Targeted Metabolomics Identifies Glucuronides of Dietary Phytoestrogens as a Major Class of MRP3 Substrates In Vivo

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BACKGROUND & AIMS: The physiologic function of the efflux transporter Multidrug Resistance Protein 3 (MRP3) remains poorly defined. In vitro, MRP3 transports several glucuronidated compounds, but the compounds transported under physiologic conditions are unknown. Knowledge of the compounds transported by MRP3 in vivo would greatly contribute to the elucidation of the physiologic function of this transport protein. **METHODS:** We used targeted metabolomics to identify substrates of MRP3 in vivo. Liquid chromatography coupled to mass spectrometry was used to specifically screen in plasma and urine of mice for compounds containing a glucuronic acid moiety. **RESULTS:** We found that several highly abundant compounds containing a glucuronic acid moiety have a much lower abundance in plasma and urine of *Mrp3*$^{(-/-)}$ than of wild-type mice. We identified these as phytoestrogen-glucuronides, and we show that MRP3 transports these compounds at high rates and with high affinity in vitro. **CONCLUSIONS: We have identified the efflux transporter MRP3 as a major factor in the disposition of phytoestrogens, a class of compounds to which mammals are exposed via food of plant origin. Our targeted metabolomics approach is not restricted to MRP3 but applicable to many other transport proteins for which knockout mouse models are available. Similar screens could be developed for sulpho- and glutathione-conjugates, further increasing the potential of identifying new physiologic transporter substrates.**

Multidrug Resistance Protein 3 (MRP3; ABCC3) belongs to the adenosine triphosphate (ATP)-binding cassette (ABC) family of membrane transporters[1] and couples the energy released by the hydrolysis of ATP to transport of a substrate over the plasma membrane out of the cell.[2,3] In vitro experiments have shown that MRP3 is a typical organic anion transporter, like other MRPs. Its substrates include (some) anionic drugs, bile acids, and compounds conjugated to a glutathione-, sulphate-, or glucuronic acid moiety (ie, phase II metabolites).[2,3] Initial studies focused on the function of MRP3 in the liver because it was hypothesized to protect the hepatocytes against the toxic accumulation of bile acids during cholestasis. After *Mrp3*$^{(-/-)}$ mice became available, this hypothesis was disproved,[4,5] and the physiologic function of MRP3 remains largely unknown.[5]

MRP3 is highly expressed in epithelial cells of liver, gut, and kidney,[6] organs also known for their high glucuronidating capacity.[7] Glucuronidation is an important detoxification mechanism, and a major function of MRP3 may therefore lie in the disposal of glucuronic acid conjugates of xenobiotics to which organisms, like humans, are constantly exposed via their diet. Indeed, several glucuronosyl conjugates of drugs have been identified as MRP3 substrates in vivo[8,9] as well as bilirubin-glucuronide and glucuronidated bile salts.[4,5]

MRP3 is localized in the basolateral membrane of epithelial cells[6] and transports its substrates toward the circulation for urinary excretion. The abundance of MRP3 substrates in plasma and urine should therefore be reduced in knockout mice lacking MRP3. We have focused on a screen for glucuronosyl conjugates that are lower in plasma and urine of *Mrp3*$^{(-/-)}$ than of wild-type (WT) mice. By this approach, we identified several new substrates of MRP3, all glucuronidated phytoestrogens. Our results show that a major physiologic function of MRP3 is to transport the glucuronic acid conjugates of phytoestrogens from the gut epithelium into the circulation, for subsequent urinary excretion.

Materials and Methods

Materials

Oasis HLB solid phase extraction (SPE) cartridges (30 mg) and the preparative Atlantis C18 reversed phase column (10 μm, 250 × 19 mm) were from Waters (Milford, MA). Analytical Luna C18(2) reversed phase columns (3 μm, 150 × 2.0 mm and 3 μm, 150 × 4.6 mm) were from Phenomenex (Torrance, CA). Creatine phos-

Abbreviations used in this paper: ABC, ATP-binding cassette; ATP, adenosine triphosphate; BSA, bovine serum albumin; cDNA, complementary DNA; CID, collision-induced dissociation; HEK, Human Embryonic Kidney cells; LC, liquid chromatography; MRM, multiple reaction monitoring; MRP3, Multidrug Resistance Protein 3; MS, mass spectrometry; m/z ratio, mass-to-charge ratio; PXR, pregnane-X-receptor; SPE, solid phase extraction; WT, wild-type.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.06.052

phate and creatine kinase were from Roche (Almere, The Netherlands). [^{3}H]genistein, [^{3}H]equol, and [^{3}H]uridine-diphosphate-glucuronic acid were from American Radiolabeled Chemicals (St. Louis, MO). Standard rodent diet (AM-II) and modified standard AIM-II diet containing 20% soy meal were from Arie Blok (Woerden, The Netherlands; for composition of the diets see the online Supplementary Materials and Methods). Acetonitrile and methanol were from Biosolve (Valkenswaard, The Netherlands). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO).

Animals

Mrp3$^{(-/-)}$ mice have been described.[5] All mice used were on a 99% FVB genetic background, received food and water ad libitum, and were housed in constant temperature rooms with a 12-hour light/12-hour dark cycle. Mouse handling and experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

Collection and Processing of Blood and Urine Samples of WT and Mrp3$^{(-/-)}$ *Mice*

Twenty-four-hour urine samples of 10- to 12-week-old mice fed a standard rodent diet (AM-II) or a diet containing 20% soy meal were collected in metabolism cages. Afterwards, mice were placed in their normal cages and kept there for 7 days before blood was collected by cardiac puncture using heparinized syringes. Plasma was obtained after centrifugation (1500*g*, 10 minutes, 4°C). Five hundred microliters plasma was mixed with 500 μL H_2O and 100 μL acetic acid, centrifuged at 10,000*g* for 5 minutes, and applied to a preconditioned (1 mL methanol, 1 mL H_2O) Oasis HLB SPE cartridge (1 cc, 30 mg). The cartridge was washed 2× with 1 mL 2% acetic acid in 5% methanol before elution with 2% NH_4OH in 92% methanol. For urine, 50% of the 24-hour urine samples were extracted using the above-described protocol for plasma, using an acetic acid concentration of 10%. Samples were evaporated until dry under vacuum and stored at −80°C until analysis. Prior to analysis by liquid chromatography (LC)/mass spectrometry (MS), plasma and urine samples were dissolved in 35 μL and 120 μL, respectively, of buffer A (see below under High-Pressure Liquid Chromatography heading).

Deglucuronidation of Plasma and Urine Samples of WT Mice

Two milliliters pooled WT mouse plasma was subjected to SPE on a 6-mL, 200-mg OASIS HLB cartridge using the conditions described above. The pellet was dissolved in 1800 μL phosphate buffer (75 mmol/L, pH 6.8), divided in 2 fractions of 900 μL to which 100 μL phosphate buffer (control) or 100 μL β-glucuronidase (25 U/μL) was added, incubated at 37°C, and processed by SPE as described above. WT mouse urine (an equivalent of 50% of the urine produced during 24 hours by 1 animal) was directly diluted in phosphate buffer to a final volume of 1800 μL and subsequently processed as described for plasma.

High-Pressure Liquid Chromatography

For the LC/MS experiments, the analytical high-pressure liquid chromatography system consisted of 2 Series 200 micropumps (Perkin Elmer, Waltham, MA). Separations were performed on a reversed-phase Luna C18(2) (Phenomenex) 3 μm, column (150 mm × 2 mm) at a flow rate of 200 μL/min, with a total run time of 35 minutes and detection by MS (see below). The mobile phases consisted of 10 mmol/L ammonium formate, pH 8.2 (buffer A), and 10 mmol/L ammonium formate, pH 8.2, in 95% acetonitrile (buffer B). Samples were separated using the following gradient A/B (vol/vol): 0–20 minutes, 10/90 to 35/65; 20–22 minutes, 35/65 to 0/100; 22–26 minutes, 0/100; 26–28 minutes, 0/100 to 10/90; 28–35 minutes, 10/90. The injection volume was 20 μL, equivalent to 285 μL plasma or 8.3% of the amount of urine excreted by a mouse during 24 hours. The equivalent of 2.75% of the urine produced during 24 hours was injected from mice on a diet containing 20% soy meal.

MS

An API4000 Qtrap quadrupole iontrap hybrid mass spectrometer (MDS Sciex, Concord, ON, Canada) was used throughout this study and operated in the negative electrospray ionization mode. Compounds containing a glucuronic acid moiety have a specific fragmentation pattern following collision-induced dissociation (CID) because they specifically lose 176 daltons.[10] The mass spectrometer was programmed to scan for deprotonated molecules that showed a neutral loss of 176 daltons after CID, and MS parameters were optimized using commercially available glucuronides (not shown). The following parameters were used throughout the study: curtain gas: 10 psi; collision-activated dissociation gas: high; ion spray voltage: −4500 V; cone temperature: 500°C; gas flow 1: 30 psi; gas flow 2: 20 psi: decluster potential: −65 V; entrance potential: 10 V; collision energy: −39 V.

The same MS parameters were used during multiple reaction monitoring (MRM) to semiquantitatively detect compounds in biologic matrices of WT and *Mrp3*$^{(-/-)}$ mice that specifically lost 176 daltons. The MRM list was based on neutral loss scans on control or deglucuronidated plasma samples of WT mice: compounds detected in control treated samples but absent after deglucuronidation were included in the MRM list. Enhanced product spectra of glucuronides found to be differentially present in plasma or urine of WT and *Mrp3*$^{(-/-)}$ mice were made using information-dependent acquisition and enhanced product ion generation and compared with enzymatically synthesized phytoestrogen-glucuronide standards (see below) for identification.