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The buckwheat iminosugar D-fagomine

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attenuates sucrose-induced steatosis and hypertension in rats

Sara Ramos-Romero^{1,2,*}, Mercè Hereu¹, Lidia Atienza³, Susana Amézqueta⁴, Josefina Casas^{5,6}, Silvia Muñoz⁷, Isabel Medina⁷, Bernat Miralles-Pérez⁸, Marta Romeu⁸, and Josep L. Torres¹

¹Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

² Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

³ Department of Pathology, Puerta del Mar University Hospital, Cádiz, Spain

⁴ Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Barcelona, Spain

⁵ Research Unit on BioactiveMolecules (RUBAM), Department of Biological Chemistry, Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

⁶ Center for Biomedical Research on Hepatic and Digestive Diseases (CIBEREHD), ISCIII, Madrid, Spain

⁷ Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain

⁸ Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Reus, Spain

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* Dr. Sara Ramos-Romero

IQAC-CSIC, Jordi Girona 18-26,

08034 Barcelona, Spain

Phone: (+34) 934006100

Fax: (+34) 932045904

E-mail: sara.ramos@iqac.csic.es

Abbreviations

AT, Adipose Tissue CVD, Cardiovascular Disease DAGs, Diacylglycerols DNL, De Novo Liposynthesis F2-IsoPs, F_{2t} Isoprostanes FG, D-fagomine HF, High-Fat HS, High-Sucrose HS+FG, High-Sucrose with D-fagomine IGT, Impaired Glucose Tolerance IR, Insulin Resistance MetS, Metabolic syndrome MRM, Multiple Reaction Monitoring NO, Nitric Oxide OGTT, Oral Glucose Tolerance Test **OS**, Oxidative Stress qRT-PCR, Quantitative Real-Time PCR RAAS, Renin-Angiotensin-Aldosterone System SREBP-1c, Sterol Receptor Element Binding Protein-1c STD, Standard VAT, Visceral Adipose Tissue VLDL, Very-Low-Density Lipoprotein WKY, Wistar Kyoto

Abstract

Scope: This study examines the long-term functional effects of D-fagomine on sucroseinduced factors of metabolic dysfunctions and explores possible molecular mechanisms behind its action.

Methods & results: Wistar Kyoto (WKY) rats were fed a 35% sucrose solution with D-fagomine (or not, for comparison) or mineral water (controls) for 24 weeks. We recorded: body weight; energy intake; glucose tolerance; plasma leptin concentration and lipid profile; populations of Bacteroidetes, Firmicutes, bacteroidales, clostridiales, enterobacteriales, and *Escherichia coli* in feces; blood pressure; urine uric acid and F_{2t} isoprostanes (F₂-IsoPs); perigonadal fat deposition; and hepatic histology and diacylglycerols (DAGs) in liver and adipose tissue.

D-Fagomine reduced sucrose-induced hypertension, urine uric acid and F_2 -IsoPs (markers of oxidative stress; OS), steatosis and liver DAGs, without significantly affecting perigonadal fat deposition and impaired glucose tolerance. It also promoted excretion of enterobacteriales generated by the dietary intervention.

Conclusion: D-fagomine counteracts sucrose-induced steatosis and hypertension, presumably by reducing the postprandial levels of fructose in the liver.

1. Introduction

s[1]Central fat accumulation and hypertension are risk factors for cardiovascular disease (CVD). Together with insulin resistance (IR), they are part of the cluster of factors known as metabolic syndrome (MetS) [1].

Dietary-fat-induced central adiposity results in ectopic fat deposition when subcutaneous adipocytes become insulin resistant and lose their capacity to store triacylglycerols, which are then deposited at undesirable sites such as the liver, the heart, the skeletal muscle or visceral adipose tissue (VAT) [2]. Then visceral adipocytes become resistant to the antilipolytic effect of insulin and free fatty acids drain directly into the liver through the portal vein, leading to impaired liver metabolism [3]. In contrast, sucrose (glucose/fructose)-induced adiposity develops through other pathways linked primarily to the excess of fructose. Unlike glucose, fructose is almost entirely catabolized, mainly in the liver, and escapes metabolic control by insulin [4]. Apart from contributing substrates for de novo liposynthesis (DNL), fructose may increase hepatic lipid levels by activating modulators of liposynthesis such as sterol receptor element binding protein-1c (SREBP-1c) [5]. Fructose-driven DNL also contributes to liver fat accumulation by inhibiting the hepatic oxidation of endogenous and exogenous fatty acids increased levels of malonyl-coA [6]. Hepatic DNL triggers postprandial via hypertriglyceridemia, which may promote lipid deposition as VAT rather than as subcutaneous adipose tissue [7].

As adipose tissue (AT) is a source of angiotensinogen, adiposity may lead to hypertension via activation of the renin-angiotensin-aldosterone system (RAAS) [8]. Free fatty acids from VAT may stimulate the RAAS via aldosterone production independently of renin [8], and they may also trigger hypertension via activation of the sympathetic nervous system [9]. Increased levels of uric acid may contribute to hypertension through systemic and renal

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vasoconstriction by increasing intracellular oxidative stress (OS), activating the RAAS, and inhibiting the production of endothelial nitric oxide (NO) [10].

D-Fagomine (1,2-dideoxynojirimycin) is a polyhydroxylated nitrogen-containing ring structurally related to glucose and mannose. It is a minor component of buckwheat (Fagopyrum esculentum Moench, Polygonaceae) and traditional buckwheat-based foodstuffs such as noodles, pancakes, fried dough, beer, cookies and bread [11]. In rats, D-fagomine reduces elevated plasma insulin concentrations induced by a high-fat (HF) diet (Sprague-Dawley rats, 9 weeks) [12] and it counteracts fat-induced low-grade inflammation and impaired glucose tolerance (IGT) (Wistar Kyoto (WKY) rats, 13-21 weeks) via a mechanism that may involve modifications of gut microbiota [13]. As D-fagomine is poorly absorbed [14], it is likely to be largely in contact with the intestinal wall. There, D-fagomine also delays starch and sucrose digestion by inhibiting brush border glycosidases [15]. In previous work we have shown that an excess of fat or sucrose (glucose/fructose) triggers different risk factors of MetS at different times in WKY rats: an HF diet induced obesity and fast onset IR and IGT via low-grade inflammation; while a high-sucrose (HS) diet induced IGT later than the HF diet, through liver DNL from fructose. Meanwhile, only the HS diet triggered elevated blood pressure [16]. After reporting the long-term functional effect of D-fagomine on fat-induced MetS factors [13], we here examine its effects on sucrose-induced factors, namely VAT, fatty liver, IGT and hypertension.

2. Materials and Methods

2.1 Animals

A total of twenty-seven male WKY rats from Envigo (Indianapolis, IN, USA), aged 8-9 weeks were used. All animal manipulation was carried out in the morning to minimize the effects of circadian rhythms. All the procedures strictly adhered to the European Union

guidelines for the care and management of laboratory animals and were licensed by the Catalan authorities (reference no. DAAM7921), as approved by the Spanish CSIC Subcommittee of Bioethical Issues.

2.2 Experimental Design and Sample Collection

The rats were kept under controlled conditions of humidity (60%), and temperature (22 ± 2 °C) with a 12 h light-12 h dark cycle. They were randomly divided into 3 dietary groups (n = 9 per group), all fed a standard feed (2014 Teklad Global 14% Protein) from Envigo *ad libitum* with free access to water or sucrose solutions as follows: the standard (STD) group was given mineral water (Ribes, Girona, Spain); the HS group was given a 35% sucrose solution in mineral water as the only source of liquid intake; and the HS with D-fagomine (HS+FG) group was given a 35% sucrose solution in mineral water supplemented with D-fagomine (> 98% from Bioglane SLNE; Barcelona, Spain). The dosage of D-fagomine was the same used in the previous HF study (2 mg per g carbohydrate) [13].

Feed and drink consumptions were monitored daily and body weight was measured weekly throughout the experiment. Energy intake was estimated as metabolizable energy based on the Atwater factors:4 kcal per g protein, 9 kcal per g fat, and 4 kcal per g available carbohydrate. Fecal samples were collected by abdominal massage after week 20. The energy content of the feces was determined by differential scanning calorimetry (25-600 °C in an O_2 atmosphere, 10 °C min⁻¹) by means of a thermogravimetric analyzer TGA/SDTA 851e (Mettler Toledo; Columbus, OH, USA) with an integrated SDTA signal.

After week 23, the rats were placed in metabolic cages overnight for urine collection. At the end of the experiment (week 24), the rats were anaesthetized intraperitoneally with ketamine and xylazine (80 and 10 mg per kg body weight, respectively) after overnight fasting. Blood was collected by cardiac puncture then plasma was immediately obtained by centrifugation

and stored at -80 °C until analysis. Perigonadal AT, a type of VAT in rats [17], and liver were removed, weighed and cut into small pieces. One part of the liver was fixed in 10% formalin for histological analysis. The rest of the liver and adipose tissue were washed with 0.9% NaCl solution and stored at -80 °C for DAG analysis.

2.3 Oral Glucose Tolerance Test, Fasting Glucose and Plasma Insulin

After weeks 13 and 21, an oral glucose tolerance test (OGTT) was performed on overnight fasted animals. A solution of glucose (1 g per kg body weight) was administered to the rats by oral gavage. Blood glucose concentration was measured by the enzyme electrode method using an Ascensia ELITE XL blood glucose meter (Bayer Consumer Care AG; Basel, Switzerland) before the experiment and 15, 30, 45, 60, 90 and 120 min after glucose intake. Fasting blood glucose was measured by the same method after week 24 on animals fasted overnight. Plasma insulin was determined on these samples using Milliplex xMAP multiplex technology on a Luminex xMAP instrument (Millipore, Austin, TX). Milliplex Analyst 5.1 (Vigenetech, Carlisle, PA, USA) software was used for data analysis. The standard curve was generated for the range 69-50,000 pg mL⁻¹ using a five-parameter logistic curve fit.

2.4 Plasma Lipid Profile and Leptin Concentration

Total plasma cholesterol, HDL and LDL cholesterol, and triglycerides were all measured by spectrophotometric methods using the corresponding kits from Spinreact (Girona, Spain) as described elsewhere [18]. Leptin levels were measured together with insulin using the Milliplex xMAP multiplex technology (Millipore).

2.5 Fecal Microbiota

The levels of total bacteria and Bacteroidetes, Firmicutes, Enterobacteriales, and Escherichia coli were estimated from fecal DNA by quantitative real-time PCR (qRT-PCR). DNA was extracted from the feces using the QIAamp DNA StoolMini Kit from Qiagen (Hilden, Germany) and quantified using a Nanodrop 8000 Spectrophotometer (Thermo Scientific; Waltham, MA, USA). were qRT-PCR was carried out in triplicate on diluted DNA samples (20 ng μ L⁻¹), using a LightCycler 480 II (Roche; Basel, Switzerland). Eachreaction mixture contained DNA solution (2 μ L) and a master mix (18 μ L) made of 2XSYBR (10 μ L), the corresponding forward and reverse primer (1 μ L each), and water (6 μ L). Both Nontemplate controls (water) and positive controls were included. The primers and annealing temperatures are detailed in Table S1, Supporting Information. The PCR cycling conditions were: 10 s at 95 °C, then 45 cycles of 5 s at 95 °C, 30 s at the primer-specific annealing temperature (Table S1, Supporting Information), and 30 s at 72 °C (extension). To determine the specificity of the qRT-PCR after amplification, melting curve analysis was carried out: 2 s at 95 °C, 15 s at 65 °C, followed by atemperature gradient up to 95 °C at a rate of 0.11 °C s^{-1} , with five fluorescence readings per °C. The relative DNA abundances for the different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated in triplicate) by considering Cp values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, according to the equation: [DNAa]/[DNAb] = 2Cpb-Cpa [19]. Total bacteria was normalized as 16S rRNA gene copies per mg of wet feces (copies per mg).

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2.6 Blood Pressure, and Urine and Plasma Uric Acid

Systolic and diastolic blood pressure was measured at time 0 and after weeks 4, 9, 15 and 22 by the tail-cuff method, using a non-invasive automatic blood pressure analyzer (Harvard Apparatus, Holliston, MA, USA).

Total urine and plasma uric acid were determined by a spectrophotometric method using a uricase/peroxidase kit from BioSystems (Barcelona, Spain) via measuring the absorbance at 520 nm on a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Creatinine levels in urine were determined by a colorimetric method using a commercial kit (C-cromatest Linear Chemicals, Montgat, Spain) via measuring absorbance at 510 nm.

2.7 Measurement of Isoprostanes

F₂-isoprostanes (F₂-IsoPs) were determined in urine samples by LC/ESI–MS/MS following a previously reported procedure [12] with some modifications. Samples (500 µL) were acidified and the mixtures were incubated for 2 h at 37 °C in the presence of β-glucuronidase (90 U mL⁻¹) (Sigma, Saint Louis, MI, USA). , F₂-IsoPs were purified by SPE after adding $[^{2}H_{4}]15$ -F_{2t}-IsoP (Cayman, Ann Arbor, MI, USA) (internal standard 100 µL, 10 µg L⁻¹) and analyzed using an Agilent 1260 chromatograph coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) fitted with a Mediterranea Sea 18 column (10 cm x 2.1 mm i.d., 2.2 µm particle size) (Teknokroma, Barcelona, Spain). The instrument was operated in the negative-ion mode with a Turbo V source to obtain MS/MS data. The chromatography solvents used were [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in acetonitrile, and the solutes were separated with an increasing linear gradient (v/v) of [B]: time 0, 10% B; 7 min, 50% B; 7.1 min, 100% B; 8 min, 100% B; 8.1 min, 10% B; and 10 min, 10% B, at a flow rate of 700 µL min⁻¹ at 40 °C. F₂-IsoPs were detected by multiple

reaction monitoring (MRM). Calibration curves were prepared using seven matrix-matched standards covering the working concentration range. The LOQ was 0.4 μ g L⁻¹ for 15-F_{2t}-IsoP and 2 μ g L⁻¹ for 5-F_{2t}-IsoP. The results were expressed as ng per mg creatinine, to correct for urine dilution.

2.8 Histology of the Liver

Formalin fixed livers were dehydrated in alcohol and embedded in paraffin (Panreac Quimica SLU; Barcelona, Spain), and then cut into 3 μ m thick slices, using a steel knife mounted in a microtome (HM 355S Rotary Microtome; Thermo Fisher Scientific, Waltham, MA, USA). Sections were stained with hematoxylin (Hematoxylin solution modified according to Gill III for microscopy; Merck KGaA, Darmstadt, Germany)/eosin (Pharmacy Service of Puerta del Mar Hospital, Cádiz, Spain) then viewed under a light microscope (NIKON Eclipse 80i; NIKON Corporation, Minato, Japan). Three parameters were graded following the method described by *Taltavull et al* [20]: steatosis, 0 (<5%), 1 (5%–33%), 2 (33%–66%), or 3 (>66%); steatosis localization, 0 (absence), 1 (periportal), or 2 (non-zonal); and the presence of lipogranuloma, 0 (absence) or 1 (presence).

2.9 Diacylglycerols in Liver and Adipose Tissue

Frozen samples were thawed and homogenized by sonication on a SFX150 Sonifier (Emerson Industrial Automation, St. Louis, MO, USA). DAGs were analyzed using the method described by Simbari *et al* [21] with some modifications. The internal standard was 1,3-17:0 D5 DG (Avanti Polar Lipids Inc., Alabaster, AL, USA; 200 pmol) and the incubation conditions were 12 h at 48 °C. Then the samples were dried and suspended in methanol, centrifuged (9390 g, 3 min) and the supernatants (8 μ L) loaded into an Acquity UPLC separation system connected to an LCT Premier orthogonal accelerated time-of-flight

mass spectrometer (Waters; Milford, MA, USA), operated in positive ESI mode (LC-TOF-MS). DAG resolution was achieved using a C8 Acquity UPLC-bridged ethylene hybrid 100 x 2.1 mm i.d., 1.7 μ m column (Waters) and a binary elution system consisting of [A] 0.2% (v/v) formic acid, 2 mM ammonium formate in water and [B] the same buffer in methanol, under linear gradient conditions: 0.0 min, 80% B; 3 min, 90% B; 6 min, 90% B; 15 min, 99% B; 18 min, 99% B; 20 min, 80% B; and 22 min, 80% B, at 30 °C. The flow rate was 0.3 mL min⁻¹. Full-scan spectra from 50 to 1,500 Da were acquired, and individual spectra were summed to produce data points of 0.2 s each. To maintain mass accuracy and precision leucine enkephalin was used as an independent reference spray () via the LockSpray interface.. Quantification was carried out using the extracted ion chromatogram of each compound, with 50 mDa windows. The linear range was determined by injecting mixtures of internal standards. DAG content was calculated as DAG 16:0, 16:0 equivalents.

2.10 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). Quantitative data are expressed as mean values with their standard errors (SEM). Normal distributions and heterogeneity of the data were evaluated and statistical significance was determined by two-way ANOVA for repeated measures (OGTT). One-way ANOVA, and Tukey's multiple-comparison test were used for mean comparison. Differences were considered significant when P < 0.05. The results from qualitative measurements of histological sections are expressed in frequencies (percentage of animals that present the value, or do not) and their statistical significance was determined using contingency tables and χ^2 statistics.

3. **Results**

3.1. Feed and Drink Intakes, Energy Balance, and Body and Perigonadal Adipose Tissue Weights

Feed intake was lower and drink intake was higher in both HS (HS and HS+FG) groups than in the STD group (Table 1). The glucose/fructose intake was similar in the HS and HS+FG groups. Energy intake was higher in both HS groups than in the STD group; while energy in feces was similar in all three groups studied (Table 1).

Body weight was similar in all of the groups at the beginning (234.8 g, SEM 3.2), and no differences were observed between the STD and either HS group throughout the experiment (Table 1). Perigonadal AT weight was significantly higher in the HS and HS+FG groups (P <0.001) and (P <0.01, respectively) (Table 1).

3.2 Glycemic Status.

The OGTT test was run after weeks 13 and 21. There were no significant differences between groups after week 13 (Figure 1A). After week 21, the levels of postprandial glucose in animals that consumed an excess of sucrose (HS and HS+FG groups) were higher (P < 0.01) than those in the STD group, 30 and 45 min after glucose administration (Figure 1B). At 60 and 90 min after administration, the levels of plasma glucose were higher (P < 0.01) in the HS than the STD group; while there was no difference between the STD and HS+FG groups.

Fasting blood glucose was below 70 mg dL^{-1} in all three groups after week 21 and at the end of the study (week 24) (Figure 1, Table 2). Fasting plasma insulin was significantly higher in animals given either HS diet (HS and HS+FG groups) (Table 2).

3.3 Plasma Leptin and Lipid Profile

Plasma leptin was higher (P < 0.001) in the HS group than in the STD group (Table 2). Total and LDL cholesterol in plasma were similar in all the groups (Table 2). HDL cholesterol levels were higher (P < 0.05) in the HS and HS+FG groups than in the STD group. Plasma triglyceride levels were higher in both HS groups than in the STD group, but this difference was only significant (P < 0.05) between the HS+FG group and the STD group.

3.4 Fecal Microbiota

The proportions of bacterial phyla, orders and *E. coli* in the gut microbiota were evaluated after week 24 (Table 3). The Bacteroidetes, bacteroidales, enterobacteriales and *E. coli* populations in feces were significantly higher (P < 0.05) in both HS groups. The highest populations were recorded in the D-fagomine supplemented group.

3.5 Blood Pressure and Uric Acid in Urine and Plasma

After 23 weeks of sucrose intake, systolic (Figure 2A) and diastolic (Figure 2B) blood pressures were significantly higher (P < 0.05) in animals fed the HS diet than in those fed the STD or HS+FG diets. Animals in the two groups that consumed an excess of sucrose (HS and HS+FG) presented a significantly (P < 0.001) higher concentration of urine uric acid than those of the STD group after week 23 (Figure 2C). The group supplemented with D-fagomine (HS+FG) presented significantly (P < 0.05) lower concentration of urine uric acid than the HS group and the lowest concentration among groups in plasma (Figure 2C, D).

3.6 Urine Isoprostanes

The animals fed the HS diet presented increased concentrations of 5-F2t-IsoP and 15-F2t-IsoP (P < 0.05) compared with the STD group after 23 weeks of intervention (Figure 3A, B); while the animals supplemented with D-fagomine (HS+FG group) presented concentrations of IsoPs similar to those in the STD group (Figure 3A, B).

3.7 Liver Histology

An excess of sucrose induced significant (P < 0.001) and highly localized steatosis (Figure 4B, C, D) and lipogranuloma (P < 0.001; Figure 4E). D-Fagomine supplementation (HS+FG group) significantly (P < 0.001) reduced the grade of steatosis, from 2 (33%-66% steatosis) in the HS group to 1 (5%-33% steatosis), with no influence on its localization. Lipogranuloma was less (P < 0.001) present in animals in the HS+FG group than in those in the HS group (Figure 4E).

3.8 Liver and Adipose Tissue Diacylglycerols

The levels of DAGs 32:1, 32:2, 34:1 and 36:2 were higher in livers from rats in the HS group than from those in the STD and HS+FG groups (Figure 5 and Table S2, Supporting Information). There were no significant differences in any DAG content between the HS and HS+FG groups in AT (Table S3, Supporting Information). The present study explores the effects of D-fagomine on a rat model of sugar-induced

4. Discussion

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metabolic alterations. From previous work we knew that WKY rats given a 35% sucrose (glucose/fructose) solution as the only source of liquid intake present moderate IGT, steatosis, deposition of perigonadal AT and raised blood pressure after 21-24 weeks of intervention, while remaining normoweight [16]. Here, we show that D-fagomine can counteract this induced steatosis and the elevation in blood pressure, while it had little effect on perigonadal fat (Table 1) and IGT (Figure 1). Gonadal AT is a type of VAT in rats [17] that has been associated with low-grade inflammation and metabolic complications, mainly owing to the production of free fatty acids and proinflammatory adipokines by adipocytes [2, 22]. Therefore, the increase in VAT may account, at least in part, for the IGT observed in the groups fed HS diets. Direct disruption of insulin signaling by lipid metabolites such as DAGs may also contribute to the sucrose-induced IGT [7, 16]. Fat accumulation and generation of DAGs in the liver would result from fructose-induced DNL [4]. Fructose-induced DNL is believed to trigger fat deposition in VAT by supplying triglycerides from the liver via upregulation of very-low-density lipoprotein (VLDL) production and secretion [7]. In contradistinction to this view, our observation that D-fagomine reduced steatosis (Figure 4) and not perigonadal fat deposition (Table 1) suggests that fructose-induced visceral fat accumulation may proceed independently of liver DNL. This hypothesis is supported by previous evidence that overconsumption of fructose may lead to rapid inflammation in subcutaneous adipocytes and an increase in intracellular cortisol that stimulates the flux of fatty acids into VAT [23] independently of any effect in the liver. The fact that D-fagomine only reduced fat deposition in the liver might be connected to its inhibitory activity on intestinal sucrase [15]. As fructose is mainly accumulated in the liver [4], this organ may be more sensitive than AT to variations in fructose concentration. Therefore, as D-fagomine

consistently reduces postprandial blood glucose/fructose levels by 25% at the dose supplied via the drink (2 mg per g sucrose) [15], it may be reducing liver DNL through lowered activation of regulatory factors such as SREBP-1c [5]. This reduction in liver DNL is consistent with the observation that D-fagomine maintains the levels of liver DAGs similar to those in the STD group, while they are significantly elevated (P < 0.05) in the HS group (Figure 5). In contrast, D-fagomine did not modify DAG levels in perigonadal AT with respect to the HS group (Table S3, Supporting Information), which confirms that a reduction in postprandial fructose concentration would not significantly affect DNL in AT. The reduction of liver DAGs may account, at least in part, for the slight effect of D-fagomine on sucrose-induced IGT recorded at the end of the study (Figure 1, time 90).

D-Fagomine also affected the levels of fasting leptin: an anorectic hormone that helps to regulate energy expenditure [24], produced by white AT and other organs [25]. In agreement with studies on both rodents and humans [26, 27], fasting plasma leptin levels where significantly higher (P < 0.001) in animals given an excess of sucrose than in those given the STD diet (Tables 1 and 2). WKY rats supplement with D-fagomine presented levels of fasting leptin similar to those in the STD group (Table 2). In a similar experiment, Sprague-Dawley rats given free access to a 30% sucrose solution developed insulin sensitivity-independent leptin resistance that was rapidly reverted (days) after the excess of sucrose was eliminated from the diet [28]. This suggests that adiposity as such may not be a major contributor to leptin resistance in that model. The reduction of postprandial glucose/fructose concentration might be responsible for the effect of D-fagomine on circulating leptin.

The animals consuming an excess of sucrose without D-fagomine supplementation (HS group) showed elevated blood pressure (Figure 2) without increased body weight (Table 1) in agreement with other studies [16, 29-31]. The reduction of circulating fructose levels may also explain the D-fagomine-mediated reduction in blood pressure (Figure 2). High levels of

liver fructose triggers elevated blood pressure by generating an excess of uric acid via overexpression of fructokinase C, increased ATP consumption and nucleotide turnover [10, 16, 32]. Lower levels of postprandial fructose in the supplemented group would be consistent with the reduction in the levels of urine and plasma uric acid (Figure 2), which may explain, at least in part, the effect of D-fagomine on lowering blood pressure. The levels of uric acid did not increase in plasma (Figure 2). This may be because urine concentration was measured in fasted animals while the physiologically relevant high levels of plasma uric acid are likely to occur in the postprandial period. In fasted animals, the levels of uric acid in urine are more likely to reflect chronic effects of fructose consumption. Additionally to affecting uric acid, D-fagomine may counteract the possible hypervolemic effect induced by a chronic high intake of sucrose [29], also by lowering the circulating levels of glucose/fructose. D-Fagomine also prevented the sucrose-induced systemic OS, which was monitored by measuring urine F₂-IsoPs (Figure 3). This late (week 24) OS does not seem to be a direct consequence of IR or IGT [16], while it may be related to elevated blood pressure via generation of uric acid [33].

The role of intestinal microbiota in host homeostasis is increasingly being revealed [34]. At the phylum level, a reduced Bacteroidetes/Firmicutes ratio compared to the lean phenotype has been associated with the obese phenotype in both humans and mice [35, 36]. In our experiment, the HS diet (HS and HS+FG groups) did not affect the populations of the phylum Firmicutes in the gut microbiota of WKY rats while it triggered a slight increase in Bacteroidetes that was more evident in the supplemented group (Table 3). This is consistent with the absence of weight gain differences between the STD, HS and HS+FG groups (Table 1). The populations of Enterobacteriales, and particularly *E. coli*, were higher (P < 0.05) in the HS group than the STD group (Table 3). Previously, we have suggested elsewhere that this may be connected to an increase in uric acid excreted via feces, which would foster the proliferation of Enterobacteriales [16]. The inhibitory activity of D-fagomine on epithelial adhesion of *E. coli* [15] would therefore explain the increase in excreted Enterobacteriales in the supplemented group (Table 3).

In conclusion, D-fagomine counteracted sucrose-induced fatty liver and elevated blood pressure in rats. The maintenance of DAG levels in the liver, though not in VAT, together with the effect of lowering uric acid concentrations suggest that D-fagomine selectively influences liver function, probably by reducing the levels of postprandial fructose as a result of its inhibitory activity on intestinal sucrase. The selective action of D-fagomine also suggests that fat deposition in VAT is not a direct consequence of liver DNL in rats fed an excess of sucrose. As D-fagomine has been proven to lower postprandial blood glucose/fructose levels in healthy volunteers (sucrose loading test, clinical trial accessible at https://clinicaltrials.gov/ct2/show/NCT01811303), it is reasonable to expect a protective action against in humans consuming an excess of sucrose. The amount of D-fagomine consumed daily by the rats in this study (ca. 98 mg kg⁻¹ body weight) would translate to 15.5 mg kg⁻¹ in humans by following the conversion proposed by Reagan-Shaw et al. [37]. This dose is higher than the amount provided by a diet rich in buckwheat-based foodstuffs [14] therefore, it should be reached by dietary supplementation. The present results, together with the previous report of the action on fat-induced low-grade inflammation, IR and IGT, are suggesting that D-fagomine may be effective at preventing MetS by acting on different risk factors triggered by different components of unhealthy Westernized diets, such as excessive intake of saturated fat and refined sugar.

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Tables

Table 1.- Feed, drink, and energy intake; and body and perigonadal AT weights of WKY rats fed the different diets for 24 weeks. Also, residual excreted energy in feces after 20 weeks of intervention.

	ST	Ď	Н	S	HS+	FG	
	Mean	SEM	Mean	SEM	Mean	SEM	
Feed intake (g per day per 100 g body weight)	5.4	0.2	2.8*	0.1	2.8*	0.1	
Drink intake (mL per day per 100 g body weight)	7.4	0.2	10.5*	0.2	9.5*	0.2	
Fructose intake (g per day per 100 g body weight)	a		1.4	0.0	1.4	0.2	
Total energy intake (kcal per day per 100 g body weight)	15.6	0.4	22.6*	0.5	21.5*	0.5	
Energy in feces kcal °C g ^{-1b}	310	20	290	20	260	20	
Body weight (g)	380	10	370	10	390	10	
Perigonadal adipose tissue weight (g)	8	0	13***	1	12**	1	

Data are presented as means with their standard errors of the mean; n = 9 per group. Comparisons were performed using one-way ANOVA and Tukey's multiple-comparison test. * P < 0.05, ** P < 0.01, *** P < 0.001 vs STD group

^a Fructose intake as part of the 2014 Teklad Global feed is negligible compared to that in the HS diets.

^b Integrated STDA signal (proportional to energy.

Table 2.- Fasting glucose and insulin, lipid profile and leptin in plasma of WKY rats fed

 different diets for 24 weeks.

	ST	Ď	HS		HS+FG	
	Mean	SEM	Mean	SEM	Mean	SEM
Fasting glucose (mg dL^{-1})	69	2	65	3	62*	1
Fasting insulin (ng mL ⁻¹)	0.4	0.1	0.8*	0.2	1.3*	0.5
Leptin (ng mL ⁻¹)	2.5	0.3	5.4***	0.6	3.9	0.4
Total cholesterol (mg dL^{-1})	135	4	132	3	132	2
HDL cholesterol (mg dL^{-1})	49	1	54*	2	53*	1
LDL cholesterol (mg dL ^{-1})	23	2	21	1	22	1
Triglycerides (mg dL ⁻¹)	60	6	90	10	110*	10

Data are presented as means with their standard errors of the mean; n = 9 per group.

Comparisons were performed using one-way ANOVA and Tukey's multiple-comparison test.

* P < 0.05, and *** P < 0.001 vs STD group

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Table	3	Excreted	intestinal	bacteria	(%	copies	per	total	bacteria)	from	rats	fed	different
diets fo	or 24	4 weeks.											

	ST	Ď	HS	5	HS+F	G	
	Mean	SEM	Mean	SEM	Mean	SEM	
Bacteroidetes	30	3	80**	10	110***	10	
Firmicutes	37	4	57	10	38	2	
Bacteroidales	40	4	60*	6	73***	5	
Clostridiales	12	2	15	2	14	2	
Enterobacteriales	0.01	0.00	0.07*	0.03	0.14*** ^δ	0.02	
E. coli	0.004	0.003	0.040*	0.012	0.135*** ^δ	0.032	

Data are presented as means with their standard errors of the mean; n = 9 per group.

Comparisons were performed using one-way ANOVA and Tukey's multiple-comparison test.

* P < 0.05, and *** P < 0.001 vs STD group; $\delta P < 0.05$ vs HS group



Figure 1. Time course of plasma glucose concentration after administration of a single dose of glucose (1 g per kg body weight) to WKY rats fed a standard (STD), high-sucrose (HS), or high-sucrose supplemented with D-fagomine (HS+FG) diet for 13 (A) and 21 (B) weeks.

Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests or two-way ANOVA. ** P < 0.01 and *** P < 0.001 vs STD group.





Figure 2. Systolic (A) and diastolic (B) blood pressure, and uric acid in urine (C) and plasma (D) in rats fed a standard (STD, \circ), high-sucrose (HS, \Box), or high-sucrose with D-fagomine (HS+FG, \blacksquare) diet for 24 weeks. Results are represented as means with their standard errors.

Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparison test. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs STD group; $\delta P < 0.05$, $\delta \delta P < 0.01$ vs HS group. **Nrticle** ccepted



Figure 3. 5- F_{2t} isoprostane (A) and 15- F_{2t} isoprostane (B) in urine from WKY rats fed a standard (STD), high-sucrose (HS), or high-sucrose with D-fagomine (HS+FG) diet for 24 weeks of nutritional intervention.

Results are represented as means with their standard errors. Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparison test. * P < 0.05 vs STD group

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Figure 4. Histological sections (hematoxylin/eosin stained) from the livers of WKY rats fed a standard (STD) (A, 10x), high-sucrose (HS) (B, 10x), or high-sucrose supplemented with Dfagomine (HS+FG) (C, 10x) diet for 24 weeks and estimation of steatosis (D) and lipogranuloma (E).

Values are in frequencies (percentage of animals that present the value, or do not). Comparisons were performed using χ^2 statistics. ** P < 0.01 and *** P < 0.001 vs STD group; δδδ P < 0.001 vs HS group.

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Figure 5. Hepatic levels of DAG 32:1 (A), DAG 32:2 (B), DAG 34:1 (C) and DAG 36:2 (D) in WKY rats fed a standard (STD), high-sucrose (HS), or high-sucrose supplemented with D-fagomine (HS+FG) diet for 24 weeks.

Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests. * P < 0.05 vs STD group.

TT1C 6 Accepted

The buckwheat iminosugar D-fagomine reduces body weight gain and delays prediabetes in rats fed a high-fat diet. It is now reported that D-fagomine reduces high-sucrose-induced hypertension, urine uric acid and F2-IsoPs (markers of oxidative stress), steatosis and liver DAGs, without affecting perigonadal fat deposition.

We suggest that D-fagomine counteracts sucrose-induced steatosis and hypertension, by reducing the postprandial levels of fructose in the liver.

