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Basic nutritional investigation

Fructose-induced metabolic syndrome decreases protein expression and activity of intestinal P-glycoprotein

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ABSTRACT

Objectves: Metabolic syndrome (MetS) is a health disorder that increases the risk for cardiovascular complications such as heart disease and type 2 diabetes. Some drugs used in patients with MetS are substrates of intestinal P-glycoprotein (P-gp), one of the most important efflux pumps that limit the absorption of xenobiotics. Thus, their bioavailability could be affected by changes in this transporter. Because one of the major causes of MetS in humans is excessive sugar intake, the aim of this study was to evaluate the effect of a fructose-rich diet on intestinal P-gp activity and protein expression in male Sprague-Dawley rats.
Methods: Fructose-drinking animals received standard chow and 15% (w/v) fructose in the drinking water over 8 wk; control rats were fed on standard chow and tap water.
Results: Ileal protein expression of P-gp was 50% lower in fructose-drinking rats than in control animals. This reduction was confirmed by immunofluorescence microscopy. These results correlated well with the decrease of about 50% in the transport rate of the substrate rhodamine 123 in everted intestinal sacs. Finally, an increase of 62% in the intestinal absorption of digoxin, a P-gp substrate used as therapeutic drug, was observed in vivo, in fructose-drinking animals.
Conclusion: The present study demonstrated that MetS-like conditions generated by enhanced fructose intake in rats decreased the protein expression and activity of ileal P-gp, thus increasing the bioavailability of P-gp substrates.

Introduction

P-glycoprotein (P-gp), also known as multidrug resistance protein-1 (MDR1, ABCB1), is a 170 kDa protein that belongs to the ATP-binding cassette gene family. This protein was named P-gp because its presence modifies cell membrane permeability to chemotherapeutic drugs, increasing the resistance to cancer treatment in ovarian cells [1]. P-gp is an ATP-dependent efflux pump that decreases intracellular accumulation of numerous compounds. Among them there are some endogenous substrates and a wide range of hydrophobic and cationic xenobiotics. It is constitutively expressed at the apical membrane of the intestinal tract epithelial cells, renal proximal tubules, and the biliary pole of hepatocytes. P-gp is also present in endothelial cells of special barriers that protect highly sensitive systems such as brain, fetus, and testis.

Due to this localization, P-gp is one of the most important efflux proteins in the small intestine and colon [2], thus decreasing the intestinal absorption of many orally administered xenobiotics and modifying their pharmacokinetics [3–6].

Metabolic syndrome (MetS) is a high-prevalence condition that affects ~25% of the world's adult population [7]. This

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syndrome is a combination of cardiovascular risk factors such as hyperglycemia, hypertriacylglycerolmia, hyperinsulinemia, and insulin resistance, all of which increase the risk for developing heart diseases and type 2 diabetes [8,9].

To our knowledge, the effect of MetS-like conditions produced by high-fructose intake on the expression and activity of intestinal P-gp has not been studied. However, there are several reports related to P-gp changes on different models of type 2 diabetes and obesity [10–13] with discrepant results. Although some of them describe an induction of intestinal P-gp [10,11], others find a decrease of this transporter in the same tissue [12,13].

Changes in intestinal P-gp could modify the bioavailability of drugs that are substrates of this transporter and are frequently used in patients with MetS or type 2 diabetes. Examples of these drugs are some statins [14], β-adrenoceptor antagonists [15], and linagliptin, which is a selective, competitive inhibitor of dipeptidyl peptidase-4 recently approved for the treatment of type 2 diabetes [16]. Moreover, these patients could present other diseases that require medications (antibiotics, antiarrhythmics, cytostatics, etc), so the pharmacokinetics of many drugs that are P-gp substrates could be affected.

Because one of the major causes of metabolic diseases in humans is excessive sugar intake [17–19], the aim of this study was to evaluate the effect of MetS-like conditions, generated by administration of 15% fructose in the drinking water, on intestinal P-gp protein expression and activity in rats. Taken together, our data demonstrated that MetS-like conditions decrease ileal P-gp protein, preserving its typical distribution. The activity of this transporter, evaluated in intestinal sacs, also decreased. Finally, an increase in the bioavailability of a known P-gp substrate such as digoxin was demonstrated, indicating a decrease in the intestinal barrier capacity.

Materials and methods

Chemicals

OptiPhase liquid scintillation cocktail and [^{3}H] digoxin (1.48 TBq/mmol) were purchased from Perkin Elmer Life Science Products (Boston, MA, USA). Unlabeled digoxin was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Verapamil, rhodamine 123, 2-thiobarbituric acid, adrenaline bitartrate, and hydrogen peroxide were from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade purity.

Animals

Procedures involving animals were conducted in accordance with National Institutes of Health (NIH) guidelines. Protocols were approved by the Internal Animal Care and Use Committee. Male Sprague-Dawley rats, 70 to 80 d old (220–240 g) were purchased from the vivarium facilities of the Faculty of Veterinary Sciences (University of Buenos Aires). They were maintained in a room with controlled temperature (19°C–22°C) and a 12-h light–dark cycle. Fructose-drinking animals received standard chow and 15% (w/v) fructose in the drinking water over 8 wk, and control rats were fed on standard chow and tap water. During the final week of treatment, daily intake of water was measured; total daily drinking volume of two animals housed together was averaged and considered as a single datum.

Specimen collection and brush-border membrane preparation

Rats (n = 6 per group, 12-h fasting) were anesthetized with urethane (1.2 g/kg intraperitoneally [IP]). After an abdominal incision, blood was collected from the aorta and the last portion of the small intestine close to the ileocecal valve (20 cm) was removed. After a rinse with ice-cold saline, a small fraction was either kept at −70°C until used for immunofluorescence microscopy studies, or fixed in 70% ethanol for hematoxylin and eosin staining. The rest of the segment was immediately used for the isolation of brush-border membranes [20,21] and used for Western blot assays. Epididymal fat pads were excised and weighed.

Western blotting

Immunoblotting for detection of P-gp in brush-border membranes was performed using antihuman MDR1 rabbit polyclonal antibody (H-241; 1:1000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). According to the manufacturer specification, H-241 is recommended for detection of P-gp in mice, rats, and humans. Rabbit polyclonal antiactin (1:1000; Sigma-Aldrich) was used as load control and goat antirabbit horseradish peroxidase-conjugated immunoglobulin G was used as secondary antibody (1:2000; Sigma-Aldrich), as previously described [22]. Densitometric analysis of immunoreactive bands was performed with NIH ImageJ free software. Protein concentration in membrane preparations was measured by a previously described method [23] using bovine serum albumin as standard.

Immunofluorescence microscopy

Intestinal slices (15 μm) were prepared with a Shandom FE Thermo cryostat and fixed for 10 min in cold metanol (−20°C). Immunohistochemistry assays were performed as previously described [24], using anti-MDR1 antibody (1:100) and rhodamine-red X-conjugated goat antirabbit as secondary antibody (1:1000; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA, USA). Fluorescence images were captured on a Nikon ECLIPSE 50 i microscope (Tokyo, Japan).

Assessment of P-gp activity in intestinal sacs

The distal portion of the ileum of urethane-anesthetized rats (n = 6 per group, 12-h fasting) was removed, gently rinsed with ice-cold saline, and immediately used to test P-gp activity in vitro. Intestinal sacs (10 cm, two per animal) were everted. One sac was incubated in 5 mL Krebs-Henseleit buffer (KH; 40 mM glucose, pH 7.4) at 37°C, bubbled with 95% O_2 to 5% CO_2. The second one was incubated in the same buffer containing verapamil (100 μM), a known P-gp inhibitor. After a 10-min preincubation, the serosal compartments were filled with KH containing the P-gp substrate rhodamine 123 (R123; 15 μM). Aliquots of 100 μL were sampled from the mucosal compartment every 5 min over a 40-min period. Then, the sacs were gently dried and weighed. The concentration of R123 in the samples was determined spectrofluorometrically (excitation and emission wavelengths: 488 and 550 nm, respectively). The rate of mucosal transport of R123 was estimated from the slope of the linear regression fitting, and expressed as nmol R123/g tissue/min^{-1} [25].

Assessment of intestinal absorption of digoxin in vivo

Rats (n = 5 per group, 12 h fasting) were anesthetized with urethane (1.2 g/kg IP) and the jugular and portal veins were cannulated with polyethylene tubing (PE50 and PE10, respectively). The bile duct was ligated to prevent enterohepathic recirculation and a cannula was introduced into the duodenum, as previously described [24]. Digoxin, a well-known P-gp substrate, was administered directly into the intestine (25.6 nmol/kg). [^{3}H]-Digoxin was used as marker (17.0 μmol of [^{3}H]-digoxin/mol of unlabeled digoxin). Blood samples from the portal vein were drawn every 5 min, and up to 30 min after digoxin administration. Appropriate volumes of 5% bovine serum albumin in saline were administered via the jugular vein to replenish body fluids. At the end of the experiment the liver was removed, weighed, and homogenized in saline solution. [^{3}H]-Digoxin concentrations in liver homogenate and portal blood were determined by liquid scintillation counting.

Biochemical determinations

Glycemia was measured in blood obtained from a tail incision with a One-Touch UltraMini meter (Johnson-Johnson, Brazil). Serum triacylglycerols and cholesterol were determined with colorimetric assay kits (Wiener, Rosario, Argentina) in blood obtained from the abdominal aorta of animals used for isolation of brush-border membranes and for P-gp activity in vitro. Glucose tolerance test was performed in some animals in treatment week 7. A glucose bolus (2 g/kg in saline solution, IP) was administered to conscious animals (12-h fasting). Basal glycemia, determined before glucose injection, was substracted from the values obtained 15, 30, 60, and 90 min after injection and the results expressed as area under the curve (AUC), as previously described [26]. After a 7-d washout period, the animals were used for other experiments.

Lipid peroxidation was estimated by measuring thiobarbituric acid-reactive substances (TBARS) using a previously described procedure [27], with minor modifications. Plasma samples mixed with 0.2 M phosphoric acid and 0.11 M 2-thiobarbituric acid (1:1:0.15 volume ratio) were incubated at 100°C over 30 min. After cooling, *n*-butanol and saturated NaCl solution were added to each reaction tube. Tubes were then centrifuged and the absorbance of the separated organic phase was measured at 535 nm. TBARS concentration was calculated using 1,1,3,3-tetramethoxypropane as standard and expressed as nmol/mg protein.

Superoxide dismutase (SOD) activity was determined spectrophotometrically at 480 nm by measuring the inhibition of the autoxidation of 0.3 mM adrenaline in 50 mM glycine buffer (pH 10.2) [28]. Enzymatic activity was calculated as the inverse amount of protein that inhibits the rate of adrenochrome formation by 50%, and was expressed as units/mg protein [29].

Catalase activity was determined by measuring the decrease in absorbance at 240 nm of 25 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.2) [30]. Enzymatic activity was calculated taking into account the molar absorption coefficient for H_2O_2 (43.6 M^{-1} cm^{-1}) and was expressed as mM H_2O_2/mg protein/min.

Calculations and statistical analysis

Data are presented as means ± SEM. Calculation of AUC and linear regression slopes, as well as statistical comparisons between mean values by unpaired Student's *t* test or one-way analysis of variance followed for Bonferroni post hoc test, were performed with GraphPad Prism 5 software. $P < 0.05$ was considered to be statistically significant.

Results

Validation of the MetS model

Table 1 shows that rats receiving 15% fructose presented higher water intake than control animals. Fructose feeding did not modify the gain of total body weight, but significantly increased the weight of epididymal fat. Plasma glucose concentration and serum triacylglycerols levels were significantly higher in fructose-drinking animals than in the control group, whereas total serum cholesterol values were not modified by the treatment. The area under the glycemia versus time curve after an overload of glucose was significantly greater in fructose-drinking rats than in control animals, thus indicating a reduced glucose tolerance. Moreover, the high-fructose diet increased plasma levels of TBARS, an index of lipid peroxidation, as well as the activity of the antioxidant enzyme SOD. Plasma catalase activity, however, did not change with the treatment.

Effect of a high-fructose diet on ileal P-gp protein expression

Because P-gp levels vary along the small intestine [31], we studied its presence in the distal segment (next to the ileocecal valve) where its expression is higher. Ileal P-gp protein amount was about 50% lower in fructose-drinking rats than in control animals, as estimated by Western blot (Fig. 1A).

Table 1
Physiological and biochemical parameters in fructose-drinking rats

	Control	n	Fructose	n
Water intake (mL/d)	48.2 ± 2.2	9	130.1 ± 4.1***	9
Body weight at end of treatment (g)	518 ± 17	9	534.6 ± 12.2	9
Δ Body weight (g)	289.3 ± 16.2	9	307.7 ± 18.3	9
Epididymal fat (% body weight)	1.3 ± 0.1	9	1.7 ± 0.1*	9
Triglyceridemia (mg/dL)	68.6 ± 7.2	9	188.1 ± 7***	9
Total serum cholesterol (mg/dL)	70.7 ± 6.7	9	60.2 ± 5.4	9
Glycemia (mg/dL)	106.7 ± 2.7	9	150.3 ± 3.4***	9
AUC (Δ mg glucose/dL in 90 min)	8046 ± 618	9	10 702 ± 672*	9
Plasma TBARS (pmol/mg protein)	39.6 ± 5.2	5	102.6 ± 11.9**	5
Plasma SOD activity (units/mg protein)	1.6 ± 0.1	5	4.2 ± 0.8*	5
Plasma catalase activity (μmol H_2O_2/min/mg^{-1} protein)	6 ± 2	5	5.5 ± 0.8	5

AUC, area under the curve; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances
Shown are mean values ± SEM; Δ body weight was calculated as the weight gained during the 8 wk of treatment. n = number of animals, except for the first line where each data is the average of two rats housed together. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ versus control (unpaired Student's *t* test).

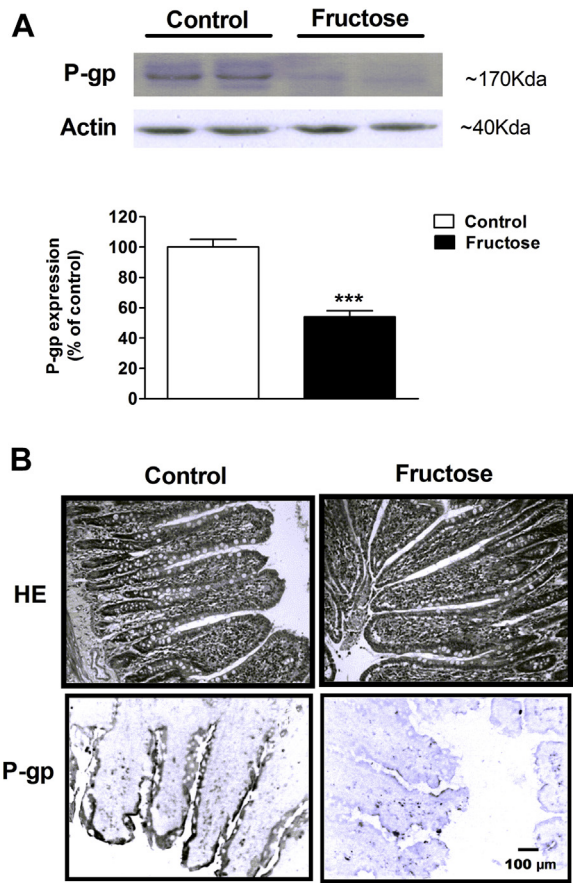
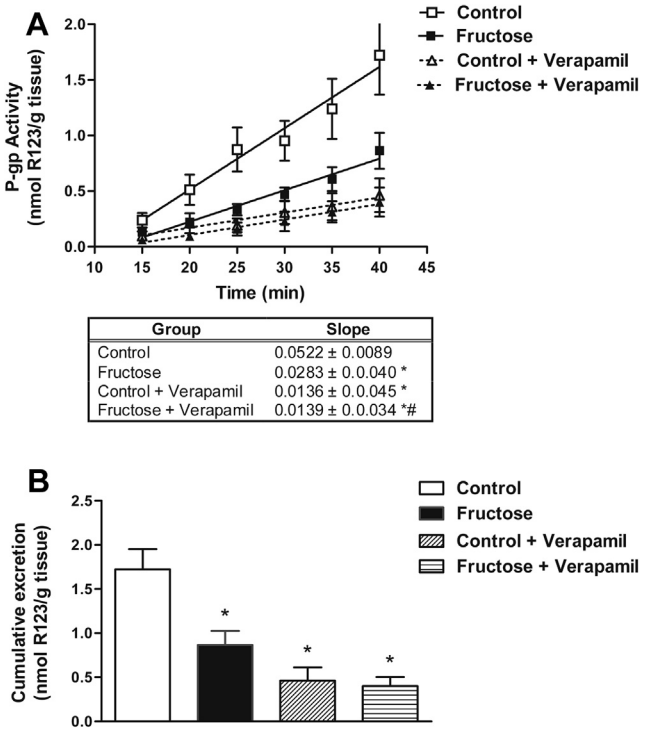


Fig. 1. Effect of a high-fructose diet on ileal P-gp protein expression. (A) P-gp Western blot assay was performed in brush-border membranes prepared from the distal 20-cm segment of ileum. 40 μg Protein were loaded per lane. Bars represent P-gp protein levels as percentages of the average value in the Control group. Results are expressed as mean ± SEM of six rats per group. *** $P < 0.001$ compared with control (unpaired Student's *t* test). (B) Structure of intestinal villi and localization of P-gp. Shown are representative images for hematoxylin and eosin staining (HE) and P-gp immunodetection in the ileum of a control (left side) and a fructose-drinking rat (right side). Similar staining pattern was observed in four independent preparations per group. P-gp, P glycoprotein.

This result was further confirmed by immunofluorescence microscopy. As shown in Figure 1B, P-gp immunoreactivity was decreased in fructose-drinking animals with conservation of its typical localization, mainly at the surface of the intestinal villi. Additionally, an anatomopathologic study of the intestinal tissue was performed. No changes in the intestinal mucosa structure were observed, showing that treatment with fructose-preserved tissue integrity.

Effect of a high-fructose diet on intestinal P-gp transport activity in vitro

Serosal to mucosal transport of R123, a typical P-gp substrate, was evaluated in everted intestinal sacs. As shown in Figure 2A, R123 excretion was lineal up to 40 min. Excretion rate, estimated as the slope of the regression line, was reduced by half in fructose-drinking rats compared with control animals. In the presence of the P-gp inhibitor verapamil (100 μM), the efflux of R123 decreased in both groups to similar values (Fig. 2B). These results indicate that high-fructose intake modifies P-gp activity, but not R123 basal efflux rate.



Group	Slope
Control	0.0522 ± 0.0089
Fructose	0.0283 ± 0.0.040 *
Control + Verapamil	0.0136 ± 0.0.045 *
Fructose + Verapamil	0.0139 ± 0.0.034 *#

Fig. 2. Effect of a high-fructose diet on intestinal P-gp activity in vitro. (A) Intestinal sacs were filled with 15 μM R123 (serosal side) and incubated in Krebs-Henseleit with or without verapamil (100 μM). The cumulative dye excretion into the outside compartment (mucosal side) was assessed every 5 min for 40 min. The mucosal efflux rates were estimated from the linear regression slopes and expressed as nmol R123/g tissue/min^{-1}. (B) Cumulative excretion of R123 after 40 min incubation was plotted to compare total R123 transport. Data are expressed as mean ± SEM of four to six rats per group. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ versus fructose (one-way analysis of variance and Bonferroni post hoc test). P-gp, P glycoprotein.

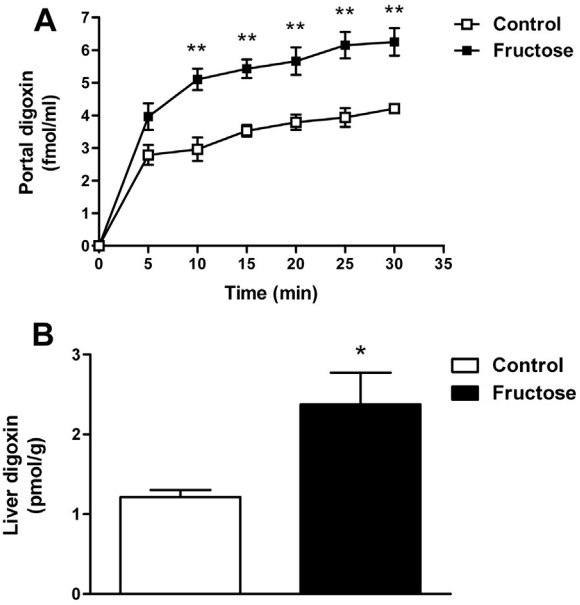


Fig. 3. Effect of a high-fructose diet on intestinal absorption of digoxin. (A) Net intestinal absorption of digoxin was estimated by changes in portal blood concentration over a 30-min period. Digoxin (25.6 nmol/kg) was administered into the duodenum of control and fructose-drinking rats. Portal blood concentration of drug was assayed every 5 min, and up to 30 min after drug administration. (B) Liver digoxin accumulation was measured at the end of the experiment. Data are expressed as mean ± SEM of five rats per group. $^{*}P<0.05$; $^{**}P<0.01$ vs. Control (unpaired Student's *t*-test).

Effect of a high-fructose diet on intestinal P-gp transport activity in vivo

To assess the transport activity of intestinal P-gp in vivo, the time course of digoxin concentration in portal blood was determined after intraluminal administration of a digoxin bolus. As shown in Figure 3A, portal digoxin concentration was significantly higher in fructose-drinking rats than in control animals beginning at 15 min. This result is consistent with a lower intestinal P-gp mediated efflux of the drug. Moreover, a significantly greater hepatic accumulation of digoxin was observed in fructose-treated rats (Fig. 3B). This could be related to the higher concentration of digoxin observed in the portal blood.

Discussion

High-fructose consumption has been associated with the development of MetS features in humans [18] and is extensively used as an animal model of this disorder. In agreement with previous reports [26,32,33], the present study shows that fructose-drinking rats had glucose intolerance, higher daily water intake, and higher values of glycemia and triacylglycerolmia than control animals. No changes in serum total cholesterol levels were observed in this study, as were previously reported for 10% of fructose-drinking rats [26]. No differences were found in total body weight gain between groups, however, larger epididymal fat depots were observed in the fructose-treated rats, which suggests an increase in body adipose tissue, probably due to adipocyte hypertrophy [34].

In agreement with the current knowledge on the metabolic effects of high-sugar diets, both in animals and humans [17,35], a prooxidant status was observed in fructose-drinking rats. This was estimated from the higher plasma levels of lipid peroxidation end products and higher activity of an antioxidant enzyme, SOD, in treated animals.

Intestinal P-gp is one of the main transporters that regulate the intestinal barrier function by reducing the net absorption of many xenobiotics, including therapeutic drugs [36]. The present study shows that fructose treatment reduces intestinal P-gp protein expression, with no detectable changes in the intracellular localization of the transporter or in the structure of the villi. The reduced expression of ileal P-gp in fructose-drinking rats correlated well with the lower transport rate of R123, a nontherapeutic P-gp substrate. The participation of P-gp in R123 transport was confirmed by inhibition with verapamil, a known inhibitor of the transporter. Additionally, a reduction in P-gp activity was detected in vivo using digoxin, a therapeutic drug substrate of this transporter. Our data show a higher rate of digoxin absorption as a consequence of a decrease in the intestinal barrier capability, mediated by P-gp.

The present findings are in accordance with some previous studies related to metabolic diseases. One of them showed a decrease in intestinal P-gp protein expression in genetically obese Zucker rats [13]. In a second, the intestinal absorption of nelfinavir, a P-gp substrate, was found to be lower in rats receiving a high-fat diet over an 8-wk period than in control rats [12]. On the contrary, it was reported that P-gp protein expression increases in the jejunum of mice receiving monosodium glutamate at birth, a model of obesity associated with hyperglycemia and hyperinsulinemia, due to the high toxicity of

glutamate in the hypothalamic neurons [10]. Besides, an enhancement of jejunal P-gp protein expression was found in Goto-Kakizaki rats, a model of genetic type 2 diabetes, although transport activity was reduced. The influence of different parameters, such as the levels of glucose, insulin, cytokines and inducible nitric oxide synthase (iNOS) on the expression of P-gp under diabetic conditions was extensively reviewed previously [37]. However, to our knowledge, this is the only study that describes changes in intestinal P-gp protein expression and activity in MetS-like conditions produced by high fructose intake. Most importantly, the question arises on weather fructose overconsumption in humans could modify the bioavailability of xenobiotics, including pharmaceutical drugs that are substrates of P-gp. This overconsumption actually occurs whenever the intake of high-fructose corn syrup incorporated into soft drinks and manufactured foods becomes excessive.

At this stage of our study, we cannot assess the mechanisms involved in the decreased expression of intestinal P-gp protein in fructose-drinking rats. However, it has been proposed that the lower expression of intestinal P-gp in obese Zucker rats could be associated with the enhanced production of proinflammatory factors, such as tumor necrosis factor (TNF)-α, interleukin-6, and iNOS [13]. Moreover, it was reported that TNF-α reduces the expression and activity of P-gp in Caco-2 cells [38]. There is also evidence that iNOS activity is involved in the reduction of P-gp protein expression in the intestine of type 1 diabetic rats [39] and human retinal pigment cells cultured in high-glucose conditions [40]. On the other hand, it was reported that oxidative stress could have a role in the down-regulation of ileal P-gp protein expression under ischemia-reperfusion conditions [41]. Therefore, it could be suggested that one or several of these biochemical mediators, or even other molecules, such as circulating glucose and insulin [37], could be involved in the lower expression and activity of small intestine P-gp reported in the present study. In this regard, there is evidence from the present data (Table 1) and from previous reports that high-fructose diets increase the production of reactive oxygen species [32,35] and proinflammatory factors [35,42], but more studies will be necessary to investigate the precise intracellular mechanisms involved in the decrease of intestinal P-gp in our experimental model.

Conclusion

These results demonstrate that MetS-like conditions generated by high fructose intake in rats decrease the expression and activity of ileal P-gp, thus increasing the bioavailability of orally administered P-gp substrates. This study may suggest that it is necessary to pay further attention to therapeutic drug dosing of polymedicated patients with MetS.

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