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ROS changes evoked by the natural sweetener Rebaudioside A in a neuronal system

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Abstract

Current water treatment methods are unable to eliminate artificial sweeteners, which can lead to their accumulation in the environment. Due to this problem, natural sweeteners can be used instead, but it is important to understand their effects in biological systems. Rebaudioside A, one of the main components of stevia, causes an increase in both ROS and in FAD linked autofluorescence in hippocampal CA3 area. These effects may be due to the insulin-mimetic properties of steviol glycosides, with the results suggesting that they cause enhancements in glycolysis and in OXPHOS, with this metabolic pathway being the possible source of the rise in ROS. In excess, these molecules may cause damage to brain cells through oxidative stress. As leftovers from Stevia's purification process can be used as biomass, with applications ranging from energy production to fertilization, continuous accumulation in the environment should be avoided in order to prevent undesired effects in the ecosystem.

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Keywords: Autofluorescence; CA3; OXPHOS; Reactive oxygen species; Stevia; Steviol Glycosides

1. Introduction

Artificial sweeteners have been reported to appear in tap water, suggesting that the current methods of water treatment are not capable of eliminating some molecules like sucralose or aspartame [1]. This could be problematic since they can bioaccumulate, leading to negative effects in ecosystems. A possible negative impact is a decrease in the antioxidant system of the brain [2]. The answer to this problem may not reside in changing the treatment of

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residues itself, but in changing artificial for natural sweeteners that are decomposed in the environment. One of those natural substances is stevia, which is obtained from a plant with the same name and has known insulin-mimetic properties. Since stevia is decomposable in nature and has less side effects than artificial sweeteners [3], its use, which may not be confined to the food industry, would have a lower ecological footprint. Some brain diseases, such as certain forms of Alzheimer, have been suggested to have origin in cells with an abnormal glycolytic activity [4] and with an excessive production of reactive oxygen species (ROS) [5]. Recently, it was suggested that steviol, the central constituent of steviol glycosides, may have chemotherapeutic potential [6].

Stevia rebaudiana is a plant from which it is possible to obtain the previous extract that is used as a non-caloric high-potency sweetener and as a sugar substitute. Its use is increasing due to the growing awareness and incidence of health problems related to nutrition, such as diabetes and obesity. The substances with those properties that are extracted from the mentioned plant are known as steviol glycosides (SG). Use of large quantities of water are often involved in the SG purification process, even in new proposals for new methods of extraction [7], which can be associated with direct impact in effluents [8]. However, since it is a natural product, it is completely decomposed in a matter of days, which is an advantage against artificial sweeteners. However, continuous release is still a concern, especially in the vicinity of the area it occurs. Biomass rich in SG can be obtained [9], either directly by harvesting the plant with this objective or as a result of the purification process. The uses of this biomass range from energy production to soil fertilizer [10]. Due to the ever-increasing amounts of stevia being consumed in current times, it is more important than ever to assess the effects that high levels of SG can have in biological systems and, as result, in the ecosystems.

The aim of this work was to characterize the effect of rebaudioside A, a steviol glycoside that can pass through the blood brain barrier [11], in neuronal ROS formation, more specifically at the synaptic system mossy fibers-CA3 pyramidal cells of the hippocampus. The CA3 hippocampal area is critically involved in epilepsy and might play a role in early Alzheimer disease, which is considered by some authors as a type 3 diabetes [12], originated when some brain cells develop insulin resistance. It has been proposed that SG could bypass the insulin resistance of those cells [13], thus preventing the development of that disease. For rebaudioside A, some studies suggest that the properties associated with SG are largely plasma glucose level dependent, requiring high glucose levels [14]. Despite the large number of studies on stevia and SG, very little has been reported concerning the cellular and molecular mechanisms behind their effects.

Reactive oxygen species (ROS) are formed during normal metabolism. However, they can also be formed in the presence of certain stimuli, that can lead to high concentrations that may have a negative effect in biological systems, such as DNA damage [15]. Cells do have natural defenses against these molecules, more commonly in the form of specific antioxidant enzymes, like catalase and flavoproteins. When the balance between ROS and antioxidant defenses is destabilized, oxidative stress occurs.

The brain is prone to be affected by excessive ROS, due to reduced levels of antioxidant defenses and high levels of fatty acids, which consume large amounts of oxygen, and this has been associated with several neurodegenerative diseases as well as brain tumors [16].

Cells exhibit metabolism-linked autofluorescence upon excitation with UV/Vis radiation, thus allowing the study of metabolic processes taking place in mitochondria and other loci. For example, because the oxidized species of the flavin nucleotide redox couple FAD/$FADH_2$, FAD, is fluorescent, flavin autofluorescence may be used to monitor mitochondrial oxidative metabolism linked to stimulation of the glycolytic pathway. Both FAD and the reduced form of pyridine nucleotides, NADH, contribute to autofluorescence [17]. However, at the excitation wavelength of 480 nm, all of the autofluorescence originates from FAD [18]. An increase in autofluorescence can also be associated with an increase of glycolysis and with ATP production [19].

2. Materials and methods

The experiments were carried out in rat brain slices (400 μm thick), from adult Wistar rats (8–16 weeks old) at the mossy fiber synaptic system from hippocampal CA3 area. The slices were exposed to an oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF), which contained (in mM): glucose 10, NaCl 124, KCl 3.5, $NaHCO_3$ 24, $NaH2PO_4$ 1.25, $MgCl_2$ 2, $CaCl_2$ 2 [20]. For the ROS measurements, the slices were previously incubated, for one hour, with the permeant fluorescent ROS indicator, 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) (20 mM), while being oxygenated. The autofluorescence studies were made in non-incubated slices. The incident light (480 nm) was focused on the mossy fiber synaptic system from hippocampal CA3 area, using a fluorescence microscope (Zeiss,

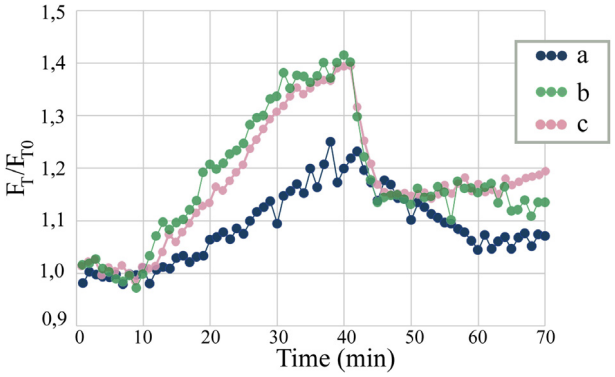

Fig. 1. Fluorescence changes from hippocampal CA3 area detected from brain slices containing the ROS indicator H_2DCFDA. In the first 10 min the brain slices were in ACSF. From the 10th to the 40th min, the ACSF medium contained rebaudioside A. Then the slices were exposed again to ACSF, for 30 min. Concentrations of rebaudioside A: a–0.15 mM; b–1 mM; c–10 mM (n = 3). F_T, total fluorescence, F_{T0}, basal total fluorescence.

Axioskop) and a tungsten-halogen lamp (12 V, 100 W). Changes in fluorescence were detected from that area by means of a photodiode (Hamamatsu, 1 mm^2), an emission high pass filter (>500 nm) and a data acquisition system (National Instruments, 16 bits). The solutions perfused in the chamber were switched from ACSF (30 minutes, the first 20 minutes to stabilize the slice, the last 10 minutes to read the signal) to ACSF with varying concentrations of rebaudioside A (0.15 mM. 1 mM and 10 mM) for 30 minutes and then back to ACSF (30 minutes), to see if the changes in the signal were reversible. The perfusion rate was 1.6 ml/min, at 32 °C.

Rebaudioside A (98%) was used because it is one of the steviol glycosides with most sweetening power and because it is one of the most abundant SG in the plant, thus being of particular importance for the industry.

H_2DCFDA was used to detect reactive oxygen species produced by cellular activity. This fluorogenic dye measures peroxyl, hydroxyl, and other ROS activity within the cell. After diffusion into the cell, H_2DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2′,7′-dichlorofluorescein (DCF). DCF is a highly fluorescent compound that can be detected by fluorescence spectroscopy.

Rebaudioside A 98% was obtained from Provital, Portugal, and H_2DCFDA from Invitrogen by Thermo Fisher Scientific, USA. All experiments were carried out in accordance with the Directive 2010/63/EU of the European Parliament and Council. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

3. Results and discussion

Several assays were conducted using slices incubated with the fluorescent ROS indicator H_2DCFDA, to determine ROS signals in the presence of different concentrations of rebaudioside A. Fig. 1 contains averages of experiments where the brain slices were exposed to 0.15, 1 and 10 mM of rebaudioside A diluted in ASCF.

Comparing Fig. 1a and b, it is clear that the increase in the concentration of rebaudioside A from 0.15 mM to 1 mM caused a rise in the total fluorescence values, with maxima around 1.2 and 1.4, respectively, having the effect doubled for 1mM.

A similar result is observed comparing Fig. 1b and c, suggesting that the maximum response evoked by rebaudioside A had already been achieved at the concentration of 1 mM.

The results thus show an increase in the total fluorescence values for different concentrations of rebaudioside A. In the presence of 0.15 mM, and of 1 and 10 mM of this compound, the signals were enhanced by about 20% and 40%, respectively, in relation to the baseline values.

According to the results, since the values have peaked, with the slices exposed to the two higher concentrations having a similar response, the maximum effect should start to occur at a concentration between 0.15 mM and 1 mM. The signals from Fig. 1 come from both autofluorescence and ROS, the later detected by the used probe, and the following assays were made in order to assess the real contribution of each one.

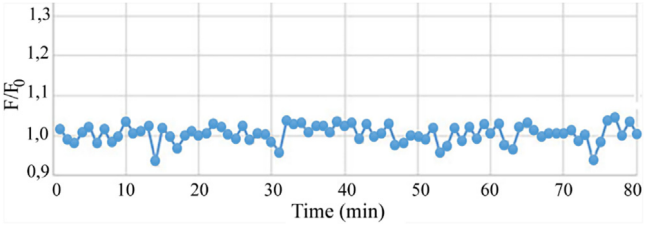

Fig. 2. Fluorescence changes from a piece of parafilm with the same opacity as an average brain slice, detected with the ROS indicator H_2DCFDA. In the first 20 min the parafilm piece was in ACSF. From the 20th to the 50th min, the ACSF medium contained rebaudioside A (1 mM). The parafilm was exposed again to ACSF in the last 30 min (n = 2). F, fluorescence, F_0, basal fluorescence.

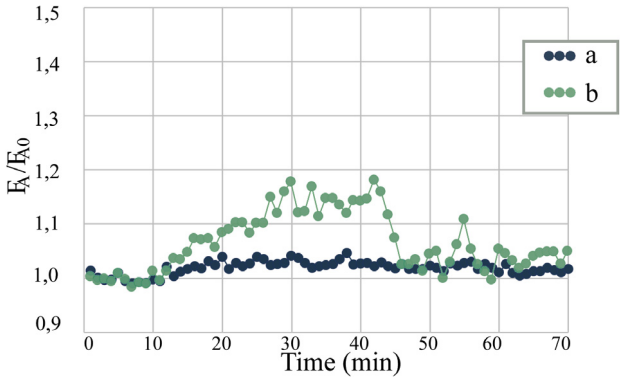

Fig. 3. Autofluorescence changes from hippocampal CA3 area, detected from nonincubated brain slices. In the first 10 min the slices were in ACSF. From the 10th to the 40th min, the ACSF medium contained rebaudioside A. Then the brain slices were exposed again to ACSF, for 30 min. Concentrations of rebaudioside A: a–0.15 mM; b–1 mM (n = 3). F_A, autofluorescence, F_{A0}, basal autofluorescence.

To serve as a control for the fluorescence of rebaudioside A on its own, a version of the main experiment was performed placing a piece of parafilm with the same opacity, measured in mV, as an average slice, in its place (Fig. 2).

The values from Fig. 2 show that the fluorescence values being measured remain constant. This suggests that rebaudioside A, on its own, does not have any fluorescence in the conditions tested. So, any fluorescence in the slice experiments should come from the brain slice's biologic activity and not from this molecule alone.

Since the detected fluorescence signals may be partially due to FAD autofluorescence, similar assays were conducted with unincubated brain slices, and so without H_2DCFDA, being the results shown in Fig. 3.

Once autofluorescence is linked to FAD and this molecule is produced during the oxidative phosphorylation (OXPHOS) metabolic pathway in mitochondria, the increase in autofluorescence values suggests an increase in the activation of this pathway. OXPHOS is known to be a major source of ROS in cells. Thus, the results suggest that the formation of these highly reactive molecules may be mediated by an increase in OXPHOS, indicating that SG stimulated this pathway in the conditions tested.

The real contribution of reactive oxygen species to the total fluorescence signal can be obtained subtracting, for each concentration of Rebaudioside A, the signals from H_2DCFDA-loaded slices (Fig. 1) from those of indicator-free slices (Fig. 3). Those results can be observed in Fig. 4.

In Fig. 4, it is shown the time course of the true ROS signals in this neuronal system. The signal is stronger for the graph corresponding to the concentration of 1 mM (Fig. 4b). The values from these experiments suggest changes in the normal cell respiration process, detected through the fluorescence of flavoproteins. This provides direct measures of oxidative and glycolytic metabolism. There is data suggesting that steviol glycosides could act by modulating GLUT1 and GLUT4 translocation through the PI3K/Akt pathway [21], by binding to the insulin receptors and initiating a cascade of events leading to the release of those glucose transporters, acting as insulin-mimetic molecules. We suggest that this mechanism caused by SG, depending on its quantity and time of exposure to those molecules, may lead to a lactic acidosis, due to an overstimulation of glycolysis, that will cause a metabolic

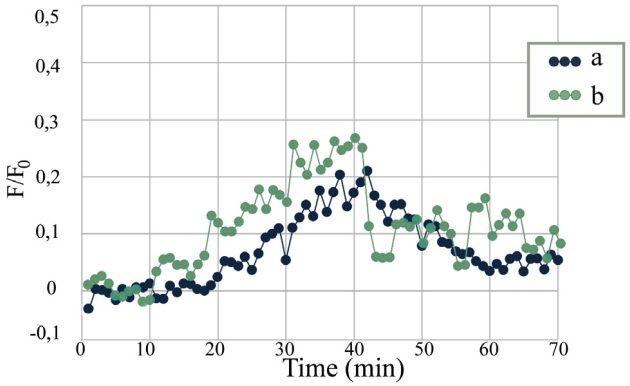

Fig. 4. ROS signals detected in hippocampal CA3 area, with the ROS indicator H_2DCFDA, corrected for autofluorescence. In the first 10 min the brain slices were in ACSF. From the 10th to the 40th min, the ACSF medium contained rebaudioside A. Then the brain slices were exposed again to ACSF, for 30 min. Concentrations of rebaudioside A: a–0.15 mM (blue); b–1 mM (green) (n = 3). F, fluorescence, F_0, basal fluorescence.

shift from glycolysis to OXPHOS [22]. Thus, this kind of treatment would lead to an increase in cell activity regarding the glycolytic activity and OXPHOS and, consequently, in the ROS levels as the data in Fig. 4 suggests. It is also noteworthy that the fluorescence levels did not fully recovered back to the baseline values when the solution was swapped back to normal ACSF, thus removing Rebaudioside A from the system, which could hint at some type of cell damage or dysfunction, probably due to the increased ROS levels, specially in the higher concentration of Rebaudioside A.

The observed ROS increase evoked by rebaudioside A also raises concern that it may cause unwanted effects in the environment, especially in animal tissues such as the brain, producing oxidative stress that can lead to several problems including DNA damage and potentiating brain-related ailments. Since large amounts of leaf residues from stevia extraction industries still contain much rebaudioside A and other SGs, from wastewater to biomass, Xu et al. [10], it would be important to further study the environmental impact of these substances.

However, in the right dose, since this is a natural substance used in the food industry with no serious side effects at lower concentrations, it could be of interest for a hypothetical medicinal use in situations were a stimulation of glycolysis or OXPHOS would be beneficial.

4. Conclusions

The results show that rebaudioside A, for the tested model of a neuronal system, increases autofluorescence and enhances ROS production. This is in line with steviol glycosides having insulin mimetic properties, which enhances glucose uptake and glycolytic activity. The observed changes are likely mediated by increases in the cellular pools of reduced flavin nucleotides. A possible source of this can be an increase in OXPHOS, a main source of ROS, in mitochondria, suggesting that SG also stimulated this metabolic pathway in the assays performed and, thus, induced oxidative stress.

Because of these effects, and even though stevia can be decomposed in nature, continuous accumulation of residues from stevia processing factories, as biomass or wastewater, may cause undesired effects in the environment during long exposure times. Thus, further studies should be made in order to know the effects that rebaudioside A causes, with emphasis on animal ecosystems, via an increase in oxidative stress, as suggested by the present results.

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