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Effect of steviol, steviol glycosides and stevia extract on glucocorticoid receptor signaling in normal and cancer blood cells

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ABSTRACT

The use of steviol glycosides as non-caloric sweeteners has proven to be beneficial for patients with type 2 diabetes mellitus (T2D), obesity, and metabolic syndrome. However, recent data demonstrate that steviol and stevioside might act as glucocorticoid receptor (GR) agonists and thus correlate with adverse effects on metabolism. Herein, we evaluated the impact of steviol, steviol glycosides, and a Greek-derived stevia extract on a number of key steps of GR signaling cascade in peripheral blood mononuclear cells (PBMCs) and in Jurkat leukemia cells. Our results revealed that none of the tested compounds altered the expression of primary GR-target genes (*GILZ*, *FKPB5*), GR protein levels or GR subcellular localization in PBMCs; those compounds increased *GILZ* and *FKPB5* mRNA levels as well as GRE-mediated luciferase activity, inducing in parallel GR nuclear translocation in Jurkat cells. The GR-modulatory activity demonstrated by stevia-compounds in Jurkat cells but not in PBMCs may be due to a cell-type specific effect.

1. Introduction

Stevioside and rebaudioside A are natural diterpenoid glycosides isolated from the leaves of *Stevia rebaudiana bertoni*. Steviol, which constitutes their major metabolite, is produced as a result of enzymatic hydrolysis by intestinal microflora (Yadav and Guleria, 2012). Due to the growing incidence of type 2 diabetes mellitus (T2D), obesity, and metabolic syndrome, steviol glycosides are widely used as non-caloric sweetener because of their low glycemic index (GI) (Chatsudthipong and Muanprasat, 2009; IDF, 2015; Van Vliet-Ostaptchouk et al., 2014). They are authorized for use in food industry since 2011 while their safety has been evaluated by many organizations, including the World Health Organization (WHO), and an ADI (Acceptable Daily Intake) has been established (Aguilar et al., 2010; Gupta et al., 2013; JECFA, 2009).

In addition to their sweetness, it has been suggested that steviol glycosides may also exert therapeutic benefit since they demonstrate anti-inflammatory, anti-hyperglycemic, anti-hypertensive, anti-tumor, anti-diarrheal, diuretic, and immunomodulatory activity (Boonkaewwan and Burodom, 2013; Brahmachari et al., 2011; Chatsudthipong and Muanprasat, 2009; Gupta et al., 2013; Sehar et al., 2008; Wang et al., 2014). Steviol glycosides, and steviol belong to diterpens and possess a similar-to-steroids' structure (Fig. 1). Recent data demonstrated that stevioside and steviol exert progesterone receptor-mediated effects and act as glucocorticoid agonists (Shannon et al., 2016).

Glucocorticoids (GCs) are a subclass of steroid hormones which are well established for their immunosuppressive effects and are considered to be one of the most effective therapeutic approaches for inflammatory diseases (Chrousos and Kino, 2005). However, among others, they exert detrimental effects on metabolism and bone cells (Cooper et al., 2016; Moutsatsou et al., 2012). GCs mediate their action via binding on the glucocorticoid receptor (GR), an intracellular hormone receptor, which in absence of a stimulus remains inactive in the cytoplasm being bound to heat shock proteins and immunophilins. After GC binding, GR undergoes

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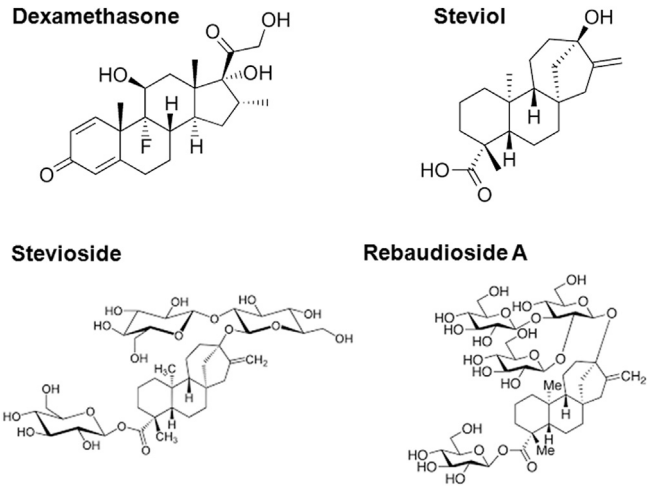

Fig. 1. Chemical structure of dexamethasone, steviol, stevioside, and rebaudioside A.

conformational changes and translocates into the nucleus where it homodimerizes and acts as a transcriptional regulator of many target-genes by binding on glucocorticoid response elements (GRE) located to their promoter. The GC-GR complex can also interfere with pro-inflammatory transcription factors, via protein-protein interactions, such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-kB) and thus prevent the activation of their target-genes. This negative regulatory mechanism is known as trans-repression (Chrousos and Kino, 2005; Oakley and Cidlowski, 2011). Most of the anti-inflammatory effects of GCs seem to result from transrepression whereas their adverse side effects on metabolism and bone cells stem from the GRE–mediated pathway (Cooper et al., 2016; Moutsatsou et al., 2012; Vegiopoulos and Herzig, 2007). It is worth mentioning that Chang et al., 2006 claim that stevioside presents GRE-mediated effect similar to dexamethasone (a synthetic glucocorticoid) in mouse blood cells (macrophages). These data imply that stevioside may be a GR modulator which could exert adverse effects on metabolism. Based on the above and considering that patients with diabetes mellitus, obesity, and metabolic syndrome are considered to be a high-consuming group of steviol glycosides enriched products, we decided to further investigate the possible glucocorticoid receptor modulatory activity of steviol glycosides, steviol, and a Greek-derived stevia extract, in normal human peripheral blood mononuclear cells (PBMCs) and Jurkat cells (human leukemic T-cells).

For this purpose we investigated their activity on GR signaling parameters such as 1) GR expression levels, 2) GR subcellular localization, 3) the expression of Glucocorticoid-induced leucine zipper (*GILZ)* and FK506 binding protein 5 (*FKBP5*) (primary GR-target genes with GREs in their promoter) (Ayroldi et al., 2014; Galigniana et al., 2012) and 4) a GRE-mediated luciferase reporter gene assay. We also assessed the effect of the aforementioned natural sweeteners on *GILZ* and *FKBP5* expression levels in whole blood from healthy volunteers following an oral consumption of a mixture of steviol glycosides.

2. Materials and methods

2.1. Preparation of crude Greek-derived stevia extract

The dry crude extract, derived from a water-based Stevia leaf extract was produced by a Timatic multi extractor at 55 °C and pressure up to 8 bars. After overnight cooling and filtration, the extract was processed with a Micro Filtration Unit (MFU) using as a first step an ultrafiltration membrane of 4-kDa molecular weight cut-off (MWCO) with diafiltration, in order to receive more of the desired molecules. The permeate from this first step was then used in a second step for concentrating the product by a nanofiltration membrane of MWCO of 400–500 Da without diafiltration. The retentate from this membrane was finally freezed-dried to receive the dry crude extract.

2.2. High-performance liquid chromatography (HPLC)

Analytical HPLC was performed on a HPLC system (Agilent Technologies 1100 series, Agilent) equipped with a ZORBAX Eclipse XDB-C18 column (4.6 × 250 mm × 5 μm, Agilent). The dry crude stevia extract was dissolved in solvent extraction (30/70; acetonitrile/water). The extract and standard solutions were then filtered through a syringe filter 0.45 μm (Millpore, Billerica, MA) and 10 μl of the samples were injected into the HPLC system for analysis of steviol glycosides. The separation was monitored by UV absorption at 200 nm. The mobile phase consisted of 32/68 acetonitrile and 10 mmol sodium phosphate adjusted to a pH value of 2.6 with phosphoric acid. The column and sample temperature were maintained at 40 °C and 10 °C, respectively, while the flow rate was 1.0 ml/min. The identification of steviol glycosides contained in the extract was achieved through comparison of their retention times and absorption maxima in the scanned spectrum with those of standard solutions (Sigma-Aldrich, Fluka, St. Louis, MO, USA). Their content was expressed as g/100 g (%).

2.3. PBMCs isolation and culture

Blood samples were obtained from four healthy subjects as part of a voluntary blood donation (Blood Donation Centre, General Hospital of Athens "Laiko"). These samples were collected after leufkapheresis was performed, using LCR5 filters. PBMCs were isolated from LCR5 leukoreduction filters as described elsewhere (Meyer et al., 2005). Briefly, filters were back-flashed with 200 ml elution medium which was then transferred onto Biocoll separating solution (Biochrom) and centrifuged at 1200×*g* for 20 min. PBMCs were collected from the interphase and washed with Phosphate buffered saline with Ethylenediaminetetraacetic acid (PBS/EDTA). Red blood cells were lysed with BD Pharm Lyse Buffer (BD Biosciences).

In order to determine the number and cell viability of PBMCs after their isolation from LCR5 filters and prior to cell culture, we performed the Trypan Blue exclusion method. Briefly, 50 μL of cell suspension were diluted 1:20 in (1×) PBS and subjected to Trypan Blue staining. 15 μL of the diluted cell suspension were then mixed with 15 μL of 0.5% (w/v) Trypan Blue solution (Thermo Scientific, Logan, UT) and the final mixture was incubated for 5 min at room temperature. The concentration of Trypan Blue positively stained cells was determined using a hemocytometer.

PBMCs were cultured in RPMI-1640 medium (Thermo Scientific, Logan, UT) containing the reducing agent glutathione, supplemented with 15% fetal bovine serum (FBS) and 1% of penicillin-streptomycin solution at concentration of 5 × 10^5 cells/ml. Cells were then incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air and were left overnight to adapt to culture conditions. In order to evaluate *GILZ* and *FKBP5* expression levels by real-time PCR, PBMCs were incubated with three concentrations of steviol, stevioside, and rebaudioside A (0.1, 1 and 10 μM) (Sigma Chemical Co., St. Louis, MO, USA) or Greek-derived stevia extract (50, 100, 200, and 500 μg/ml) in absence and in presence of 0.1 μM dexamethasone (DEX) (Sigma Chemical Co., St. Louis, MO, USA) for 12 and 24 h. The effect of mifepristone (0.1 μM) (RU-486) (Sigma Chemical Co., St. Louis, MO, USA) was also assessed. To ascertain the