The reduction of arterial tension produced by stevioside is dependent on nitric oxide synthase activity when the endothelium is intact

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Abstract

In endothelium-intact rat aortic ring preparations pre-contracted with norepinephrine or KCl, N^G-nitro L-arginine (L-NOARG, 0.1 mM) and 1H-[1,2,4] oxidiazolo [4,3-a] quinoxalin-1-one (ODQ, 10 μM) antagonized the reduction of the vascular tone induced by stevioside, but this antagonism did not occur when the experiment was performed with endothelium-denuded aortic rings. The data indicates that the vasodilatation produced by stevioside is dependent on nitric oxide synthase and guanylate cyclase activities when the endothelium is not damaged.

Key words: *Stevia rebaudiana (Bert)* Bertoni, Compositae, stevioside, nitric oxide, aortic ring

Introduction

Stevioside, a sweet-tasting glycoside isolated from the herb *Stevia rebaudiana (Bert)* Bertoni (Compositae), has been used as a sugar substitute (Soejarto *et al.*, 1982). However, it has also been reported that stevioside reduces arterial blood pressure when administered orally or intravenously (Hsu *et al.*, 2002; Hsieh *et al.*, 2003). The efficiency of the oral administration of stevioside in the reduction of arterial blood pressure in untreated patients with essential hypertension has not been completely established (Ferri *et al.*, 2006) due to a low gastrointestinal absorption (Geuns *et al.*, 2003; Ferri *et al.*, 2006). However, there is no controversy about the efficiency of stevioside in the reduction of arterial pressure when administered intravenously (Melis and Sainati, 1991; Liu *et al.*, 2003).

As stevioside induces hypotension by causing dilation of peripheral vessels (Liu *et al.*, 2003), the intravenous administration of this compound could be useful in hypertensive emergencies (Levy, 2005). However, the vascular mechanism that induces the hypotensive

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effect of stevioside has not been clarified. Since the activity of the nitric oxide (NO) synthesis pathway is an important factor in vascular relaxation (Ignarro *et al.*, 1987), we investigated the effects of stevioside in aortic ring preparations of rats pre-contracted by either norepinephrine or KCl and the effects of treatment with inhibitors of NO synthesis (N^G-nitro L-arginine, L-NOARG), or with inhibitors of guanylate cyclase (GC) 1H-[1,2,4] oxidiazolo [4,3-a] quinoxalin-1-one (ODQ).

Materials and methods

Rats were killed by decapitation after intraperitoneal injection of sodium thiopental (36 mg/Kg) and urethane (600 mg/Kg). A medial laparotomy was performed immediately to excise the thoracic aorta. The vessel was cut into 3-mm rings and gently dissected free of fat and connective tissue. Rings were then mounted into 30-ml organ baths filled with oxygenated (95% O_2, 5% CO_2) Krebs buffer (in mmol/L: NaCl, 118; KCl, 4.75; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; EDTA 0.03, and glucose, 11) at 37°C, and the pH of the solution was maintained at 7.4. The Ethics Committee of the State University of Maringá approved the procedures used in the current study.

The preparation was connected to strain gauges (Grass, FT 03) and isometric tension was recorded on a computer equipped with the chart software of Powerlab (AD Instruments, Pty, Ltd., Castle Hill, NSW, Australia). After the rings were allowed to equilibrate for 2h, the preparations were stretched gradually to an optimal resting tension (1g). The endothelium was considered to be intact when the addition of acetylcholine (1.0 μM) produced 100% relaxation of the pre-contraction caused by norepinephrine (0.01 μM). In the experiments using endothelium-free preparations, the endothelium was removed by gentle rubbing. The successful removal of the endothelium was confirmed by the failure of 1.0 μM acetylcholine to relax the rings pre-contracted by norepinephrine (0.01 μM). After the resting tension stabilized, KCl (20 mM) or norepinephrine (0.01 μM) was administered to induce a rapid increase of vascular tone, which was followed by a stable vasoconstriction (tonic contraction). Norepinephrine was the drug chosen to produce pre-contraction of vessels because it is an endogenous agonist and when compared with epinephrine has higher affinity for alpha-adrenoceptors (Westfall and Westfall, 2006). The concentration of norepinephrine we used had produced about 50% of maximal tension in isolated rat aortic rings preparations devoid of endothelium (Testai *et al.*, 2005). The concentration of KCl used produced contraction of aortic rings preparations without, however, impairing the action of agents considered as possible K^+ channel openers (Nelson and Quayle, 1995) as such mechanism of action and/or the blockade of Ca^{2+} channels might explain the vessel relaxation induced by stevioside (Lee *et al.*, 2001). Since the preliminary experiments showed that the effect of stevioside obeyed an all-or-nothing response, the lowest concentration of stevioside to produce reduction of the vascular tone induced by KCl or norepinephrine was used in the current work. The maximum relaxing effect of stevioside was determined 15 min after its application in all the experiments. Fifteen min was chosen as norepinephrine-induced contraction started to decrease its tension after 30 min. In the experiments with L-NOARG or ODQ, each agent was administered to the bath 20 min before

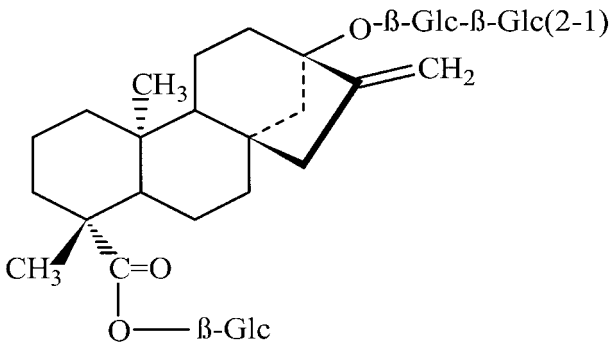

Fig. 1. Chemical structure of stevioside.

stevioside. The tension recorded at t=15 min was expressed as the percentage of that of KCl or norepinephrine (control). 1H-[1,2,4] oxidiazolo [4,3-a] quinoxalin-1-one (ODQ, 10 μM) (Crauwels *et al.*, 2000) and N^G-nitro L-arginine (L-NOARG, 0.1 mM) (Moore *et al.*, 1990) were used in the current studies to block the guanylate cyclase or NOS, respectively. Data were analysed to ANOVA, followed by the Bonferroni test with the level of significance set at P<0.05. Stevioside (Fig. 1) was purified (99.9%) according to Dacome *et al.* (2005). Other agents, L-NOARG, ODQ, norepinephrine and urethane were purchased from Sigma (St. Louis, MO, USA) and sodium thiopental from Cristália (SP, Brazil).

Results

The lowest concentration of stevioside able to reduce the increase of tension produced by 0.01 μM norepinephrine was 1 mM in preparations with or without intact endothelium (Fig. 2, A, B and C). The relaxant effect of 1 mM stevioside was lower in endothelium-denuded aortic ring preparations than in endothelium-intact preparations pre-contracted with norepinephrine (Fig. 2C). In contrast, the lowest concentrations of stevioside able to reduce the increase of tension produced by 20 mM KCl were 0.01 mM and 1 mM in preparations with and without intact endothelium, respectively (Fig. 2, A, B and D).

Both L-NOARG (0.1 mM) and ODQ (10 μM) increased norepinephrine-induced tension to a similar extent in preparations with or without intact endothelium (Fig. 3A), but such agents only increased the tension produced by KCl in preparations with intact endothelium (Fig. 3B). L-NOARG (0.1 mM) and ODQ (10 μM) antagonized the relaxation induced by stevioside in the endothelium-intact rat aortic ring preparations pre-contracted by norepinephrine or KCl (Fig. 3, A and B), but such agents did not modify the relaxation induced by stevioside in endothelium-denuded aortic ring preparations pre-contracted by norepinephrine or KCl (Fig. 3, A and B).

Discussion

This study has confirmed a previous report (Crauwels *et al.*, 2000) that part of the relaxing effect of stevioside is not dependent on an intact endothelium, as the relaxation induced by

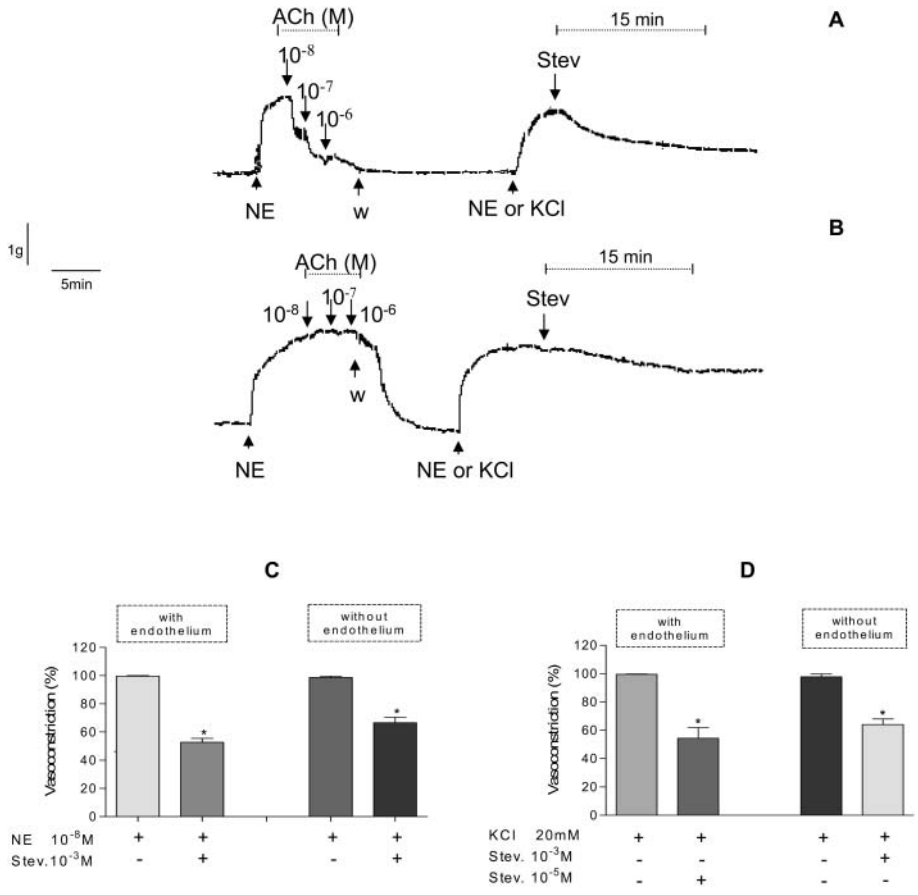

Fig. 2. Relaxing effect produced by acetylcholine (ACh) (A, B) and stevioside (Stev, 10^{-3} M, A, B, C and D; 10^{-5} M, A and D) in rat aortic ring preparations with (A, C, D) or without (B, C, D) endothelium pre-contracted by 10^{-8} M norepinephrine (NE) or 20 mM KCl. Stevioside produced maximal relaxing effect 15 min after application. The effect of stevioside obeyed an all-or-nothing response and 10^{-5} M Stev induced relaxation only in endothelium-intact aortic ring preparations pre-contracted by KCl. Columns indicate relative tension (mean ± SEM of 6 to 8 experiments) taking NE or KCl-induced contraction as 100%. *, $P<0.05$ different to control (NE or KCl alone) (ANOVA; Bonferroni post hoc test). **, $P<0.05$ different to combined administration of NE and Stev in endothelium-intact aortic ring preparations (ANOVA; Bonferroni post hoc test).

stevioside was obtained in preparations both with and without endothelium and pre-contracted with either norepinephrine or KCl. Additionally, pre-treatment of rat aortic ring preparations with L-NOARG or ODQ impaired the relaxation induced by stevioside only in the endothelium-intact aortic ring preparations pre-contracted by norepinephrine or KCl. These data suggest that the reduction of vascular tone induced by stevioside is influenced by the activities of NO-synthase and guanylate cyclase when the endothelium is intact, but it does not depend on the activities of such enzymes when the endothelial tissue is damaged. The influence of NOS activity in endothelial cells in the relaxation caused by stevioside in the preparations pre-

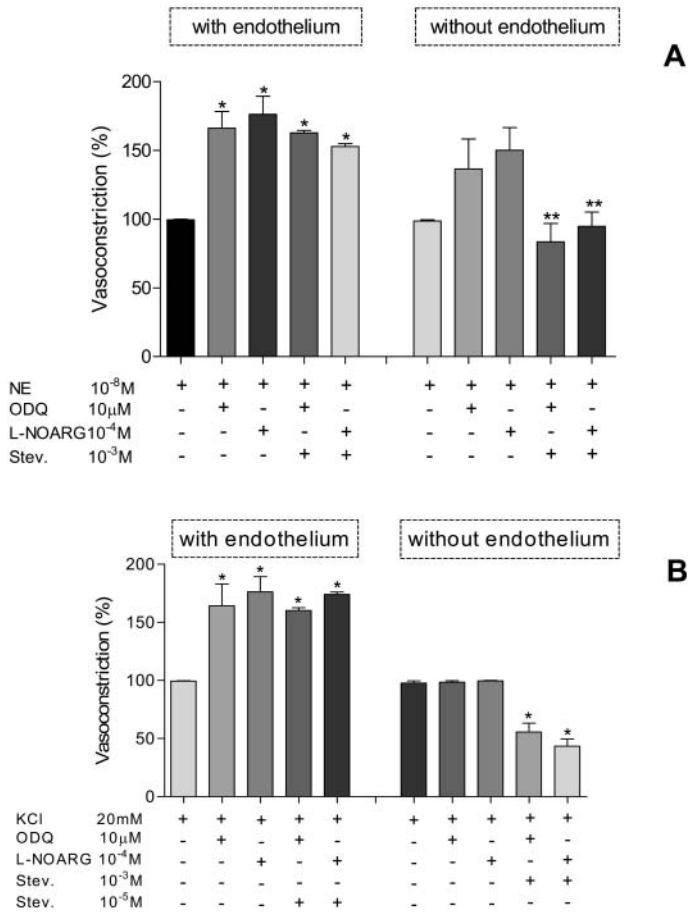

Fig. 3. Effects of stevioside (Stev) combined with L-NOARG or ODQ in rat aortic ring preparations with and without intact endothelium and pre-contracted with norepinephrine (NE) (A) or 20 mM KCl (B). Heights of columns indicate the percentage of vasoconstriction (mean ± SEM) of 6 to 8 experiments. *, $P<0.05$ different to control (NE or KCl alones). **, $P<0.05$ different to vasoconstriction induced by the combined administration of L-NOARG or ODQ with NE or KCl (ANOVA; Bonferroni post hoc test).

contracted by norepinephrine or KCl seems strong, as even higher concentrations of stevioside (up to 100 mM, not shown) were not able to reduce the vascular tension. It is unlikely that the antagonism by either L-NOARG or ODQ of the vasodilatation induced by stevioside in preparations with intact endothelium had been due to strong vasoconstriction generated by the combined administration of L-NOARG or ODQ with norepinephrine or KCl, as such intense vasoconstriction induced by the combined administration of those agents with norepinephrine also occurred in endothelium-denuded aortic ring preparations, in which stevioside induced relaxation under such experimental conditions. It has been shown that NOS exists in the endothelium and arterial smooth muscle cells of rats (Gurdal *et al.*, 2005), and that the vasoconstriction induced by norepinephrine may be depressed by NO released through

stimulation of alpha-adrenoceptors (Kaneko and Sunano, 1993; Gurdal *et al.*, 2005). Thus, the increment which resulted from application of L-NOARG and ODQ in the vasoconstriction induced by norepinephrine in preparations both with or without intact endothelium could have been the result of reductions in the synthesis of NO in the endothelium and arterial smooth muscle cells thus decreasing guanylate cyclase activity, and thereby reducing the inhibitory effects of the gas on the vasoconstriction mediated by alpha-adrenoceptors. However, it is possible that the combined administration of L-NOARG or ODQ with norepinephrine has induced the release of other endothelial vasoconstrictor substances, as the vascular relaxation induced by stevioside in endothelium-denuded aortic preparations treated with a combined administration of these agents was not obtained in endothelium-intact aortic ring preparations, despite the application of higher concentrations of stevioside (up to 100 mM, not shown). It has been demonstrated that endothelial cells can release the vasoactive peptide endothelin (Lüscher, 2007; Yanagisawa *et al.*, 1988). Endothelin is a substance that, in contrast with NO and prostacyclin, increases vascular tone (Lüscher, 2007). It is produced only in endothelial cells and has its production inhibited by both endothelium-derived NO and cGMP and potentiated by inhibitors of NOS or guanylate cyclase (Boulanger and Lüscher, 1990). Thus, stevioside was not able to reduce the increment caused by L-NOARG and ODQ in the vasoconstriction induced by norepinephrine in endothelium-intact aortic ring preparations because in such experimental conditions, in contrast with that utilizing the endothelium-denuded preparations, endothelin could also be causing vasoconstrictor effects. This hypothesis is strengthened by data obtained in previous studies that had indicated that the vasorelaxant effect of stevioside is dependent on the blockade of Ca^{++} channels (Lee *et al.*, 2001), and that such mechanism of action of these drugs is not able to reverse the vasoconstriction induced by endothelin (Vanhoutte *et al.*, 1989). In contrast, the increment caused by L-NOARG and ODQ in the vasoconstriction induced by cellular depolarization with KCl would be the result of a specific reduction in NO synthesis in endothelial cells bringing about a decrease in the inhibitory effect of the gas and increasing the effect of endothelin on arterial smooth muscle cells. This was verified by the finding that the vasoconstriction induced by KCl was only incremented by L-NOARG and ODQ in preparations with intact endothelium. The preferential activation by KCl of NOS activity in endothelial tissues could have resulted in the concentration of stevioside that reduced the increase of tension in the intact aortic rings preparations being found to be lower in preparations pre-contracted by KCl than in the preparations pre-contracted by norepinephrine.

As a whole, the data indicate that the stevioside-induced vasodilatation is not dependent on the activities of NOS and guanylate cyclase when the vascular endothelium is damaged, but that it depends on the activities of these enzymes when the endothelium of the aortic ring preparations is intact. The influence of NOS activity in endothelial cells and in arterial smooth muscle cells on the stevioside-induced vasodilatation depends on the type of agent used to produce the previous vasoconstriction. Since the intravenous administration of stevioside has been proposed to be useful in hypertensive emergencies (Levy, 2005), it must be taken into account that stevioside, after its intravenous administration, may be metabolized to isosteviol (Cardoso *et al.*, 1996), a diterpene agent that induces vasodilatation by opening potassium

channels ($SK_{Ca^{2+}}$ and K_{ATP}) (Wong *et al.*, 2004). As the vasodilatation produced by isosteviol is not influenced by NOS activity (Wong *et al.*, 2004), the arterial actions of isosteviol could counterbalance the influences of the endothelium on the hypotensive effect of stevioside.

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