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Regulation of expression and activity of multidrug resistance proteins MRP2 and MDR1 by estrogenic compounds in Caco-2 cells. Role in prevention of xenobiotic-induced cytotoxicity

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ABSTRACT

ABC transporters including MRP2, MDR1 and BCRP play a major role in tissue defense. Epidemiological and experimental studies suggest a cytoprotective role of estrogens in intestine, though the mechanism remains poorly understood. We evaluated whether pharmacologic concentrations of ethynylestradiol (EE, 0.05 pM to 5 nM), or concentrations of genistein (GNT) associated with soy ingestion (0.1–10 μM), affect the expression and activity of multidrug resistance proteins MRP2, MDR1 and BCRP using Caco-2 cells, an in vitro model of intestinal epithelium. We found that incubation with 5 pM EE and 1 μM GNT for 48 h increased expression and activity of both MRP2 and MDR1. Estrogens did not affect expression of BCRP protein at any concentration studied. Irrespective of the estrogen tested, up-regulation of MDR1 and MRP2 protein was accompanied by increased levels of MDR1 mRNA, whereas MRP2 mRNA remained unchanged. Cytotoxicity assays demonstrated association of MRP2 and MDR1 up-regulation with increased resistance to cell death induced by 1-chloro-2,4-dinitrobenzene, an MRP2 substrate precursor, and by paraquat, an MDR1 substrate. Experiments using an estrogen receptor (ER) antagonist implicate ER participation in MRP2 and MDR1 regulation. GNT but not EE increased the expression of ERβ, the most abundant form in human intestine and in Caco-2 cells, which could lead in turn to increased sensitivity to estrogens. We conclude that specific concentrations of estrogens can confer resistance against cytotoxicity in Caco-2 cells, due in part to positive modulation of ABC transporters involved in extrusion of their toxic substrates. Although extrapolation of these results to the in vivo situation must be cautiously done, the data could explain tentatively the cytoprotective role of estrogens against chemical injury in intestine.

1. Introduction

The major functions of the intestine are to absorb nutrients and to protect the body against xenobiotics including carcinogens. Gastrointestinal absorption of xenobiotics is decreased by the presence of export pumps at the apical membrane of the enterocyte. These transporters belong to the ABC transporter family and actively mediate cellular extrusion, consequently restricting absorption of potentially toxic compounds contributing to the barrier function of the intestine (Dietrich et al., 2003). The most relevant members of these proteins are multidrug resistance-associated protein 2 (MRP2, ABCC2), multidrug resistance protein 1 (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) (Mottino et al., 2000; Dietrich et al., 2003).

Abbreviations: MRP2, multidrug resistance-associated protein 2; MDR1, multidrug resistance protein 1; BCRP, breast cancer resistance protein; EE, ethynylestradiol; GNT, genistein; ER, estrogen receptor; AP-1, activating protein-1; Sp1, specificity protein-1; PQ, paraquat; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, dinitrophenyl-S-glutathione; DNP-CG, dinitrophenyl-cysteinyl glycine; DMSO, dimethyl sulfoxide; CypA, cyclophilin A; PVDF, polyvinyl difluoride; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretazolium bromide; FBS, fetal bovine serum; PXR, pregnane X receptor; HBSS, Hank's balanced salt solution; PCR, polymerase chain reaction; P, probenecid; V, verapamil.

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MRP2 actively exports amphipathic organic drugs as well as hydrophilic anions in the form of glucuronide, sulfate and glutathione conjugates. It is preferentially expressed in the proximal intestine and the expression gradually decreases from the jejunum to the distal ileum (Mottino et al., 2000). MDR1 has broad substrate specificity for structurally and pharmacologically unrelated hydrophobic compounds and it is highly expressed on the apical surface of ileal and colon enterocytes, with decreasing expression proximally into the jejunum, duodenum and stomach (Takano et al., 2006). BCRP also has a role in limiting oral bioavailability of wide range of compounds with either negative or positive charge. Together with MDR1, BCRP expression is higher in the ileal enterocytes and shows similar distribution in mice and rats along duodenum, jejunum and colon (Enokizono et al., 2007).

Over the last years, epidemiological studies suggest a protective role of estrogens (Di Leo et al., 2001) and phytoestrogens such as genistein (GNT) (Barone et al., 2012) in chemical-induced colon injury and colorectal cancer development. Animal studies offer further evidence supporting the hypothesis that estrogens play a protective role against chemical injury at the intestine level (Guo et al., 2004; Weige et al., 2009). The origin of the estrogen protective effect remains unknown. Intracellular accumulation of xenobiotics and their biotransformation derivatives is minimized by ABC transporters. Considering that intestinal epithelium is constantly exposed to oral xenobiotics and the strategic localization of MRP2, MDR1 and BCRP at the apical membrane of the enterocyte, we postulate that their positive regulation by estrogens could contribute to estrogen protection, e.g. by preventing cellular damage.

We used the Caco-2 cell line, an established in vitro model for intestinal drug transport studies, to evaluate the effect of pharmacological concentrations of ethynylestradiol (EE) and concentrations of GNT associated with soy ingestion, on the expression and activity of MRP2, MDR1 and BCRP. We further determined if changes in expression and activity of these transporters protect against cytotoxicity produced by xenobiotics, substrates of these same transporters, and whether estrogen receptors are implicated as mediators.

2. Materials and methods

2.1. Chemicals

EE, GNT, probenecid, rhodamine 123 (Rh123), verapamil, leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, CDNB, paraquat dichloride (methyl viologen; PQ), NADPH, 2-vinylpyridine, dithionitrobenzoic acid, glutathione reductase and glutathione were obtained from Sigma Chemical Co (St. Louis, MI, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, HE, Germany). All other chemicals were of analytical grade purity and used as supplied. Dinitrophenyl-S-glutathione (DNP-SG) was synthesized with the use of 1-fluoro-2,4-dinitrobenzene (Mottino et al., 2001).

2.2. Cell culture and treatments

The human Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, USA). Cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, PAA, Pasching, Austria), 1% (v/v) of L-glutamine, 1% (v/v) of a mixture of antibiotics (10,000 units/ml penicillin and 10,000 μg/ml streptomycin), 1% (v/v) of non-essential aminoacids (Cat number 11140050, Invitrogen, Carlsbad, CA, USA) and 6 ng/ml of amphotericin B (PAA, Pasching, Austria). They were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. For studies to quantitate protein and mRNA expression, cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and maintained in culture for 15 days. After that, the medium was changed to phenol red-free DMEM medium supplemented with 10% (v/v) charcoal-dextran treated FBS (Hyclone) (treatment media) and EE (0.05, 0.5, and 5 pM or 0.05, 0.5 and 5 nM) or GNT (0.1, 1, and 10 μM) were added, dissolved in DMSO. Only DMSO was added to control cells (C). The treatment time was 48 h and the treatment medium was renewed every 24 h. The final concentration of DMSO in the culture media was always below 0.1%. Alternatively, cells were pretreated for 30 min with the ER antagonist 7a,17b-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182/780, 1 μM) (Tocris Cookson Inc., Ellisville, MO, USA) followed by treatment with EE or GNT for 48 h. The inhibitor was present during the treatment time. Cell viability after all the treatments was assessed measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretazolium bromide (MTT) to its formazan, which was quantified spectrophotometrically ($\lambda_{measurement}$ = 530 nm, $\lambda_{reference}$ = 630 nm) using a microplate reader LD-400 (Beckman Coulter, Brea, CA, USA). We found that the rate of MTT conversion was not statistically different between cells treated with estrogens and the respective controls (data not shown).

2.3. Western blot studies

At the end of incubations, cells were washed twice with cold PBS and scraped using RIPA buffer (Thermo Scientific, Rockford, IL, USA) supplemented with phenylmethylsulfonyl fluoride (1 mM), leupeptin (5 mg/ml) and pepstatin A (5 μg/ml) as protease inhibitors. Lysates were passed 20 times through a 25-G needle and subjected to protein concentration determination. Expression of MRP2 and MDR1 were additionally determined in total cellular membranes, which were prepared as described (García et al., 2001). Western blotting was performed as previously described (Ruiz et al., 2005) using anti-MRP2 (M2 III-6, Alexis Biochemicals, Carlsbad, CA, USA), anti-MDR1, anti-BCRP, anti-ERα, anti-ERβ, anti-c-FOS, anti-c-JUN and anti-Sp1(H-241, BXP-21, H-184, H-150, H-79, sc-420, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Equal loading and transference of protein was systematically checked by both detection of β-actin using a monoclonal antibody to human β-actin (Sigma–Aldrich, St. Louis, MO, USA), and staining of the membranes with Ponceau S. The immunoreactive bands were quantified with the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

2.4. RNA isolation and quantitative real-time PCR

At the end of incubations, cells were washed twice with cold PBS and scraped using TRIzol® reagent (Invitrogen Carlsbad, CA, USA) and total RNA was isolated according to the manufacturer's instructions. cDNA was produced using the Superscript Preamplification System for the first strand cDNA synthesis using random hexamers, according to the manufacturer's instructions (Invitrogen Carlsbad, CA, USA). Real-Time quantitative PCR was performed on cDNA samples using the Platinum SYBR Green qPCRSuperMix-UDG (Invitrogen Carlsbad, CA, USA) and reactions were carried out on a Stratagene Mx3000P (Stratagene, La Jolla, CA, USA). Sequences of primer pairs and conditions for MRP2, MDR1, Cyclophilin A (CypA) and rRNA 18S were designed to optimally detect the respective mRNAs. For MRP2 mRNA amplification, the forward and reverse primer sequences were, respectively, 5′AGGTTTGCCAGTTATCCGTG3′ and 5′AACAAAGCCAACAGTGTCCC3′. For MDR1 mRNA amplification, the forward and reverse primer sequences were 5′CCAAAGACAACAGCTGAAA3′ and 5′TACTTGGTGGCACATAAAC3′, respectively. Results were normalized to CypA or rRNA 18S as housekeeping genes, using CypA forward and reverse primers, respectively, 5′ GTCAACCCCACCGTGTTCTTC3′ and 5′TTTCTGCTGTCTTTGGGACCTTG3′ and forward and reverse rRNA 18S primers 5′CGCCGCTAGAGGTGAAATTC3′ and 5′TTGGCAAATGCTTTCGCTC3′ respectively. Relative levels of MRP2 and MDR1 mRNAs normalized to CypA or rRNA 18S were calculated based on the $2^{-\Delta\Delta Ct}$ method.

2.5. Transport activities

The activity of MRP2 was determined by the ability of Caco-2 cells to extrude DNP-SG, a model substrate, into the incubation medium. This methodology was previously used to evaluate Mrp2 activity in rat intestinal epithelium (Mottino et al., 2001). Briefly, cells were cultured in 96-well plates and treated with EE or GNT (5 pM or 1 μM, respectively; 48 h), as described above. The treatment medium was replaced with fresh medium containing DNP-SG (100 μM, dissolved in PBS) and cells were incubated at 37 °C for 30 min. At the end of the incubation, the medium was removed, cells were washed twice with PBS and immediately incubated with Hank's balanced salt solution (HBSS) at 37 °C for 30 min, in the presence or absence of probenecid (P, 1 mM), to confirm MRP2 participation (Bakos et al., 2000). Cells were then scraped with HBSS and sonicated. Cell lysates and incubation media were treated with 10% perchloric acid and centrifuged (2 min, 14,000 × g, 4 °C). Supernatants were used for detection of DNP-SG and dinitrophenyl-cysteinyl glycine (DNP-CG) by HPLC (Waters 600; Waters, Milford, MA, USA) as described (Mottino et al., 2001). DNP-CG is the result of γ-glutamyltransferase action on DNP-SG at the apical membrane of Caco-2 cells (Vermeulen et al., 2011). The activity of MRP2 was determined through calculation of the relationship between the amount of DNP-SG + DNP-CG accumulated in the medium after 30 min of incubation and the total amount of DNP-SG available to be extruded. The total amount available to be extruded was estimated as the sum of the amount of DNP-SG remaining in the cells at the end of incubation and that of DNP-SG + DNP-CG extruded into the medium along the 30-min period.

The activity of MDR1 was estimated as described by Rigalli et al. (2011), through assessment of intracellular retention of the model substrate Rh123, which inversely correlates with the ability of the cells to extrude the probe. Briefly, cells were treated with EE or GNT (5 pM or 1 μM respectively; 48 h), and at the end of incubation, treatment medium was replaced with fresh medium containing Rh123 (5 μM, dissolved in DMSO), with or without verapamil (100 μM, dissolved in DMSO), as MDR1 inhibitor (Kota et al., 2010). Cells were incubated for 2 h to allow the probe to enter