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Intestinal multidrug resistance-associated protein 2 is down-regulated in fructose-fed rats☆

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Abstract

Expression and activity of jejunal multidrug resistance-associated protein 2 (Mrp2) and glutathione-S-transferase (GST) were examined in fructose fed Wistar rats, an experimental model of metabolic syndrome. Animals were fed on (a) control diet or (b) control diet plus 10% w/vol fructose in the drinking water. Mrp2 and the α class of GST proteins as well as their corresponding mRNAs were decreased, suggesting a transcriptional regulation by fructose. Confocal microscopy studies reaffirmed down-regulation of Mrp2. Everted intestinal sacs were incubated with 1-chloro-2,4-dinitrobenzene in the mucosal compartment, and the glutathione-conjugated derivative, dinitrophenyl- S-glutathione (DNP-SG; model Mrp2 substrate), was measured in the same compartment to estimate Mrp2 activity. Excretion of DNP-SG was substantially decreased by fructose treatment, consistent with simultaneous down-regulation of Mrp2 and GST. In addition, the effect of fructose on intestinal barrier function exerted by Mrp2 was evaluated *in vivo* using valsartan, a recognized Mrp2 substrate of therapeutic use. After intraduodenal administration as a bolus, intestinal absorption of valsartan was increased in fructose-drinking animals. Fructose administration also induced oxidative stress in intestinal tissue as demonstrated by significant increases of intestinal lipid peroxidation end products and activity of the antioxidant enzyme superoxide dismutase, by a decreased GSH/GSSG ratio. Moreover, fructose treatment conduced to increased intestinal levels of the proinflammatory cytokines IL-β1 and IL-6.

Collectively, our results demonstrate that metabolic syndrome-like conditions, induced by a fructose-rich diet, result in down-regulation of intestinal Mrp2 expression and activity and consequently in an impairment of its barrier function.

Keywords: Fructose-rich diet; Metabolic syndrome; Insulin resistance; Intestine; Mrp2; GST

1. Introduction

Multidrug resistance protein 2 (Mrp2, ABCC2) is a drug efflux pump belonging to the ATP-binding cassette (ABC) transporter superfamily. It is constitutively expressed at the apical membrane of hepatocytes, enterocytes, renal tubule cells and other epithelial cells [1–3]. In small intestine, Mrp2 coupled to metabolizing enzymes, such as Glutathione S-Transferase (GST; EC 2.5.1.18), plays a crucial role as intestinal biochemical barrier to prevent absorption of food contaminants and drugs incorporated orally [4]. Mrp2 expression is concentrated at the tip of the villus, with significantly higher expression in proximal jejunum respect to distal ileum [5], thus playing a major role as a "first line of defense" against the action of potentially harmful xenobiotics. Likewise, Mrp2 is of clinical relevance since it modulates the pharmacokinetics and consequently determines the safety and efficacy of many orally administered drugs by limiting its absorption and distribution [6].

The metabolic syndrome (MetS), also called *insulin resistance syndrome*, is a pathological condition characterized by a cluster of

Abbreviations: ABC, ATP-binding cassette; Bcrp, Breast cancer resistance protein; BBM, border membrane; b.wt., body weight; CAT, Catalase; CDNB, 1-chloro-2,4-dinitrobenzene; C, control; DNP-SG, dinitrophenyl- S-glutathione; FRU, fructose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione reduced; GSSG, glutathione oxidized; GST, glutathione S-transferase; HOMA, homoeostasis model assessment; IR, insulin resistance; LPO, lipid peroxidation; Mrp2, multidrug resistance-associated protein 2; HDL, high-density lipoprotein; HPLC, High-performance liquid chromatography; P-gp, P-glycoprotein; SOD, Superoxide Dismutase; TAG, triacylglycerol; TBARS, thiobarbituric acid reactive substances; IL-1β, Interleukin-1β; IL-6, Interleukin-6.

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metabolic abnormalities including elevated blood pressure, elevated triglycerides, reduced high-density lipoprotein (HDL)-cholesterol serum, hepatic steatosis, glucose intolerance, hyperinsulinemia and insulin resistance, with the last one being considered the common etiologic factor in this syndrome [7–9]. MetS is rapidly growing worldwide and is associated with increased risk to multiple chronic pathologies including cardiovascular disease and Type 2 diabetes. Consumption of fructose has increased throughout the world, contributing to increased total caloric intake and resulting in an increase in the incidence of MetS [7–10]. Many investigations have demonstrated that administration of a fructose-rich diet to normal rats induces several features of the MetS, associated with increased indices of inflammation and oxidative stress, affecting multiple tissues and organs [11–13]. Accordingly, fructose administration along 21-days period to normal rats induces MetS-like conditions, as demonstrated by development of insulin resistance, impaired glucose tolerance, hyperlipidemia, as well as a significant enhancement of oxidative stress markers in several organs [14–17].

Pharmacotherapy targeting different components of the MetS and associated comorbidities has been generally accepted as appropriate management of high-risk patients [18]. Since these drugs are mostly administered orally, the intestinal barrier function strongly influences their bioavailability and therefore their efficacy and safety. In particular, the MetS represents a predisposing factor for development of toxicity induced by drugs, though the underlying mechanism remains essentially uncertain [19–21]. Interestingly, some of these drugs are at the same time Mrp2 substrates, and therefore, a modification in the activity of Mrp2 may be one of the factors contributing to their adverse effect.

The expression and activity of Mrp2 can be regulated under specific physiological and pathological conditions, either at posttranscriptional and transcriptional levels [1,22,23]. Whether MetS-like conditions generated by high-fructose consumption affect intestinal Mrp2 expression and activity and, in consequence, its role as biochemical barrier is currently unknown. The aim of our study was to evaluate the effect of the administration of 10% fructose with the drinking water along a 21-days period on intestinal Mrp2 expression and activity in rats.

2. Materials and methods

2.1. Chemicals

Fructose was obtained from Laboratorio Cicarelli (BsAs, Argentina). Leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, 3-isobutyl-1-methylxanthine, glutathione, dithiothreitol, 1-chloro-2,4-dinitrobenzene (CDNB), MK571 (MK), valsartan, 2-thiobarbituric acid, nitroblue tetrazolium and hydrogen peroxide (H_2O_2) were obtained from Sigma–Aldrich (St. Louis, MO, USA). 2-Methylbutane was obtained from Acros Organics (Pittsburgh, PA, USA), and 2-vinylpyridine was obtained from Fluka Chemical Corp (Milwaukee, WI, USA). All other chemicals and reagents used were commercial products of analytical-grade purity.

2.2. Animals and treatments

Adult male Wistar rats (220–250 g; 70-day old), received standard commercial diet *ad libitum* and tap water (control group), or tap water with 10% (w/v) fructose (FRU), for 21 days [14–17]. Animals were grouped (two animals per cage) and kept under controlled conditions (23±2 °C) with a fixed 12-h light–dark cycle (06:00–18:00 h). Liquid intake was monitored every other day and body weight once per week throughout the duration of the treatment. Total drinking volume of animals housed together was averaged and considered as a single data. All the experimental protocols were performed according to the Regulation for the Care and Use of Laboratory Animals (Expedient 6109/012 E.C. Resolution 267/02) and were approved by the Institutional Animal Use Committee of the National University of Rosario, Argentina.

2.3. Specimen collection

Fasting animals were anesthetized (between 08:00 and 09:00 h) with an intraperitoneal dose of ketamine [100 mg (0.42 mmol)/kg b.wt.]/xylazine [15 mg (0.07 mmol)/kg b.wt.]. After an abdominal incision, blood samples were taken through cardiac puncture and placed into heparinized tubes to measure plasma glucose, TAG (triacylglycerol) and immunoreactive insulin levels. For collection of jejunum specimens, the first 15 cm starting from the pyloric valve and corresponding to the duodenum were excluded, and the following 30 cm were taken and considered as the proximal jejunum. This segment was carefully rinsed with ice-cold saline and dried with filter paper. For Western blot studies, the jejunum was immediately opened lengthwise, the mucus layer was carefully removed and the mucosa was obtained by scraping, weighed and used for brush border membrane (BBM) or cytosol preparations. For confocal microscopy analysis of Mrp2 localization, small rings were cut from this same region of the intestine, gently frozen in liquid nitrogen-cooled 2-methylbutane and kept at−70 °C until use in slice preparation or frozen in liquid nitrogen and kept at−70 °C until use in total RNA isolation by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. For Mrp2 transport studies *in vitro*, 3-cm segments of the proximal jejunum were immediately used in the everted sac preparation. Aliquots of proximal jejunum were homogenized in saline (1:2) for assessment of glutathione content, lipid peroxidation and antioxidant enzyme activities or in ice-cold phosphate-buffered saline (pH: 7.40) (1:2) for assessment of IL-1β and IL-6. In addition, epididymal fat pads were excised and weighed.

2.4. Biochemical assays

Plasma glucose and TAG levels were determined by spectrophotometric methods. Both were measured with a commercial kit (Wiener Laboratorios, Rosario, Argentina) in a Lambda 9 (Perkin Elmer) spectrophotometer. Plasma insulin levels were measured with a commercial kit (Rat insulin RIA Kit, Millipore Corporation, Billerica, MA, USA), and radioactivity was measured with a solid scintillation counter, Alfanuclear Cmos (Buenos Aires, Argentina) [24].

Glucose tolerance test was performed a day before the animals were sacrificed. A glucose bolus (2 g/kg pc in saline solution, ip) was administered to conscious animals (12-h fasting). Glycemia was measured, in blood taken from the tail, prior to injection of glucose (time 0) and at 15 min, 30 min, 60 min and 90 min postinjection. For the calculation of the area under the curve (GraphPad Prism5software), baseline glycemia value was subtracted from subsequent readings. The area under the curve was expressed in mM/min.

Serum insulin and fasting blood glucose values were used to estimate IR (insulin resistance) by HOMA (homoeostatic model assessment)–IR index, using the equation: serum insulin (μUI/ml) × fasting blood glucose (mmol/l)/22.5 [25]. When the value of HOMA–IR increases, IR augments, thus indicating a decrease in insulin sensitivity.

2.5. Western blot studies

BBM were prepared from mucosa samples as described by [5] Mottino *et al.* (2000). Cytosolic fractions were obtained from intestinal mucosa by ultracentrifugation methodology [26]. Protein concentration was measured by using bovine serum albumin as standard [27]. Aliquots of the BBM and cytosol preparations were kept on ice and used the same day in Western blot studies. Apical Mrp2, P-glycoprotein (P-gp), breast cancer resistance protein (Bcrp) and villin were detected in BBMs as described previously [5,28]. The expression of the major GST classes present in intestine was evaluated in citosol as described previously [29]. Equal loading and transference of protein was systematically checked by both detection of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively, and staining of the membranes with Ponceau S. Primary antibodies used were: MRP2, M2 III-6 (Alexis Laboratories, San Diego, CA, USA), P-glycoprotein, H-241; Bcrp, BXP-21; villin, H-60; GAPDH, FL-335 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), GSTα, GS9; GSTμ, GS23 (Oxford Biomedical Research, Oxford, MI), GSTπ (Immunotech, Marseille, France) and β-actin, A-2228 (Sigma–Aldrich). Finally, the immunoreactive bands were quantified with Gel-Pro Analyzer software (Media Cybernetics, Inc., Bethesda, MD, USA).

2.6. Microscopy studies

For *in situ* immunodetection of Mrp2, intestinal rings from jejunum were sectioned (thickness, 5 μm) and fixed as described previously [28]. Mrp2 was detected with the respective antibody, and the cell nuclei were detected with 4,6-diamino-2-phenylindole blue fluorescence as described previously [28]. All confocal studies were performed with a Nikon (Tokyo, Japan) C1 Plus microscope. To ensure comparable staining and image capture performance for the different groups belonging to the same experimental protocol, intestinal slices were prepared on the same day, mounted on the same glass slide and subjected to the staining procedure and microscopy analysis simultaneously. In addition, sectioned intestinal rings were stained with hematoxylin and eosin for light microscopy examination.

2.7. Real-time polymerase chain reaction studies

Quantitative real-time PCR studies of Mrp2 and GST mRNAs were performed as described previously [29] using the following primer pairs: forward, 5′-accttccacgtagtgatcct-3′ and reverse, 5′-acctgctaagatggacggtc-3 for Mrp2; forward, 5′-gattgacatgtattcagagggt-3′ and reverse, 5′-tttgcatccatggctggctt-3′ for GSTYa2 belonging to GST class Alpha; and forward, 5′-gtaacccgttgaaccccatt-3′ and reverse, 5′-ccatccaatcggtagtagcg-3′ for 18S rRNA (housekeeping gene).

2.8. Assessment of Mrp2 activity in vitro

To characterize the effect of the fructose-rich diet on intestinal Mrp2 efflux activity, the *in vitro* model of everted sacs was chosen. The everted sacs, prepared from proximal jejunum, were incubated in the presence of 100 μM CDNB in the mucosal compartment as described previously [30], for 30 min. After diffusion of CDNB into the enterocyte and further endogenous conjugation with glutathione, the product dinitrophenyl-*S*-glutathione (DNP-SG) was detected by high-performance liquid chromatography in the same mucosal compartment [30]. All experiments were performed in the presence or absence of 10 μM MK571 [31] in both the mucosal and serosal sides to inhibit Mrp2 activity.

2.9. Assessment of Mrp2 activity in vivo

Rats were anesthetized, and the femoral 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 [32]. Valsartan, a specific angiotensin II receptor blocker and well-known Mrp2 substrate, was administered directly into the duodenum (10 mg/kg) [33]. Blood samples from the portal vein were drawn every 5 min and up to 30 min after valsartan administration. Appropriate volumes of 5% bovine serum albumin in saline were administered *via* the femoral vein to replenish body fluids. Valsartan concentrations in portal blood were determined by HPLC. Samples were deproteinized by mixing 120 μl of portal plasma aliquots and 500 μl of methyl alcohol. After centrifugation at 16,099 *g* for 10 min, the upper organic layer was transferred into a polythene tube and was concentrated to dryness under a gentle stream of nitrogen at 37 °C. The dried residue was dissolved in 100 μL of mobile phase solution and subject to HPLC analysis as described previously [34].

2.10. Assessment of GST activity

The glutathione-conjugating activity toward CDNB was assayed in the cytosol from the proximal jejunum as described previously [26].

2.11. Determination of intestinal glutathione content and lipid peroxidation (LPO)

Two volumes of proximal jejunum homogenates were mixed with 1 volume of 10% sulfosalicylic acid and centrifuged at 5000 *g* for 5 min, and the supernatants were immediately used in the assessment of total glutathione [reduced (GSH) + oxidized (GSSG)] and GSSG [35]. GSH values were determined from total glutathione and GSSG concentrations, and oxidative stress index was calculated from the GSH/GSSG ratio.

LPO was estimated by measuring thiobarbituric acid reactive substances (TBARS) using the procedure of [36] Ohkawa *et al.* (1979). Intestinal homogenates were mixed with 10% trichloroacetic acid and then centrifuged at 3000 *g* for 10 min. The clear supernatant was mixed with 0.7% thiobarbituric acid and heated at 95 °C for 1 h. Samples were cooled at room temperature, and the corresponding absorbance was measured at 532 nm against a blank consisting in reaction mixture without intestinal homogenate but subjected to the same procedure.

2.12. Assessment of antioxidant enzyme activities

After homogenization of jejunum in saline solution, insoluble material was removed by centrifugation (2000 *g*, 5 min), and the supernatants were subjected to enzyme assays. Protein concentration was measured by using bovine serum albumin as standard [27]. Superoxide dismutase (SOD) assay was based on the method of [37] Beauchchamp and Fridovich (1971) with modifications of [38] Donahue *et al.* (1997), which measures the SOD inhibition of photochemical reduction of nitroblue tetrazolium. The enzyme activity was expressed as % of controls. Catalase (CAT) activity was determined by monitoring the rate of decomposition of H_2O_2 as a function of decrease in absorbance at 240 nm for 2 min [39]. The enzyme activity was also expressed as % of controls.

2.13. Assessment of interleukins

Jejunum homogenates were sonicated with an ultrasonifier [Sonics (Newtown, CT, USA) Vibra-cell VCX 130] by six cycles (20 s sonications and 40 s pause on ice), centrifuged (15,000 *g*, 10 min, 4 °C), and the supernatants were subjected immediately to IL-1β and IL-6 determination [40]. They were measured by the enzyme-linked immunosorbent assay (ELISA) method according to the protocol provided by the manufacturer (eBioscience, San Diego, CA, USA, for IL-1β; Biosource International, Camarillo, CA, USA, for IL-6).

2.14. Statistical analysis

Data are presented as mean ± S.D. All statistical analysis was performed by using unpaired Student's *t* test, except for studies of Mrp2 activity *in vitro* in which we performed one-way analysis of variance, followed by Bonferroni's test. Values of $P<0.05$ were considered statistically significant.

Table 1
Serum markers of MetS

	C	FRU
Plasma glucose (mmol/l)	5.88±0.66	6.15±0.23
Plasma triglycerides (mmol/l)	0.56±0.15	1.05±0.18 *
Plasma insulin (ng/ml)	1.18±0.24	2.02±0.42 *
HOMA-IR	7.50±1.42	13.49±2.30 *
Area under curve (mM/min)	1.89±0.20	2.79±0.32 *

The HOMA-IR was calculated with the formula: serum insulin (μUI/ml) × fasting blood glucose (mmol/l)/22.5. An increment in the HOMA–IR index indicates an insulin resistance state in FRU rats.
Data are expressed as means ± S.D. of six-eight rats per group.
* Significantly different from C, $P<0.05$.

3. Results

3.1. Effect of fructose on body weight and water intake

After the 21-days treatment period, body weight did not differ between FRU and control animals (317±16 *vs.* 306±29 g, respectively), whereas the weight of epididymal fat was significantly higher in FRU versus control group (2.32±0.09% *vs.* 1.92±0.07% of body weight, respectively, $P<0.05$). Liquid consumption was also significantly higher for FRU animals than for controls (89.82±4.05 *vs.* 38.04±1.54 ml/day, respectively; $P<0.001$). In contrast, food consumption was higher for control rats (32.39±1.27 *vs.* 27.10±0.05 g/day for control and FRU groups, respectively; $P<0.05$).

3.2. Validation of the MetS model induced by FRU administration

The results on biochemical determinations in serum are shown in Table 1. There were no significant differences in blood glucose levels between groups. However, the concentrations of triglycerides and insulin in plasma were significantly increased by fructose administration. The HOMA-IR values demonstrated development of insulin resistance in FRU versus control group. Consistent with this, the area under the curve in the glycemia versus time graphic was significantly greater for FRU rats than for controls.

3.3. Effect of fructose on Mrp2 expression

Fig. 1A shows that Mrp2 expression in the FRU group was substantially decreased (−89%) with respect to controls, whereas P-gp and Bcrp, other major transporters expressed in the surface of the intestine, were preserved. The expression of villin, a Ca^{2+}-regulated actin-binding protein known to be specifically associated with the cytoskeleton of the brush border, was neither affected suggesting preservation of the microvilli structures.

The effect of fructose administration on expression of Mrp2 at the villus was also analyzed *in situ* by confocal immunofluorescence microscopy. The nuclei of the enterocytes are shown in blue. Fig. 1B shows that Mrp2 (red) is mostly expressed at the luminal surface of the villus in both experimental groups, suggesting that fructose treatment did not affect its normal localization. However, the Mrp2 immunoreactivity signal is weaker in FRU animals when compared to controls, consistent with Western blot studies. In addition, the histological study of the intestinal tissue showed no apparent changes in villus structure between groups, suggesting preservation of tissue integrity.

To establish whether down-regulation of Mrp2 protein was related to the decreased mRNA levels, we further performed real-time PCR studies. The data reveal that fructose administration decreased the Mrp2 mRNA levels by 47% respect to controls (53±14% and 100±23% for FRU and C, respectively, $P<0.05$, $n=8$), suggesting participation of a transcriptional mechanism.

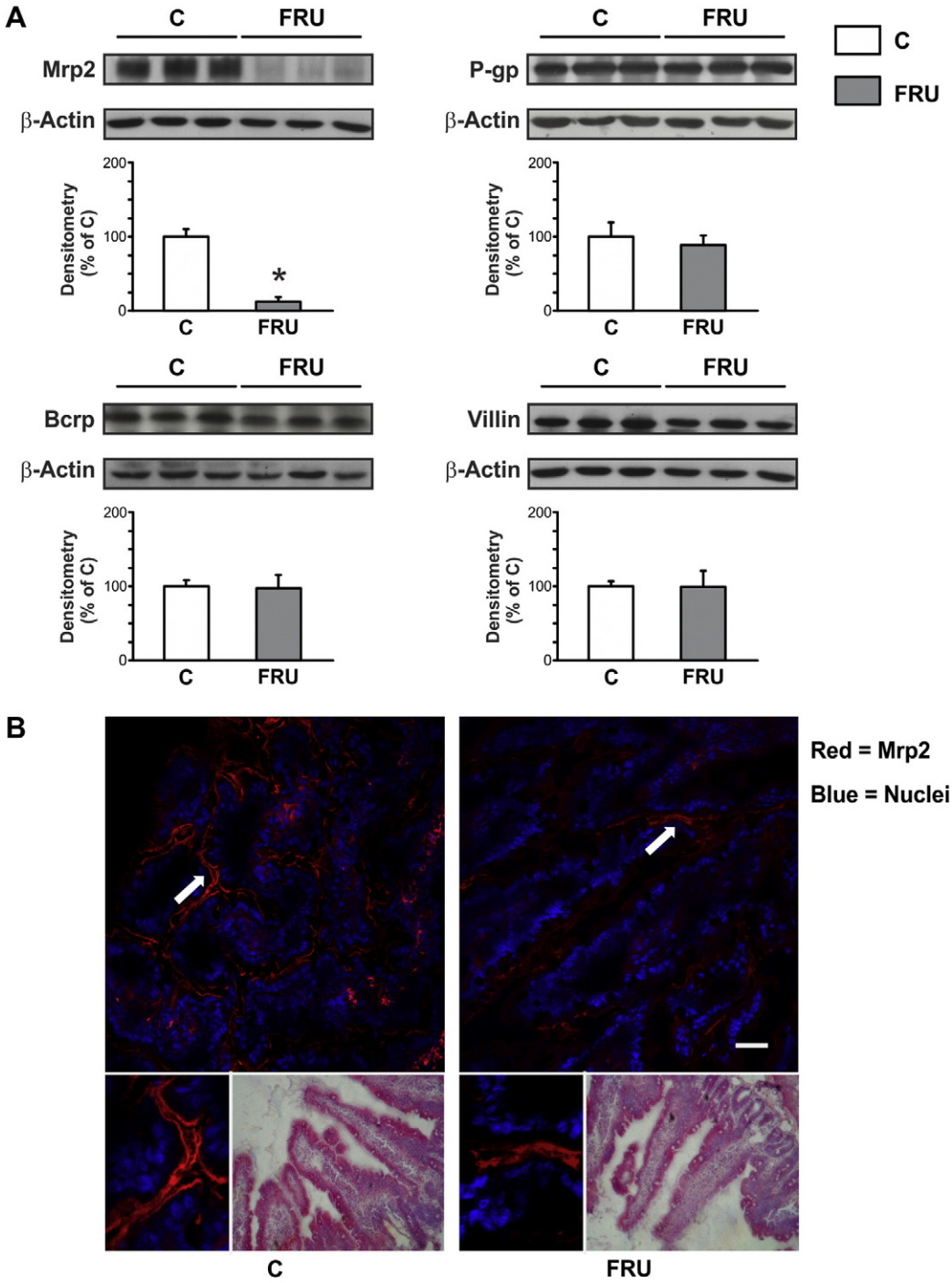

Fig. 1. Effect of fructose on Mrp2 expression. (A) Western blot analysis of Mrp2, P-gp, Bcrp and Villin in BBM vesicles from proximal jejunum. Equal amounts of total protein (20 μg) were loaded in the gels. Uniformity of loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S and detection of β-actin. Densitometry data were related to β-actin and presented as % of controls (C). (B) Localization of Mrp2 and structure of the intestinal villus. Confocal immunofluorescence analysis revealed that the localization of Mrp2 (red) was restricted to the apical membrane of jejunum enterocytes and preserved in the FRU group compared with controls (C). Small bottom panels show in more details the regions indicated with arrows. The nuclei are shown in blue fluorescence. Similar patterns of staining were observed in six independent preparations per group. Representative microscopic images from hematoxylin–eosin staining show a similar structure of intestinal villus in both groups (small bottom panels on the right). Scale bars are indicated in white and correspond to 50 μm. Data are expressed as means ± S.D. of six–eight rats per group. *Significantly different from C, $P<0.005$.

3.4. Effect of fructose on in vitro and in vivo Mrp2 activity

To determine the functional impact of down-regulation of Mrp2 expression, we evaluated the secretion of DNP-SG, a typical substrate for Mrp2 [41], by using the model of everted intestinal sacs. In this model, CDNB diffuses passively from the mucosal compartment (intestinal lumen) into the enterocyte, and its metabolite, DNP-SG, is pumped back to the mucosal compartment by Mrp2. This measure well estimates the intestinal barrier function associated to Mrp2. Fig. 2A shows that the excretion of DNP-SG was substantially decreased by fructose treatment throughout the incubation period. At the 30 min period (inset in Fig. 2A), the amount of DNP-SG accumulated in the mucosal

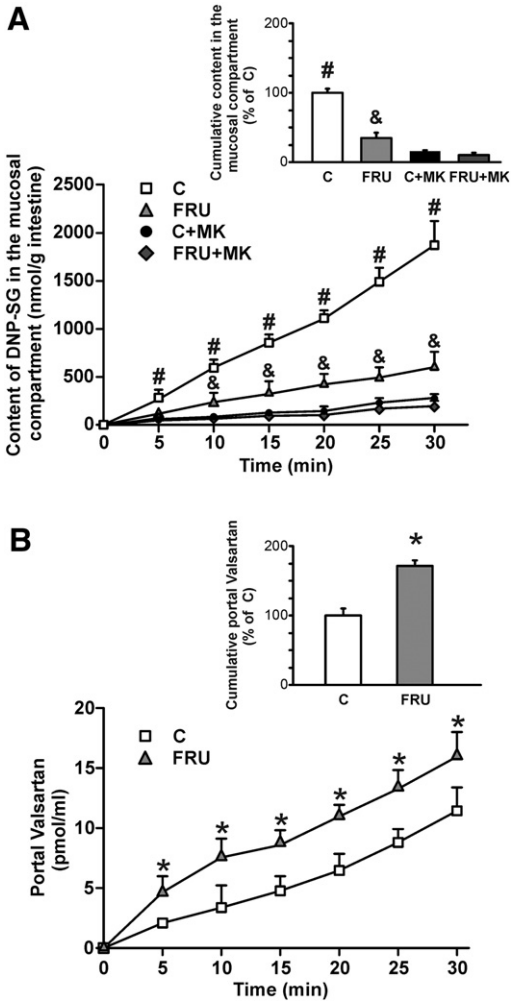

Fig. 2. Effect of fructose on *in vitro* and *in vivo* Mrp2 activity. (A) Time course of efflux of DNP-SG into the mucosal compartment. CDNB (100 μM) was added to the mucosal side in everted sacs with or without the addition of MK571 (10 μM), and accumulation of DNP-SG was detected in the same side. The inset depicts cumulative content of DNP-SG in the mucosal compartment by 30 min and was plotted to compare total DNP-SG transport. (B) Net intestinal absorption of valsartan was estimated by changes in portal blood concentration over a 30 min period. Valsartan (10 mg/kg) was administered into the duodenum of C and FRU rats. Portal blood concentration of drug was assayed every 5 min and up to 30 min after drug administration. The inset depicts the amount of valsartan accumulated in portal blood by 30 min. Data are expressed as means ± S.D. of six–eight rats per group. #, Significantly different from FRU, C + MK and FRU + MK, $P<0.005$. &, Significantly different from C + MK and FRU + MK, $P<0.005$. *, Significantly different from C, $P<0.005$.

compartment decreased by 65% with respect to controls. When the everted intestinal sacs were incubated with the MRPs inhibitor MK571 (10 μM), the secretion of the glutathione conjugate was decreased in both groups to similar basal values (Fig. 2A). These results confirm the participation of an MRP transporter in the different activity detected between FRU and control groups.

We further evaluated the effect of fructose on the role of Mrp2 as a primary barrier limiting oral bioavailability of drugs. Valsartan is a recognized substrate of Mrp2, used therapeutically to treat hypertension due to its angiotensin II AT1-receptor blocker activity. We evaluated *in vivo* the time-course of valsartan concentration in portal blood after intraduodenal administration of a single dose. Fig. 2B shows that significantly higher amount of valsartan was detected in samples taken from FRU than from control rats. This finding is consistent with a significant impairment in Mrp2 activity and its concomitant intestinal barrier function.

3.5. Effect of fructose on GST expression and activity

GST is a major Phase II biotransformation system acting in tandem with Mrp2 to generate conjugated derivatives for further extrusion from the cell. Because fructose could affect intestinal GST and thus the formation of Mrp2 substrates, including DNP-SG, we evaluated the expression of the major GST classes present in intestine as well as GST activity toward CDNB. Fig. 3A shows that expression of GSTα was significantly decreased (−50%) in FRU animals and that GSTμ and GSTπ remained unaltered. We further assessed GSTYa2 mRNA levels, the only isoform belonging to the α class detected in rat intestine [26]. The result in Fig. 3B, evidenced a significant decrease (−64%) of GSTYa2 mRNA in the FRU group suggesting transcriptional regulation of GSTα. In accordance with GSTα expression, cytosolic GST activity was decreased (−38%) by fructose (Fig. 3C).

3.6. Effect of fructose on intestinal redox balance and antioxidant defenses

The effect of fructose administration on the intestinal redox balance, evaluated through assessment of GSH/GSSG ratio and TBARS levels, is presented in Fig. 4. The GSH/GSSG ratio was significantly reduced by 41% in the FRU group (22.5±2.0) when compared to control rats (38.3± 2.7; $P<0.05$) (Fig. 4A). Consistent with this, the intestinal content of GSSG, a high-affinity endogenous substrate of Mrp2, was significantly higher in the FRU group (191±11 nmol/g intestine) than in the control group (108±14 nmol/g intestine; $P<0.01$). Detection of intestinal TBARS levels also revealed a 50% increase in the FRU group (0.18± 0.01 nmol/mg protein) with respect to controls (0.12±0.01 nmol/mg protein; $P<0.01$) (Fig. 4B). We also evaluated the antioxidant defense status and found an increased activity of SOD in FRU animals (+40%; Fig. 4C), whereas CAT activity remained unchanged (11465±890 *vs.* 10112±649 μmol H_2O_2/min/mg protein for FRU and control rats, respectively) (Fig. 4D).

3.7. Effect of fructose on intestinal proinflammatory cytokine levels

The effects of fructose administration on IL-1β and IL-6 levels were investigated in intestinal homogenates prepared from proximal jejunum. As shown in Fig. 4E–F, IL-1β and IL-6 levels increased significantly in FRU group when compared to control group (+58% and +59%, respectively, $P<0.05$), consistent with establishment of local inflammation.

4. Discussion

It has been documented that fructose consumption increased dramatically in the last decades and so the incidence of MetS. In addition, growing information coming from both human studies and animal models suggests that high dietary intake of fructose is an important nutritional factor in the development of MetS and its associated complications [11]. High-fat or high-carbohydrate diets are relevant components of the etiology of this pathological condition in humans, and thus, alteration in any of these diets constitutes an appropriate animal model to mimic the disease [8,13]. In the present study, we administered 10% of fructose in drinking water to normal rats for 21 days. In line with previous reports [14–17], our results show that fructose treatment resulted in increased daily liquid intake and triglyceridemia, in addition to glucose intolerance. In addition, FRU rats exhibited higher plasma insulin levels and HOMA-IR index respect to control rats, which indicates the establishment of an insulin resistance state. Finally, although body weight did not differ between groups, we found larger epididymal fat depots in fructose rats, which suggest an increase in body adipose tissue, possibly due to adipocyte hypertrophy [42]. This metabolic profile resembles several characteristics of the human MetS [43].

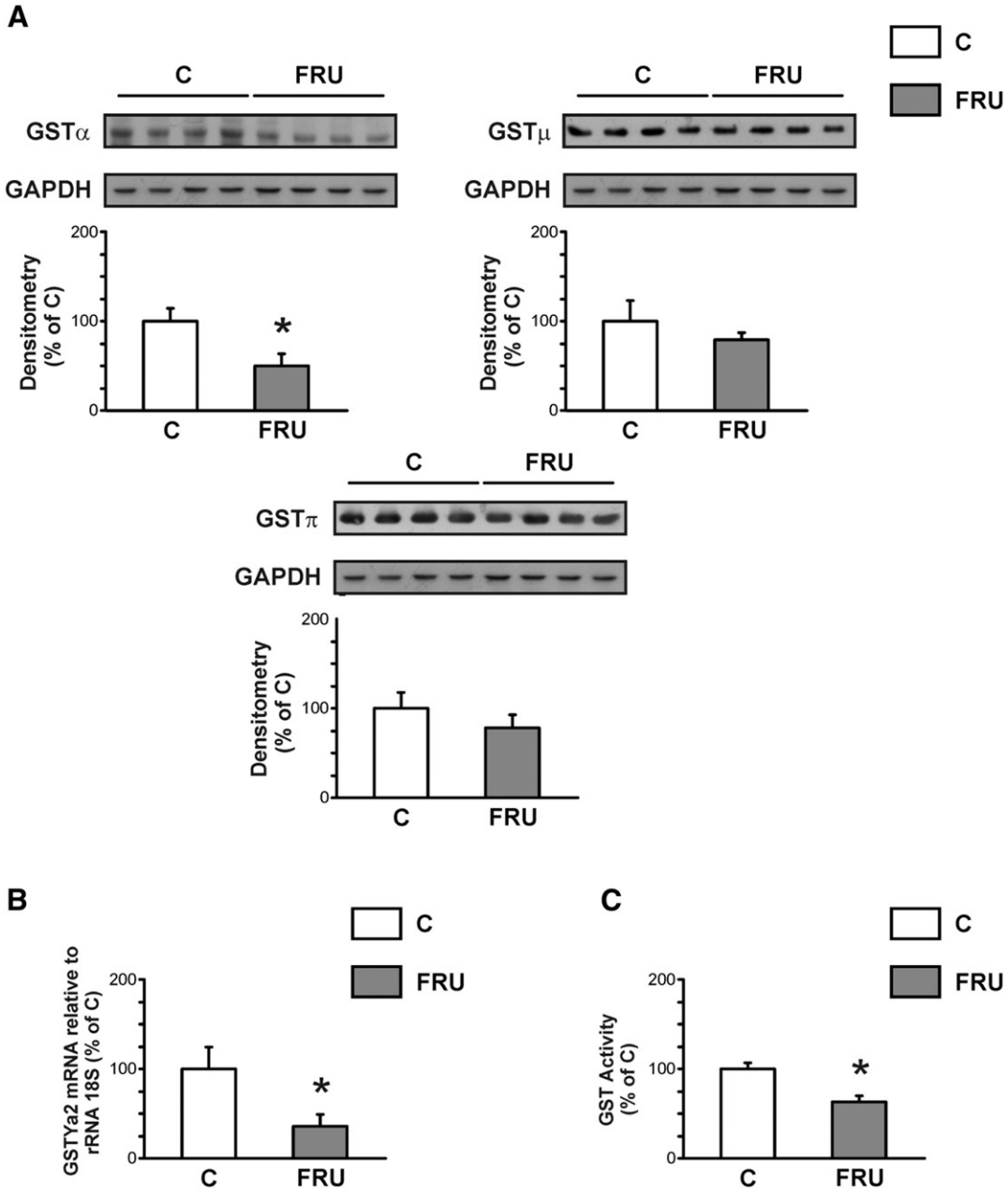

Fig. 3. Effect of fructose on GST expression and activity. (A) Western blot analysis of the major GST classes in cytosolic fractions from proximal jejunum. Equal amounts of total protein (15 μg) were loaded in all lanes. Uniformity of loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S and detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Densitometry data were related to GAPDH and presented as % of controls (C). (B) Quantitative real-time PCR assessment of GSTYa2 mRNA levels in proximal jejunum. Results were referred to 18 s rRNA and expressed as % of controls (C). (C) GST activity toward CDNB in cytosolic fractions from proximal jejunum. Data are expressed as means ± S.D. of six–eight rats per group. *, Significantly different from C, $P<0.005$.

While most of the studies under MetS-like conditions are focused on the consequences on major metabolic tissues including liver, muscle and adipose tissue, there is limited information in relation to the intestine. Notably, the small intestine is the main site of exposure and/or absorption of nutrient as well as potentially toxic food contaminants and therapeutic drugs. More importantly, the alteration of the integrity and functionality of the intestinal epithelium negatively impacts on the rest of the organism. It is well known that intestinal Mrp2 plays an important role in preventing toxicity of xenobiotics since it restricts the amount of substances that eventually get into the systemic circulation after oral incorporation [44]. We here demonstrated for the first time that the expression of Mrp2 (protein and mRNA) is significantly down-regulated in rats under fructose-rich diet. Consistent with its decreased expression, Mrp2 activity as biochemical barrier was severely affected, as demonstrated *in vitro* by experiments in everted sacs. GST activity was also decreased in fructose rats through reduction of the expression of the α class, one of the major isoforms of GST mediating conjugation of CDNB. Because GST acts in tandem with Mrp2, taken together, the data suggest altered disposition of hydrophobic compounds requiring sequential participation of both systems. This may involve compounds of therapeutic use, eventually leading to alterations in their bioavailability and safety. To test this possibility, we additionally explored Mrp2 activity *in vivo* using valsartan, an antihypertensive drug, as a model [45]. Importantly, it is mainly excreted (85% of orally administered dose) into feces in its unchanged form [46]. The present data clearly demonstrate an increase in intestinal valsartan absorption under MetS-like conditions.

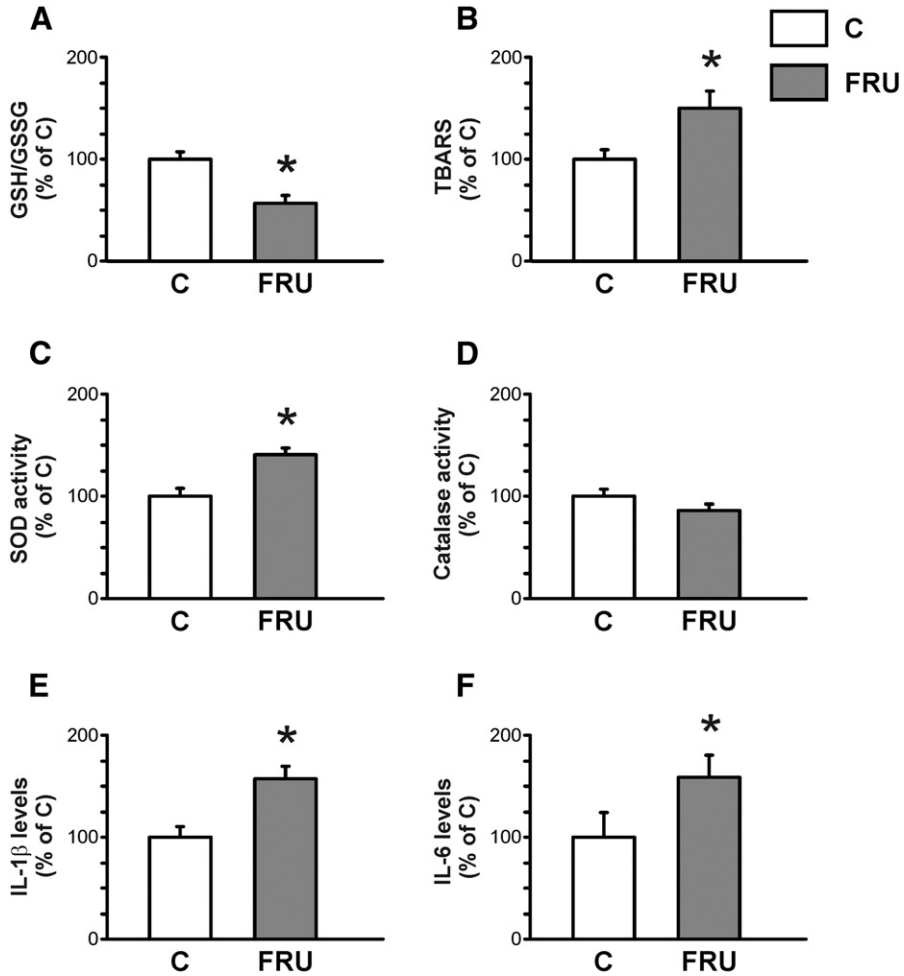

Fig. 4. Effect of fructose on intestinal redox balance, antioxidant defenses and interleukin levels. GSH/GSSG ratio (A), thiobarbituric acid reactive substances (TBARS) (B), **s**uperoxide dismutase (SOD) activity (C), catalase (CAT) activity (D), interleukin 1β (E) and interleukin 6 (F) were determined in intestinal homogenate of rats from C and FRU groups. All results were expressed as % of controls (C). Data are expressed as means ± S.D. of six–eight rats per group. *, Significantly different from C, $P<0.005$.

Thus, the impairment in Mrp2 activity constitutes an important factor increasing plasma bioavailability of valsartan under MetS-like conditions, which can result in adverse effects. Frequently, patients with MetS are treated not only for the primary disorder but also for comorbid conditions, including cardiovascular complications, hypertension and dyslipidemia. Many of the agents prescribed to treat these conditions are potential substrates of GST/Mrp2 and consequently may present altered oral bioavailability and challenge the safety of the treatment. If intestinal, MRP2 is down-regulated in patients with MetS is unknown. Interestingly, some drugs reported to present increased incidences of drug-induced toxicity in liver from patients with metabolic disease such as MetS are at the same time MRP2 substrates [19–21]. Examples of these drugs are methotrexate and cyclosporine A. Whether this finding responds to a failure in MRP2 function needs more direct demonstration.

Similar studies focusing on systems involved in drug disposition were conducted in animal models of diabetes or metabolic syndrome [47–51]. However, only [52] Mei *et al.* (2012) described changes in the expression of intestinal Mrp2. These authors used streptozotocin to induce diabetes, and in contrast to our findings, they reported an induction of intestinal Mrp2. Higher levels of total bile acids and conjugated bilirubin in plasma of diabetic animals, together with insulin deficiency, were proposed as the mechanisms underlying Mrp2 alteration. Our experimental model clearly differs from that of streptozotocin-induced diabetes, as higher levels of insulin are present and total bile acids and conjugated bilirubin in plasma are not expected to change.

In the present work, the specific mechanisms underlying the reduction of expression and activity of intestinal Mrp2 under MetS conditions have not been studied. Oxidative stress and inflammatory response have been suggested to mediate, at least partially, the action of fructose in the generation of MetS [11,16,17]. For example, high-fructose feeding rats showed increased levels of oxidative stress markers [53], plasma release of proinflammatory cytokines IL-1β, IL-6 [54] and content of tumor necrosis factor (TNF)–α in tissues [55] and reactive oxygen species in circulatory leukocytes [56]. Together, these data confirm that fructose administration is associated with an inflammatory response, which in turn, is probably mediated by increased oxidative stress. The possibility that high-fructose diet generates local inflammation and a prooxidant status in intestine, as demonstrated previously for other tissues, was also considered in the current study. This postulation was indeed confirmed, as higher levels of proinflammatory cytokines IL-1β, IL-6 were detected in intestinal tissue of fructose rats *versus* controls. Likewise, higher levels of lipid peroxidation end products and activity of the antioxidant enzyme SOD, as well as a lower value of intestinal GSH/GSSG ratio were detected in the fructose group when compared to control rats. Notably, proteins of the MRP family are involved in the efflux of glutathione conjugates as well as GSSG [57] and may play an important role in maintaining the GSH/GSSG ratio in response to an

oxidative challenge. Thus, the impairment in Mrp2 function currently reported likely constitutes an additional component leading to exacerbation of the oxidative stress found in the intestinal tissue.

Even though the main mechanisms explaining Mrp2 down-regulation in FRU rats remains uncertain, it is possible to associate the marked inflammation and oxidative stress produced in the intestine under treatment with fructose. This connection was indeed demonstrated in the liver under conditions of lipopolysaccharide-induced cholestasis and by single ethacrynic acid administration [58,59], where Mrp2 down-regulation were proposed to be at posttranscriptional level. Increased levels of proinflammatory cytokines such as IL-1β and IL-6, which were responsible for down-regulation of intestinal Mrp2 expression at transcriptional and posttranscriptional levels in obstructive cholestasis and in endotoxin-treated rats, could also explain the current findings [60–62]. These observations strongly suggest that oxidative stress and inflammation may play a pivotal role in the Mrp2 down-regulation induced by fructose administration. However, further experiments are needed to confirm this postulation and identify the specific molecular mechanism.

In conclusion, the present study demonstrated for the first time that MetS-like conditions generated in normal rats fed with 10% fructose solution for 21 days resulted in reduction in the intestinal expression and function of Mrp2, as well as in GSTα. Co-regulation of the enzyme-transporter systems is of frequent occurrence in the small intestine with important implications in drug toxicology–pharmacology. While more studies are needed to translate these findings to humans, the current results suggest that toxicity of food contaminants as well as availability of therapeutic drugs, substrates of GST/Mrp2, may be exacerbated in MetS patients.

Conflict of interest

The authors declare that they have no conflict of interest.

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