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Original Article

Cardioprotection of stevioside on stunned rat hearts: A mechano-energetical study

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ABSTRACT

Background: The sweetener and hypoglycemic properties of stevioside (STV) are well known, as the main component of the plant *Stevia rebaudiana*. Given its extensive use in diabetic patients, it was of interest to evaluate its effects on the most frequent cardiovascular disease, the coronary insufficiency.
Purpose: To study whether STV could be cardioprotective against ischemia-reperfusion (I/R) in a model of "stunning" in rat hearts.
Study design: A preclinical study was performed in isolated hearts from rats in the following groups: non-treated rats whose hearts were perfused with STV 0.3 mg/ml and their controls (C) exposed to either moderate stunning (20 min I/45 min R) or severe stunning (30 min I/45 min R), and a group of rats orally treated with STV 25 mg/kg/day in the drink water during 1 week before the experiment of severe stunning in the isolated hearts were done.
Methods: The mechano-calorimetrical performance of isolated beating hearts was recorded during stabilization period with control Krebs perfusion inside a calorimeter, with or without 0.3 mg/ml STV before the respective period of I/R. The left ventricular maximal developed pressure (P) and total heat rate (Ht) were continuously measured.
Results: Both, orally administered and perfused STV improved the post-ischemic contractile recovery (PICR, as % of initial control P) and the total muscle economy (P/Ht) after the severe stunning, but only improved P/Ht in moderate stunning. However, STV increased the diastolic pressure (LVEDP) during I/R in both stunning models. For studying the mechanism of action, ischemic hearts were reperfused with 10 mM caffeine-36 mM Na^+-Krebs to induce a contracture dependent on sarcorreticular Ca^{2+} content, whose relaxation mainly depends on mitochondrial Ca^{2+} uptake. STV at 0.3 mg/ml increased the area-under-curve of the caffeine-dependent contracture (AUC-LVP). Moreover, at room temperature STV increased the mitochondrial Ca^{2+} uptake measured by Rhod-2 fluorescence in rat cardiomyocytes, but prevented the $[Ca^{2+}]$m overload assessed by caffeine-dependent SR release.
Conclusions: Results suggest that STV is cardioprotective against I/R under oral administration or direct perfusion in hearts. The mechanism includes the regulation of the myocardial calcium homeostasis and the energetic during I/R in several sites, mainly reducing mitochondrial Ca^{2+} overload and increasing the sarcorreticular Ca^{2+} store.

Abbreviations: AUC, area under curve; C, control perfusion; F/Fo, relative fluorescence; Ht, heat rate production; I/R, ischemia/reperfusion; LDH, lactate-deshidrogenase; LVEDP, left ventricular end diastolic pressure; LVP, left ventricular pressure; mKATP, mitochondrial ATP-dependent potassium channels; mNCX, mitochondrial sodium/calcium exchanger; NCX, sarcolemmal sodium/calcium exchanger; P, maximal pressure development; PICR, postischemic contractile recovery; P/Ht, total muscle economy; R-caff-36 Na^+, reperfusion with Krebs + caffeine 10 mM and 36 mM Na^+; RyR, ryanodine receptors; SERCA, sarcoplasmic reticulum calcium pump; SR, sarcoplasmic reticulum; STV, stevioside

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Introduction

Stevioside is a non-caloric sweetener extracted from the leaves of *Stevia rebaudiana* (Bertoni) (Asteraceae), a native plant from Paraná in Brazil, Paraguay and the Northeast of Argentina. This hydrophilic diterpenoid glycoside is 300 times sweeter than sugar and concurrently a good reductor of postprandial blood glucose in type-2 diabetic patients (Gregersen et al., 2004). It was demonstrated that when orally administered to pigs stevioside was strongly converted into its aglycon steviol by the colonic bacteria (Geuns et al., 2003). It was found that steviol but not stevioside is the responsible to reduce glucose absorption (Koyama et al., 2003).

Stevioside (STV) was also described as hypotensor and vasodilator (Chan et al., 1998; Tirapelli et al., 2010), as diuretic (Melis, 1995) and bradycardic (Humboldt and Boech, 1997) in preclinical and clinical studies. It reduced the vascular resistance by inhibition of calcium influx (Melis and Sainati, 1991; Lee et al., 2001; Liu et al., 2003). On line with this, we described the intestinal antispasmodic effect of STV associated to a non-competitive blockade of Ca^{2+}-influx to smooth muscle cells (Matera et al., 2012). Both effects, bradycardia and Ca^{2+} channels blockade allow us to hypothesize that STV could reduce the Ca^{2+} overload induced during ischemia/reperfusion (I/R). Depending on the duration of ischemia (I), hearts could suffer either a ``stunning" characterized by reduced contractile recovery and the develop of diastolic contracture during R, or an infarction with necrotic areas and loss of contractile activity. Two previous studies described that isosteviol, a derivative compound, induced cardioprotection. In one of them isosteviol was perfused from 1 to 10 μmol/l in isolated guinea-pig hearts exposed to I/R, and the cardioprotection was partially attributed to the opening of the mitochondrial KATP channels (mKATP) like in myocardial preconditioning (Miura et al., 2001). In the other one, Xu et al. (2007) demonstrated that isosteviol intravenously injected before occluding the left coronary artery reduced the size of infarct and the accumulation of injury marker enzymes such as LDH and creatine-kinase. These effects observed in a drastic model of I/R were also partially inhibited by blocking the mKATP (Xu et al., 2007). Since *Stevia rebaudiana* and the STV are used as sweetener and hypoglycemic agents in diabetic patients, it was of interest to assess whether STV could prevent the consequences of brief episodes of myocardial ischemia. These episodes are frequently the result of vascular occlusions in diabetic or non-diabetic people over 60 years old, and Phytotherapy could be useful to prevent them. So, we studied the effects of STV on the mechano-energetical behaviour of isolated rat hearts exposed to two degrees of stunning. These models provide respectively medium and low contractile recovery and mitochondrial metabolic changes, which drive to alterations in muscle economy and Ca^{2+} handling. These *ex vivo* models resemble the clinical situations of a transient coronary obstruction, after which it is required a prevention to avoid further ischemic complications to myocardium.

Contractile and calorimetric output of the isolated heart provides information about the energetic of Ca^{2+} handling and muscle economy. Global I/R induces contractile and energetic dysfunction, since metabolism and calcium homeostasis are affected during R (Bolli and Marban, 1999; Consolini et al., 2007; Valverde et al., 2010; Garcia-Dorado et al., 2014). Myocardial ischemia reduces the metabolic restoration of ATP and the associated heat fraction, but also it reduces the ATP pool compartmentalized and necessary to extrude Ca^{2+} from cytosol (Guzun et al., 2015), both of which become in diastolic contracture during I (Consolini et al., 2004, 2007). Still more contracture appears during first min of R, when the concentrations of H^+, Na^+ and Ca^{2+} increases in cytosol until restoration of mitochondrial metabolism (Schafer et al., 2001; Valverde et al., 2010). With the reperfusion, the sarcoplasmic reticulum Ca^{2+} pump (SERCA), the sarcolemmal Na^+/Ca^{2+} exchanger (NCX) and the mitochondrial transporters (Ca uniporter, mNCX, mKATP and mPTP) play the main roles to restore the cellular Ca^{2+} homeostasis (Motegi et al., 2007; Murphy and Steenbergen, 2008; Bernardi and Di Lisa, 2015; Mattiazzi et al., 2015). The isolated physiologically perfused whole heart allows to study the simultaneous and continuous mechano-energetical behaviour before and during the no-flow ischemia and reperfusion. Then, the aim of this work was to evaluate the effects of STV on the stunning due to two degrees of I/R, without infarct. STV was administered either *ex vivo* and *in vivo* to see whether the effects are due to STV or to some metabolite. Also, the effects of STV on cellular Ca^{2+} handling were explored in isolated cardiomyocytes.

Methods

Animals

The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as was recommended in the Guide for Care and Use of Animals (NIH Nro publication # 85-23 revised in 1985 and 1996, National Academy Press, Washington DC, USA), according to the Resolution 1047 anexo II of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) de la República Argentina, 2005.).

Isolated heart preparation and contractile measurements

Adult Sprague-Dawley rats (200 a 250 g weight) of either sex were heparinized (non-fractioned heparine 2000 IU) and anesthetized with 25% urethane (0.6 ml by 100 g, via i.p.). The animals were spontaneously breathing during the general anesthesia. Hearts were rapidly excised and retrograde perfusion through coronary arteries by the Langendorff method was done, as previously described (Consolini et al., 2007; Ragone and Consolini, 2009). Hearts were perfused with control Krebs (C) at 37 °C and at a constant flow rate of 7 ml min^{-1} g^{-1}, by a peristaltic pump (Gilson Minipuls, France). This perfusion flow was calculated by the equation $CF = 7.43*HW^{0.56}$ (where CF is coronary flow and HW is the heart weight), valid for different species and recommended to prevent heart edema caused by high flow rate of saline perfusion (Dhein, 2005). This flow was sufficient to develop an optimal maximal pressure (P) without a significant edema, as described in other work (Colareda et al., 2016). The experimental conditions (working temperature, heart rate and I/R periods) were similar to those of previous studies (Ragone and Consolini, 2009; Consolini et al., 2011; Bonazzola et al., 2014; Ragone et al., 2015; Colareda et al., 2016) in which cellular mechanisms had been characterized during stunning of rat hearts. Atria were removed and the spontaneous beating was stopped by applying pressure on the focus in the interventricular septum. A latex balloon was introduced in the left ventricle, connected by a flexible cannula to a Bentley DEL900 or a Statham P23db pressure transducer. While continuously perfused, the heart was introduced into the calorimetrical chamber, which was closed and submerged in a bath kept at controlled temperature of 37.0 ± 0.01 °C. Rat hearts were electrically stimulated with 5 V–5 ms at 3 Hz, by means of two electrodes connected to an electrical stimulator (Letica LE12406, Spain or Grass SD9, USA). The isovolumic left intraventricular pressure (LVP) was continuously recorded at optimal volume, as well as the total heat rate. The LVP was recorded during all the experiment either in a PowerLab 2/26 two channels digital acquisition system (AD Instruments, Australia) or in a Grass polygraph of 8 channels (Grass Instruments, Quincy, MA, USA) with A/D acquisition (TL-1 DMA Axon Instruments INC., Foster City, CA, USA). The maximal pressure development (P) of a contraction was calculated from the difference between the peak in LVP recording and the diastolic level (left ventricular end diastolic pressure (LVEDP)). During I and R there were calculated the changes in diastolic pressure over the preischemic condition in Krebs-C (ΔLVEDP), in mmHg, as an estimation of diastolic contracture. Also, during I/R, the pressure development during a contraction (contractility) was expressed as a percentage of the steady