Distinct Alterations in ATP-Binding Cassette Transporter Expression in Liver, Kidney, Small Intestine, and Brain in Adjuvant-Induced Arthritic Rats

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ABSTRACT: Pathophysiological changes of infection or inflammation are associated with alterations in the production of numerous absorption, distribution, metabolism and excretion-related proteins. However, little information is available on the effects of inflammation on the expression levels and activities of ATP-binding cassette (ABC) transporters. We examined the effect of acute (on day 7) and chronic (on day 21) inflammation on the expression of ABC transporters in some major tissues in rat. Adjuvant-induced arthritis (AA) in rats was used as an animal model for inflammation. The mRNA levels of *mdr1a* and *mdr1b* encoding P-glycoprotein (P-gp) decreased significantly in livers of AA rats on day 21. Hepatic protein levels of P-gp, Mrp2, and Bcrp decreased significantly in membranes but not homogenates of AA rats after 7 days and after 21 days of treatment with adjuvant. Contrary to liver, protein levels of P-gp and Mrp2, but not Bcrp in kidney, increased significantly in membranes. The biliary excretion of rhodamine 123 was decreased in rats with chronic inflammation owing to decreases in efflux activities of P-gp. Our results showed that the expression of transporters in response to inflammation was organ dependent. In particular, hepatic and renal P-gp and Mrp2 exhibited opposite changes in membrane protein levels. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:2556–2564, 2014

Keywords: ABC transporters; active transport; disease state; disposition; efflux pumps; elimination; membrane transport/transporters; MRP; multidrug resistance transporters; P-glycoprotein

INTRODUCTION

Drug transporters, including efflux transporters [ATP-binding cassette (ABC) proteins] and uptake transporters [solute carrier (SLC) proteins], have an important impact on drug disposition, efficacy, and toxicity. P-glycoprotein (P-gp/Abcb1), multidrug resistance-associated protein 2 (Mrp2/Abcc2), and breast cancer resistance protein (Bcrp/Abcg2) remain the main ABC transporters that contribute to drug resistance in tumors and xenobiotic excretion from cells.[1] Decreases or inhibition of ABC transporter activities could lead to intracellular accumulation of drugs and/or their metabolites. For example, mdr knockout mice showed higher accumulations of arsenic,[2] digitoxin,[3] and vincristine in tissues.[4] The use of cyclosporin A resulted in the inhibition of biliary excretion of mycophenolic acid glucuronide via Mrp2,[5] and Bcrp inhibition by gefitinib resulted in increased bioavailability of oral irinotecan.[6]

Pathophysiological changes in human patients and in animal models of infection or inflammation are associated with immediate and often dramatic alterations in the production of numerous liver-derived proteins. Such inflammation-associated changes, which are collectively known as acute-phase responses, can result in profound increases in plasma concentrations of various drugs, thereby causing toxic effects.[7–11]

We have reported on the alterations in mRNA levels of cytochrome P450s and transporters in a rat model of adjuvant-induced arthritis (AA).[12,13] We showed that the total clearance of flurbiprofen was significantly increased compared with controls owing to the extensive increase in the unbound fraction of flurbiprofen in plasma, although glucuronidation activities and CYP contents were slightly impaired.[14] In AA rats, the pharmacokinetics of propranolol,[15] acebutolol,[16] and cyclosporine A[17] are altered.

Adjuvant-induced arthritis rats have been used as a model for rheumatoid arthritis for the development of anti-inflammatory medicines because they exhibit systemic inflammatory disease with changes to bone and cartilage similar to that observed in humans with rheumatoid arthritis.[18] AA rats at 7 days (AA 7 d) and 21 days (AA 21 d) after adjuvant treatment have been shown to exhibit acute and chronic inflammatory conditions, respectively. We think that AA rats are suitable model to examine the correlation between the developments of inflammation and alterations in expression of ABC transporters.

Serum levels of inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) in AA rats are significantly increased after the acute phase.[19] To evaluate alterations in drug disposition in inflammation, it is important to collect fundamental information regarding changes in the functions of ABC transporters.

However, alterations in mRNA and protein levels of ABC transporters in the liver, kidney, small intestine, and brain have not yet been exhaustively studied during the development of inflammation. This prompted us to investigate the effects of inflammation on expression levels and activities of P-gp, Mrp2, and Bcrp in the liver, kidney, small intestine, and brain. We also examined mRNA levels of inflammatory cytokines and plasma levels of nitric oxide (NO) to clarify the mechanisms for the

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Table 1. Primer Sequences Used in PCR Assays

Genes	Primer Sequences (5′-3′)
Mdr1a	CGTTGCCTACATCCAGGTTT
	TGGAGACGTCATCTGTGAGC
Mdr1b	ATGCTGCTTGTTTCCGGTTC
	ATGGCACCAAAGACAACAGC
Mrp2	CTCGGTCTTATGCGGCGTATT
	TCTGGAAACCGTAGGAGACGAA
Bcrp	TTGGACTCAAGCACAGCAAATG
	ATGGAATACCGAGGCTGGTGA
*IL-1*β	TGACCCATGTGAGCTGAAAG
	TCGTTGCTTGTCTCTCCTTG
IL-6	TGCCACTTGTGTGCTAAAGG
	CATGGTTTGTCTTCCACACACG
TNF-α	GAAAGGGAATTGTGGCTCTG
	TTCAGCGTCTCGTGTGTTTC
18S rRNA	CGCCGCTAGAGGTGAAATTC
	CCAGTCGGCATCGTTTATGG

distinct alterations in transporter expression between the liver and kidney.

MATERIALS AND METHODS

Preparation of AA Rats

Female Sprague–Dawley rats (7 weeks old), weighing 180–240 g, were purchased from CLEA Japan, Inc. (Tokyo, Japan). The animals were housed in a temperature-controlled room with free access to standard laboratory chow and water. Adjuvant was prepared from 100 mg heat-killed *Mycobacterium butyricum* (Difco Laboratories, Detroit, Michigan) suspended in 10 mL of Bayol F oil. Hindpaw volumes were measured using liquid plethysmometry. Animals were studied at 7 days (acute phase) and 21 days (chronic phase) after injection of adjuvant or Bayol F. AA rats in the acute phase exhibited local inflammation at the treated site. In the chronic phase, severe inflammation was observed in local and systemic sites. The Committee for the Care and Use of Laboratory Animals at Kinki University School of Pharmacy approved the experiments.

Real-Time Reverse-Transcription Polymerase Chain Reaction

After animals were anesthetized with diethyl ether, the liver, kidney, small intestine, and brain were removed. Following flash freezing in liquid nitrogen, all samples were stored at −80°C until used for RNA extraction.

Determination of mRNA levels was performed using real-time reverse-transcription polymerase chain reaction (RT-PCR) as previously described.[20] Total RNA (500 ng) was extracted from each sample and reverse transcribed to complementary DNA (cDNA) using a PrimeScript-RT reagent Kit (TaKaRa, Shiga, Japan). Reactions were incubated for 15 min at 37°C and 5 s at 85°C. The reverse-transcribed cDNA was used as a template for real-time RT-PCR. Amplification was performed in a 50-μL reaction mixture containing 2 × SYBR Premix Ex Taq (TaKaRa) and 0.2 mM of each primer set as shown in Table 1. PCRs were incubated at 95°C for 10 s, and then amplified at 95°C for 5 s, 55°C for 20 s, and 72°C for 31 s for 40 cycles. Data were normalized to the amount of 18S rRNA in each sample. Data were analyzed with ABI Prism 7000 SDS software (Applied Biosystems, Carlsbad, California) using the multiplex comparative method.

Western Blot Analysis

Hepatic and renal homogenates and membrane proteins were prepared and subjected to Western blot analysis. For homogenates, 100 mg of each tissue was homogenized in HEPES–Tris buffer (pH 7.4) containing 250 mM sucrose and 1% (v/v) protease inhibitor cocktail (Sigma–Aldrich, St. Louis, Missouri) at 4°C and centrifuged at 3000*g* for 10 min at 4°C. The supernatant was subsequently collected and used in the analysis. Membrane proteins were extracted using the Mem-PER eukaryotic membrane protein extraction reagent kit (Thermo, Mansfield, Massachusetts). The membrane protein extracted by Mem-PER eukaryotic membrane protein extraction reagent kit includes the minimal cross-contamination (typically less than 10%) of hydrophilic (cytoplasmic) protein into membrane protein fraction. Protein concentrations were determined using a BCA protein assay kit (Pierce, Loves Park, Illinois).

Homogenates (20 μg) and membrane proteins (5 μg) were diluted with loading buffer, denatured at 95°C for 3 min and resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7% polyacrylamide). Proteins were then transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corporation, Billerica, Massachusetts) and subjected to semidry blotting using a Transblot SD (Bio-Rad, Irvine, California). P-gp, Mrp2, Bcrp, and β-actin proteins were detected using ECL Prime Western blotting detection reagent (GE Healthcare, Piscataway, New Jersey). The following primary antibodies were used: a mouse monoclonal anti-P-gp antibody (clone C219) (GeneTex, Irvine, California), a mouse monoclonal anti-MRP2 antibody (clone M_2III-6) (Abcam, Cambridge, UK), a rabbit polyclonal anti-Bcrp antibody (clone ARP43649_T100) (Aviva Systems Biology, San Diego, California), and a mouse monoclonal anti-β-actin antibody (clone B11V08) (Acris Antibodies, San Diego, California). A peroxidase-labeled goat anti-mouse IgG antibody (KPL, Milford, Massachusetts) was used as the secondary antibody for detection of P-gp, Mrp2, and β-actin and a peroxidase-labeled goat antirabbit IgG antibody (KPL) was used for Bcrp.

Biliary and Urinary Excretion of Rho-123

Control and AA 21 d were anesthetized and catheters were inserted into the bile duct and jugular vein to collect bile and blood, respectively. Urine was collected from a polyethylene tube inserted into the urinary bladder. Livers were excised, frozen in liquid nitrogen, and stored at −80°C for subsequent analyses. After intravenous administration of Rho-123 (0.2 mg/kg) to rats, samples were collected at 20, 40, and 60 min. After 10-fold dilution of bile and urine, the levels of Rho-123 in bile, urine, plasma, and liver were determined using a fluorescence assay (Ex/Em 485/527 nm; SH-9000Lab; Hitachi High-Technologies Corporation, Tokyo, Japan).

Determination of Plasma NO Levels

Levels of NO in deproteinized 100 μL plasma samples from AA rats after 1 day (AA 1 d), AA 7 d, and AA 21 d were determined using a QuantiChrom NO Assay Kit (BioAssay Systems, Hayward, California). This kit was designed to accurately measure NO production using absorbance at 540 nm following reduction of nitrate to nitrite using an improved Griess method.