Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 40 (2016) 178–186

Journal of Nutritional Biochemistry

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

Ana Sofía Londero[a], Maite Rocío Arana[a], Virginia Gabriela Perdomo[b], Guillermo Nicolás Tocchetti[a], Felipe Zecchinati[a], Carolina Inés Ghanem[c], María Laura Ruiz[a], Juan Pablo Rigalli[e], Aldo Domingo Mottino[a], Fabiana García[d,1], Silvina Stella Maris Villanueva[a,*,1]

[a]Instituto de Fisiología Experimental (IFISE-CONICET)-Facultad de Ciencias Bioquímicas y Farmacéuticas-Universidad Nacional de Rosario. Rosario, Santa Fe, Argentina
[b]Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET)-Facultad de Ciencias Bioquímicas y Farmacéuticas-Universidad Nacional de Rosario. Rosario, Santa Fe, Argentina
[c]Instituto de Investigaciones Farmacológicas (ININFA-CONICET)-Facultad de Farmacia y Bioquímica-Universidad de Buenos Aires. Ciudad Autónoma de Buenos Aires, Argentina
[d]Facultad de Ciencias Médicas-Universidad Nacional de Rosario-CONICET-Cátedra Fisiología Humana. Rosario, Santa Fe, Argentina
[e]Department of Clinical Pharmacology and Pharmacoepidemiology, Universität Heidelberg. Heidelberg, Germany

Received 6 June 2016; received in revised form 20 October 2016; accepted 7 November 2016

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.

☆ Funding sources: This study was supported by grants from: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) [PIP 2012-0240 (to A.D.M.)], Fondo para la Investigación Científica y Tecnológica (FONCyT) [PICT 2014-0476 (to A.D.M.) and PICT 2014-1121 (to S.S.M.V.)].

* Corresponding author at: Instituto de Fisiología Experimental (CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas (UNR), Suipacha 570, (2000) Rosario, Argentina. Tel.: +54-341-4305799; fax: +54-341-4399473.
E-mail address: villanueva@ifise-conicet.gov.ar (S.S.M. Villanueva).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jnutbio.2016.11.002
0955-2863/

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).