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Stevia-derived compounds attenuate the toxic effects of ectopic lipid accumulation in the liver of obese mice: A transcriptomic and metabolomic study

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ABSTRACT

There is a close interaction between Type 2 Diabetes, obesity and liver disease. We have studied the effects of the two most abundant *Stevia*-derived steviol glycosides, stevioside and rebaudioside A, and their aglycol derivative steviol on liver steatosis and the hepatic effects of lipotoxicity using a mouse model of obesity and insulin resistance. We treated ob/ob and LDLR-double deficient mice with stevioside (10 mg·kg^{-1}·day-1 p.o., n = 8), rebaudioside A (12 mg·kg^{-1}·day-1 p.o., n = 8), or steviol (5 mg·kg^{-1}·day^{-1} p.o., n = 8). We determined their effects on liver steatosis and on the metabolic effects of lipotoxicity by histological analysis, and by combined gene-expression and metabolomic analyses. All compounds attenuated hepatic steatosis. This could be explained by improved glucose metabolism, fat catabolism, bile acid metabolism, and lipid storage and transport. We identified PPARs as important regulators and observed differences in effects on insulin resistance, inflammation and oxidative stress between *Stevia*-derived compounds. We conclude that *Stevia*-derived compounds reduce hepatic steatosis to a similar extent, despite differences in effects on glucose and lipid metabolism, and inflammation and oxidative stress. Thus our data show that liver toxicity can be reduced through several pathophysiological changes. Further identification of active metabolites and underlying mechanisms are warranted.

1. Introduction

The growing incidence, prevalence and severity of obesity and obesity-related disturbances have led to the quest for natural sweeteners that do not provide calories. Much attention has been placed on glycosides which are extracted from *Stevia rebaudiana* Bertoni. This perennial herb from the *Asteraceae* family is known to the scientific world for its sweetness caused by steviol glycosides (SG) which are synthesized by SG biosynthesis pathway operating in the leaves. Stevioside and rebaudioside A are the major metabolites (Yadav and Guleria, 2012). Stevioside is one of the major sweeteners in use in Japan and Korea (Geuns, 2003; Geuns et al., 2003). Stevioside and rebaudioside A undergo hydrolysis by the intestinal microflora to steviol, which is not further metabolized by the intestinal flora and is absorbed from the intestine. The rate of hydrolysis for stevioside is greater than for rebaudioside A (Renwick and Tarka, 2008).

Obesity, a low-grade inflammatory and oxidative stress state, is closely associated with multiple metabolic alterations, including Type 2 Diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD) and the associated toxic effects of the ectopic accumulation of lipids and oxidized lipids in tissues (Butler et al., 2006). Indeed, studies showed a positive relationship between obesity, inflammatory C-reactive protein (Visser et al., 1999), and oxidized LDL (ox-LDL) (Van Guilder et al., 2006). Moreover, we showed that ox-LDL was associated with the incidence of the metabolic syndrome and several of its components, especially visceral obesity (Holvoet et al., 2004, 2008). Stevioside was found to exert antihyperglycemic and insulinotropic effects in a non-obese animal model of type-2 diabetes (Jeppesen et al., 2002) by acting directly on pancreatic cells (Jeppesen et al., 2000). More recently, we showed that stevioside treatment of obese diabetic mice improved adipose tissue maturation, and increased glucose transport, insulin signaling and antioxidant defense in white visceral adipose tissues. Together, these increases were associated with a twofold increase of adiponectin. In addition, stevioside reduced plaque volume in

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the aortic arch by decreasing the macrophage, lipid and ox-LDL content of the plaque. Circulating adiponectin was associated with improved insulin signaling and antioxidant defense in both the adipose tissue and the aorta of stevioside-treated mice (Geeraert et al., 2010). However, the effects of steviol and rebaudioside A are not well known. We selected mice with combined leptin and LDL-receptor deficiency (double knockout [DKO] mice) because they exhibit most of the metabolic syndrome components which are associated with increased inflammation and oxidative stress, accelerated atherosclerosis and impaired cardiovascular function (Verreth et al., 2004). However, the effects of *Stevia*-derived glycosides, stevioside and rebaudioside, and their aglycol derivative steviol on liver steatosis in a mouse model of obesity and hyperlipidemia have not been studied. Importantly, DKO mice spontaneously develop liver steatosis without the use of predisposing diets, representing a model in which the storage capacity of the adipose tissue is exceeded and ectopic lipid deposition begins in other organs. This is particularly important because NAFLD is strongly linked to obesity and associated metabolic syndrome and cardiovascular disease in humans (Angulo, 2007; Rull et al., 2010). The contribution of NAFLD is generally neglected probably because there is not a clinically efficient, sensitive diagnostic procedure for the early detection of the disease, and the assessment and monitoring of response to treatment thereof. In addition, pathogenic mechanisms are not completely understood (Feuerer et al., 2009; Johnson et al., 2008; Lee et al., 2008; Nishimura et al., 2009; Rull et al., 2010; Winer et al., 2009). Because a progressive course is possible in this condition, a benign prognosis should not be made. This is particularly distressing because the prevalence of NAFLD is constantly increasing. No proven treatment for the overall management of NAFLD has yet emerged other than the obvious weight loss through diet and exercise as a primary recommendation. Therefore, we analyzed the effect of a natural sweetener on liver steatosis in a mouse model representative for the metabolic syndrome.

2. Materials and methods

2.1. Experimental design

Experimental procedures were performed in accordance with protocols of the Animal Care and Research Advisory Committees of KU Leuven. Homozygous LDL receptor knockout mice ($LDLR^{-/-}$), heterozygous *ob*/+, and C57BL6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). $LDLR^{-/-}$ mice were backcrossed into a C57BL6 background to the tenth generation and had 98.4% C57BL6 background. To obtain leptin deficiency (*ob*/*ob*) on a background of LDLR deficiency, $LDLR^{-/-}$ and *ob*/+ mice were crossed, and the F1 progeny of this mating ($LDLR^{-/+}$; *ob*/+) were then crossed to obtain mice that had zero, one, or both normal LDLR alleles and were leptin-deficient ($LDLR^{-/-}$; *ob*/*ob*, $LDLR^{+/-}$; *ob*/*ob*, and $LDLR^{+/+}$; *ob*/*ob*, respectively) as well as control $LDLR^{-/-}$, $LDLR^{+/-}$, and wild-type mice. All offspring were genotyped by polymerase chain reaction (PCR) techniques as previously described (Mertens et al., 2003). Animals were housed at 22 °C on a fixed 12/12-hour light–dark cycle and were fed regular chow diet throughout the experiment. Food and water were available *ad libitum* and intake was similar in all treatment groups. Male mice were daily treated as described (Geeraert et al., 2010) for 12 weeks starting at the age of 12 weeks, with the study compounds stevioside (10 $mg \cdot kg^{-1} \cdot day^{-1}$ *p.o.*, n = 8), or rebaudioside A (12 $mg \cdot kg^{-1} \cdot day^{-1}$ *p.o.*, n = 8), or steviol (5 $mg \cdot kg^{-1} \cdot day^{-1}$ *p.o.*, n = 8). The European Food Safety Authority established an Acceptable Daily Intake for steviol glycosides, expressed as steviol equivalents, of 4 mg/kg bodyweight/day, corresponding to 10.6 $mg \cdot kg^{-1} \cdot day^{-1}$ of stevioside and 12 $mg \cdot kg^{-1} \cdot day^{-1}$ rebaudioside A. On 11 November 2011, the European Commission allowed the usage of steviol glycosides as a food additive, establishing maximum content levels for different types of foods and beverages (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:295:0205:0211:EN:PDF). We used 12 untreated, obese, age-matched mice as a control group. After 12 weeks, all animals were sacrificed for analyses. The animals were allocated to experimental groups by computer-generated randomization schedules and investigators responsible for the assessment of outcomes had no knowledge of the experimental group to which the animals belonged. Treatments were prepared and labeled by an independent investigator according to the randomization schedule to assure allocation concealment. There were no animals excluded from analysis. Stevioside, rebaudioside A and steviol were extracted from the leaves of the *S. rebaudiana* Bertoni plant, and purified according to procedures developed by the KU Leuven (Geuns et al., 2006; Minne et al., 2004).

2.2. Biochemical measurements

Fasting blood was collected in EDTA-containing tubes. Plasma cholesterol and triglyceride concentrations were measured with standard enzymatic assays (Boehringer, Mannheim, Germany), glucose with a glucometer (Menarini Diagnostics, Zaventem, Belgium), and insulin with a mouse ELISA (Mercodia, Oxon, UK). The homeostatic model assessment index (HOMA-IR) was calculated as an estimate of insulin resistance (IR) (Matthews et al., 1985). Glucose tolerance was determined by the intraperitoneal glucose tolerance test (IPGTT). Glucose was measured in samples obtained by tail bleeding before and 15, 30, 60, 120 and 240 min after i.p. glucose administration (20% glucose solution; 2 g kg^{-1}). Interleukin-6 (IL6), adiponectin, and tumor necrosis factor-alpha (TNFα) were measured with specific mouse ELISA (R&D Systems, Uppsala, Sweden) (Geeraert et al., 2010).

2.3. Histological analyses

Livers were perfused, removed, prepared in portions and either frozen or stored at –80 °C until used or fixed for 24 h in 10% neutral-buffered formalin. Sections of 2 μm thickness were stained with hematoxylin and eosin to evaluate histological alterations. The percentage of fat in liver tissue was estimated by image analysis, by using the AnaliSYS™ (Soft Imaging System, Münster, Germany) software (Joven et al., 2007; Tiniakos, 2010), and expressed as the ratio between the area covered by lipid droplets and the total area. For each individual mouse, 30 fields from each of 3 different sections were analyzed.

2.4. Quantitative real time PCR and transcriptomic profiling

Total RNA was isolated from liver tissue of overnight fasting mice (n = 6 for each group) using the QIAcube system with spin-column kits from QIAgen (Izasa, Barcelona, Spain). TaqMan® primers and probes were obtained from validated Assays-on-Demand products (Applied Biosystems, Foster City, CA, USA) (Supplementary Table S1) and used in real time PCR (rt-PCR) amplifications on the 7900HT Fast Real-Time PCR system (Applied Biosystems). For transcriptomic data analysis we used an integrated web-based RT^2 profiler PCR array data analysis software (SABiosciences, Qiagen) (Mehta et al., 2010). The level of RNA expression of each gene was calculated by using the threshold cycle (Ct) value and normalized with the appropriate housekeeping gene (Delta Ct). Differences between stevia-derived treated animals and the control group were determined by using the fold change gene expression [2^ (-Delta Ct)], that is the normalized gene expression in each treated group divided by the normalized gene expression in the control group.

2.5. Metabolomic profiling

Portions of the left and right lobes of the liver (n = 6 for each group) were sent to Metabolon Inc. (Research Triangle Park, Durham, NC, USA), extracted and prepared for analysis using reported methods (García-Heredia et al., 2013a). Metabolites from an equivalent amount of liver tissue were extracted with methanol, and the resulting extract divided into equal fractions for analysis by ultra high performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS; separately under positive mode and negative mode) and gas chromatography–mass spectrometry (GC–MS). Metabolites were identified by comparison of ion data to a reference library of chemical standards (~2800) entries that included retention time, mass (m/z), and MS or MS/MS spectra (García-Heredia et al., 2013b; Rull et al., 2014).

2.6. Statistical analyses

Data were initially analyzed using ANOVA (single-factor or two factors) when necessary. Differences between any two groups were assessed with the Mann–Whitney *U*-test. Spearman correlation coefficients were used to evaluate the degree of association between variables. The SPSS/PC + 18.0 (SPSS, Chicago, IL) was employed for these purposes. Areas under the curve were calculated using the Graph Pad Prism version 5 program. The level of significance was set at $p \leq 0.05$. For the statistics of the transcriptomic analyses, the web-based RT^2 profiler PCR array data analysis software was used (SABiosciences, Qiagen) (Mehta et al., 2010). The p-value was calculated by using the 2^ (-Delta Ct) gene expression level. Our transcriptomic data comprised a total of 46 genes, and expecting to see approximately 2 genes meeting the $p \leq 0.05$ cut-off by random chance, no estimation of the False Discovery Rate (FDR) was considered necessary. For the statistics of the metabolomic analyses, Welch's two-sample *t*-test was used to identify biochemicals that differed significantly between the study contrast groups. An estimate of the FDR (q-value) was calculated to correct for multiple comparisons that normally occur in metabolomics-based studies (Gall et al., 2010). Our dataset comprises a total of 321 named biochemicals, so we would expect to see 16 compounds meeting the $p \leq 0.05$ cut-off by random chance. Because q-values were reasonable for $p \leq 0.05$, no q-value cut-off was established for this study. Metabolomics statistical analyses were performed with the program "R" http://cran.r-project.org/ (Metabolon Inc.).