate that enter in the gluconeogenic pathway at pyruvate step, our results suggest that pyruvate carboxylase (PC) and phosphoenol pyruvate carboxylase (PEPCK) could be inhibited by the treatment with SRB leaves. According to this view, a recent investigation showed that the PEPCK expression could be influenced by SRB [26]. In agreement, glucose production from l-glutamine which, enters in the gluconeogenic pathway before the PEPCK step, was also inhibited by the treatment with SRB leaves. However, gluconeogenesis was not totally inhibited since the glucose production from glycerol, which enters in this metabolic pathway after the PEPCK step was not influenced by the treatment with SRB leaves.

In contrast to SRB leaves, we did not observe decreased glucose production in livers from animals treated with stevioside (results not shown), even when isolated hepatocytes were employed (Fig. **3**).

Because PPARγ agonists reduce insulin resistance and inhibit the expression of the rate-limiting enzyme of gluconeogenesis, i. e., PEPCK [27], the possibility of an effect on PPARγ receptors was investigated. Pioglitazone, a synthetic agonist of PPARγ, that has been used to improve insulin sensibility in patients with type 2 diabetes by improving the cellular signalling trigged by this hormone, was used as a positive control. As shown by Fig. **4**, in contrast to the PPARγ agonist pioglitazone, which increased PPARγ transcription activity 4.8-fold, extracts from SRB leaves did not show any effect on PPARγ. In addition, similar results were obtained when STV was tested (not shown).

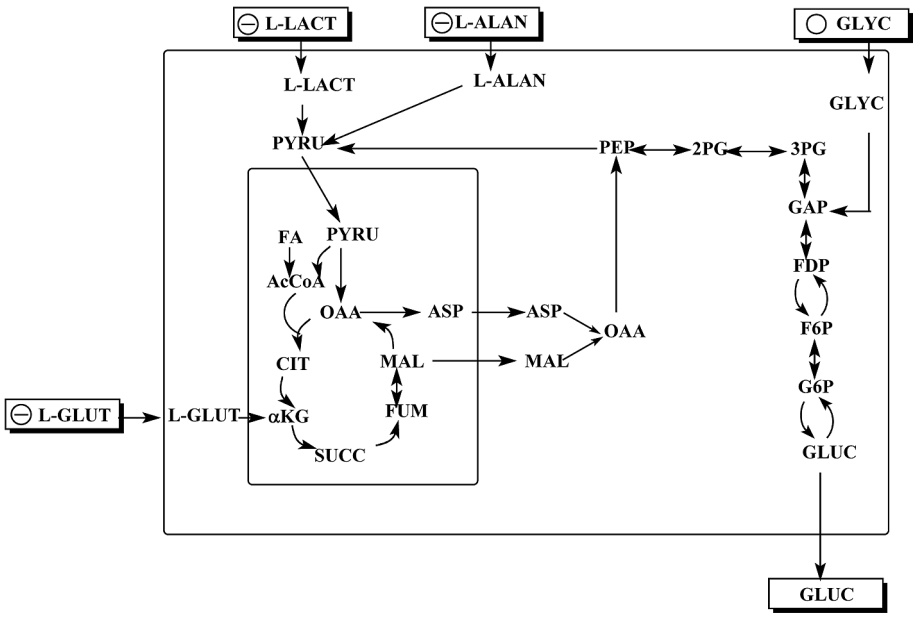

Fig. **2** Effect of the treatment with *Stevia rebaudiana* (Bert.) Bertoni leaves on gluconeogenesis. Plasma membrane is depicted by the large rectangle and the mitochondria by the small rectangle. (y) Decreased gluconeogenesis. (Θ) Absence of effect on gluconeogenesis. Abbreviations: AcCoA = acetyl-CoA; ALAN = L-alanine; ASP = aspartate; CIT = citrate; FA = fatty acid; FDP = fructose diphosphate; F6P = fructose 6-phosphate; FUM = fumarate; GAP = glyceraldehyde phosphate; G6P = glucose 6-phosphate; GLUC = glucose; L-GLUT = L-glutamine; α-KG = α-ketoglutarate; L-LACT = L-lactate; PYRU = pyruvate; MAL = malate; OAA = oxaloacetate; PEP = phosphoenol pyruvate; 2PG = 2-phosphoglycerate; 3PG = 3-phosphoglycerate; SUCC = succinate.

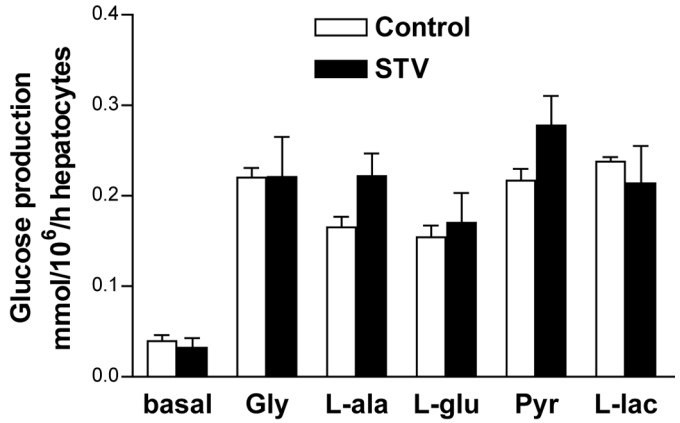

Fig. **3** Glucose production from gluconeogenic substrates in isolated hepatocyte from rats treated with stevioside (STV group) or water (control group). During 15 days the animals received STV (5.5 mg/kg × day) (STV group) or an equal volume of water (control group) by gavage. The data are the mean of 4 – 5 experiments. Abbreviations: Gly = glycerol (2 mM); L-ala = L-alanine (5 mM); L-glu = L-glutamine (5 mM); L-lac = L-lactate (2 mM).

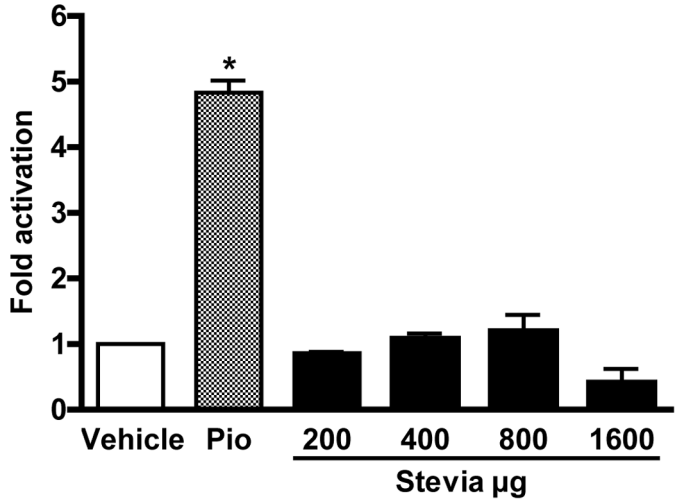

Fig. **4** Effect of pioglitazone (Pio) and increasing amounts of extracts from *Stevia rebaudiana* (Bert.) Bertoni leaves (Stevia) on the activation of PPARγ. U937 cells were co-transfected with expression vector encoding the PPARγ receptor and a reporter vector PPRE-tk-Luc. The transfected cells were treated with vehicle (ethanol:DMSO); Pio at 10^{-5} M; 200, 400, 800 or 1600 μg of Stevia and assayed for luciferase activity. * $P < 0.05$ vs. vehicle.

Our results confirmed the popular reputation of SRB leaves to reduce glycaemia. But, the possibility of effects on the reproductive system must be considered [28], [29], [30]. On the other hand, these studies were contradicted by Shiotsu [31] who did experiments using methods as similar as possible to the method used by Mazzei-Planas and Kuc [28].

In view of the fact that SRB leaves contain diterpene glycosides (stevioside, rebaudiosides A, B, C, D, steviobioside, etc) and a thousand of other compounds, including non-glycosides diterpenes, sterols, triterpenoids, flavonoids, coumarins, caffeic acid, chlorogenic acid and a variety of volatile oils [32], the identification of the hypoglycaemic molecule and/or fraction and the demonstration of the absence of toxicological potential will be necessary to open the possibility for the development of a new antidiabetic compound.

Taken together, our results showed that SRB leaves diminish the glycaemia and one of the mechanisms involved was the reduction of hepatic gluconeogenesis. Furthermore, the hypoglycaemic effect promoted by the treatment with SRB leaves was not mediated by PPARγ activation or stevioside and future studies will test new compounds and/or fractions from SRB leaves.

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