Lipopolysaccharide Increases Cell Surface P-glycoprotein That Exhibits Diminished Activity in Intestinal Epithelial Cells

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ABSTRACT:
Increasingly, it is recognized that commensal microflora regulate epithelial cell processes through the dynamic interaction of pathogen-associated molecular patterns and host pattern recognition receptors such as Toll-like receptor 4 (TLR4). We therefore investigated the effects of bacterial lipopolysaccharide (LPS) on intestinal P-glycoprotein (P-gp) expression and function. Human SW480 (P-gp+/TLR4+) and Caco-2 (P-gp+/TLR4–) cells were treated with medium control or LPS (100 ng/ml) for 24 h prior to study. P-gp function was assessed by measuring the intracellular concentration of rhodamine 123 (Rh123). To confirm P-gp-specific effects, breast cancer resistance protein (BCRP/ABCG2) and multidrug resistance-associated protein 2 (MRP-2/ABCC2) were also analyzed. Treatment of SW480 cells with LPS led to diminished P-gp activity, which could be prevented with polymyxin B (control: 207 ± 16 versus LPS: 402 ± 22 versus LPS + polymyxin B: 238 ± 26 pmoles Rh123/mg protein, p < 0.05 control versus LPS). These effects could be blocked by using polymyxin B and were not seen in the P-gp+/TLR4– Caco-2 cell line (control: 771 ± 28 versus LPS: 775 ± 59 pmoles Rh123/mg protein). Total cellular levels of P-gp did not change in LPS-treated SW480 cells; however, a significant increase in cell surface P-gp was detected. No change in activity, total protein, or apically located MRP-2 was detected following LPS treatment. Sequence analysis confirmed wild-type status of Caco-2 and SW480 with respect to TLR4.

P-glycoprotein (P-gp) is arguably one of the best studied members of the ATP-binding cassette (ABC) transporter superfamily. In the last two decades, great advances have been made toward the elucidation of regulatory mechanisms controlling P-gp expression and function in tissues such as the intestinal epithelium (Geick et al., 2001; Scotto, 2003; Altermann et al., 2005; Burk et al., 2005). Genetic factors such as polymorphisms in the ABCB1 gene and concomitant administration of P-gp inducers and inhibitors influence the level of expression and functionality of the transporter (Hoffmeyer et al., 2000; Geick et al., 2001). One area that requires greater elucidation relates to the local intestinal environment. Diet has been purported to influence P-gp expression, yet more research is required before distinct associations can be made (Lo and Huang, 1999). In addition to diet, there are undoubtedly other environmental factors that may play a role.

One important environmental interaction that is not well characterized and may influence P-gp relates to the interface of intestinal epithelial cells with commensal bacteria. The human body is constantly exposed to microorganisms that reside in the gastrointestinal tract as part of the commensal flora (Hooper et al., 1999; Eckburg et al., 2005; Gill et al., 2006). These commensals are vital for both health and disease and serve many important functions, including important metabolic activities. Examples include serving as a functional barrier against pathogens and priming the intestinal immune system (Hooper and Gordon, 2001; Hooper et al., 2001; MacDonald and Gordon, 2005; Ley et al., 2006; Turnbaugh et al., 2006).

The capacity to discriminate between commensal and pathogen relies in large part on a family of evolutionarily conserved receptors designated as pattern recognition receptors (PRR) (Caro and Podolsky, 2005). One important family of PRR is the Toll-like receptor (TLR) family. To date, eleven TLR have been identified, each with specific ligands (Akira and Takeda, 2004). For example, the classic ligand that TLR4 recognizes is lipopolysaccharide (LPS) from Gram-negative bacteria (Poltorak et al., 1998; Akira et al., 2000). Upon LPS binding, a complex signaling cascade is initiated, leading to the activation or repression of a number of genes important for host response. Recent

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ABBREVIATIONS: P-gp, P-glycoprotein; ABC, ATP-binding cassette; PRR, pattern recognition receptors; CDFDA, 5-(and-6)-carboxy-2'-7'-dichlorofluorescein diacetate; TER, transepithelial electrical resistance; MK-571, [(E)-3-[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-[3-dimethylamino]-3-oxopropyl]thio[methyl][thio]-propanoic acid; BCRP, breast cancer resistance protein; HRP, horseradish peroxidase; LPS, lipopolysaccharide; MRP-2, multidrug resistance-associated protein 2; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonylfluoride; Rh123, rhodamine 123; TLR4, Toll-like receptor 4.
data indicate that microbial ligands signaling through TLRs are not limited to those from pathogenic organisms but also from nonpathogenic bacteria that make up the commensal flora (Rakoff-Nahoum et al., 2004). Therefore, it is essential to understand how commensal flora regulate homeostasis in the intestine through TLR signaling.

In the current study, we sought to elucidate the consequences of intestinal epithelial cell exposure to LPS on P-gp. The hypothesis was that LPS activation of TLR4 would lead to alterations in P-gp function. Our results indicate that LPS treatment leads to increased plasma membrane P-gp, which exhibits attenuated transport ability. These results shed light on a potentially novel regulator of P-gp in the intestine.

Materials and Methods

Cell Lines and Reagents. SW480 and Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Ultra-pure LPS from Escherichia coli 0111:B4 strain was from InvivoGen (San Diego, CA). Rhodamine 123 (Rh123) and 5-(and-6)-carboxy-2’,7’-dichlorofluorescein di-acetate (CFDA) were purchased from Invitrogen (Carlsbad, CA). The primary antibodies against human P-gp were MRK-16 (Kamiya Biomedical, Seattle, WA) and JSB-1 (Signet Laboratories, Dedham, MA). The primary antibody for multidrug resistance-associated protein 2 (MRP-2/ABCC2) was obtained from Kamiya. Species specific secondary antibodies were purchased from Invitrogen (Alexa Fluor 488) and Sigma-Aldrich (St. Louis, MO) (HRP-conjugated). SuperSignal West Dura Extended Duration Substrate was used for chemiluminescent detection (Pierce Biotechnology, Rockford, IL). The MRP-2 inhibitor MK-571 was obtained from EMD Biosciences (La Jolla, CA). All other chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific (Hampton, NH).

Cell Culture. For fluorescent probe uptake and confocal microscopy studies, cells were seeded in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) with or without glass coverslips. SW480 cells were studied 7 to 9 days after seeding, and Caco-2 cells were studied 14 days after seeding. Cells used for immunoblotting were grown in six-well plates and studied at similar time periods. All cell lines were maintained in a humidified atmosphere at 37°C. SW480 cells were maintained in Leibovitz’s L-15 medium containing 10% (v/v) fetal bovine serum and 2 mM L-glutamine. Caco-2 and growth medium consisted of Dulbecco’s modified Eagle’s medium without sodium-pyruvate. For the measurement of transepithelial electrical resistance (TER), SW480 cells were seeded on porous (0.4 μm) Transwell inserts (Costar, Cambridge, MA) in six-well plates. On the day of study, TER was measured using a Millipore-ERS Electrical Resistance System (Millipore, Bedford, MA) as we have described previously (Chiu et al., 2003).

Uptake of Fluorescent Probe Substrates. To evaluate the effect of LPS on intestinal epithelial P-gp, SW480 and Caco-2 cells were used. Both SW480 and Caco-2 cells express P-gp, yet only SW480 cells can signal through TLR4 (Vora et al., 2004). Because of the poor expression of the TLR4 complex in Caco-2 cells, they serve as an excellent control. SW480 and Caco-2 cells were exposed to 100 ng/ml LPS or medium control for 24 h and fixed in 3.7% (v/v) formaldehyde in PBS. In selected wells, polymyxin B (10 μg/ml) was added to antagonize the effects of LPS. Slides were washed twice with PBS and permeabilized by incubating with 0.2% Triton X-100 in PBS for 5 min at 4°C. These slides were then washed with PBS three times and blocked with 0.1% (v/v) bovine serum albumin in PBS for 30 min. Five micrograms of MRK-16 was added in a total volume of 100 μl to the coverslip and kept for 2 h at room temperature. Cells were washed three times with PBS followed by incubation with anti-mouse Alexa Fluor 488 secondary antibody (1:100) for 1 h before a final washing. For controls, selected wells were incubated with the mouse anti-human MRP-2 antibody. Experiments were conducted in triplicate and were repeated on two to three separate occasions.

Confocal Microscopy. SW480 cells grown on coverslips were exposed to 100 ng/ml LPS or medium control for 24 h and fixed in 3.7% (v/v) formaldehyde in PBS. In selected wells, polymyxin B (10 μg/ml) was added to antagonize the effects of LPS. Slides were washed twice with PBS and permeabilized by incubating with 0.2% Triton X-100 in PBS for 5 min at 4°C. These slides were then washed with PBS three times and blocked with 0.1% (v/v) bovine serum albumin in PBS for 30 min. Five micrograms of MRK-16 was added in a total volume of 100 μl to the coverslip and kept for 2 h at room temperature. Cells were washed three times with PBS followed by incubation with anti-mouse Alexa Fluor 488 secondary antibody (1:100) for 1 h before a final washing. For controls, selected wells were incubated with the mouse anti-human MRP-2 antibody. Experiments were conducted in triplicate and were repeated on two to three separate occasions.

Sequencing of BCRP/ABCG2 around Position 482. To confirm that changes in Rh123 transport in LPS-treated SW480 cells were due to changes in BCRP activity, the ABCG2 gene was sequenced to determine the amino acid at position 482. The BCRP wild-type allele accession number is XM_032424 and served as the basis for primer design (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer). Total RNA from of SW480 was isolated using the TRIzol reagent (Invitrogen) according to manufacturer’s instructions. Two micrograms of total RNA was reverse transcribed, and full-length cDNA was amplified using the Qiagen One Step RT-PCR Kit (Qiagen, Valencia, CA). ABCG2 primers were as follows: (F) 5’-GCCACGATCTGCAGTACCC-3’ and (R) 5’-AACATTGTGCTGCTGGCAAC-3’. A 1:10 dilution of the first round of ABCG2 cDNA was amplified a second time with nested primers: (F) 5’-CTGAGATCCTGAGCCTTTGG-3’ and (R) 5’-GGCAAGGGAACAGAAACAGAAAACAA-3’. To amplify the region spanning position 482, the primers (F) 5’-CTCGGATCCATCTACCTTGGGCTAAAAATGATTCC-3’ and (R) 5’-GGCAATCGCCAGAATCCGAGCAGCCACGTC-3’ were used. The amplified PCR product was sequenced at the University of Tennessee Health Science Center Molecular Resource Center using the sequencing primer 5’-GATTATTTACCATGGAGGATGTGTCACACG-3’. The sequence was analyzed and aligned with the human ABCG2 cDNA using Applied Biosystems (Foster City, CA) pairwise alignment software.

Statistical Analysis. Data were analyzed using a one-way analysis of variance using SigmaStat Statistical Software 2.03 (SPSS, Chicago, IL). If significant differences were detected, pair-wise comparisons were made using a Tukey post-hoc test. Significance was defined as p < 0.05 for all analyses.
Altered Uptake of Rh123 Is Not Due to Changes in BCRP or MRP-2. To rule out contributions of BCRP or MRP-2, we assessed these two transporters. We sequenced the BCRP gene (ABCG2) to confirm wild-type status at position 482 since this isoform cannot target P-gp. Polymyxin B alone did not affect localization of P-gp (data not shown). Lastly, LPS did not alter P-gp localization or protein levels in Caco-2 cells (data not shown).

Altered Uptake of Rh123 Is Not Due to Changes in BCRP or MRP-2. To rule out contributions of BCRP or MRP-2, we assessed these two transporters. We sequenced the BCRP gene (ABCG2) to confirm wild-type status at position 482 since this isoform cannot transport Rh123. We also evaluated MRP-2 by immunoblot, confocal microscopy, and uptake of a fluorescent substrate to confirm that changes in this protein did not occur with LPS treatment. Sequence analysis confirmed that SW480 cells were wild type at position 482 encoding for arginine. No changes in MRP-2 total protein, apical distribution, or function were detected in SW480 cells treated with LPS for 24 h compared with vehicle (medium) controls (Fig. 4).

Discussion

One of the best characterized roles for P-gp in the gastrointestinal tract relates to decreased absorption of orally administered substrates (Cascorbi, 2006). Despite this knowledge, the specific physiologic roles of P-gp are still unclear. Understanding the normal physiological mechanisms that regulate the expression and function of intestinal P-gp are therefore required. Given the close proximity of the microflora to P-gp on the apical membrane of intestinal epithelial cells, it is
reasonable that these bacteria may affect the expression and function of this important transporter. Recently, important contributions of the intestinal microflora to epithelial homeostasis have been recognized (Hooper et al., 1999; Rakoff-Nahoum et al., 2004; Shirkey et al., 2006). One mechanism by which commensal bacteria modulate epithelial physiology is through TLRs, which interact with ligands including LPS (Rakoff-Nahoum et al., 2004; Vora et al., 2004; Fukata et al., 2005). In the current investigation, we hypothesized that activation of intestinal TLR4 receptors by LPS may affect P-gp expression and function. Our findings indicate that exposure of intestinal epithelial cells to LPS results in increased cell surface localization of P-gp, which exhibits attenuated transport ability. No alterations in total cellular P-gp were detected, suggesting that the increase in surface expression of P-gp was the result of cellular redistribution rather than increased synthesis.

Previous investigations have evaluated the effects of LPS on P-gp expression in rodents and have found decreased expression or function of the transporter (Goralski et al., 2003; Kalitsky-Szirtes et al., 2004; Hartmann et al., 2005; Wang et al., 2005). LPS administered via the intraperitoneal (I.P.) route resulted in dramatic reduction in mdr1a mRNA levels in the brain, heart, liver, and small intestine. In addition to transcriptional alterations, significant decreases in the transport of a number of known P-gp substrates were demonstrated. Maezono and colleagues employed an LPS-induced intestinal damage model using excised rat intestinal segments to assess P-gp activity. Similar to the studies described above, they found decreased P-gp activity (Maezono et al., 2005). Once again, however, LPS was administered I.P., prior to segment excision. Thus, it is impossible to separate the local effects of LPS on intestinal epithelial cells from global ischemia which, in turn, may have affected the intestine. Although these studies were eloquent in design, they do not shed light on the direct effects of LPS on P-gp expression and function. Rather, they may reflect the effects of systemic inflammation and/or LPS-induced hypotension on P-gp in peripheral organs. Certainly, limitations are inherent with in vitro studies as well; however, use of a TLR4+/P-gp+ system such as the SW480 cell line circumvents the difficulties presented with systemic administration of LPS.

Exposure of SW480 cells to LPS led to decreased P-gp activity demonstrated by increased intracellular accumulation of Rh123 at 24 h. Involvement in TLR4 signaling was supported by the findings that coinubation of polymyxin B prevented these changes, and no alterations in Rh123 accumulation occurred in P-gp+/-TLR4+/-Caco-2 cells. LPS did not appear to totally abolish P-gp transport activity since MRK-16 treatment could further increase the intracellular accumulation of Rh123. One could postulate that increases in intracellular Rh123 may be due to increased permeability from leaky tight junctions caused by LPS. However, TER values did not change in LPS-treated cells and, therefore, this explanation is unlikely.

One difficulty in assessing the functional characteristics of P-gp is that the protein exhibits broad substrate affinity with considerable overlap with substrates for other membrane transporters. Many so-called P-gp substrates are also in fact transported by other transporters, making the ability to draw conclusions about P-gp specificity difficult (Sarkadi et al., 2004). Besides P-gp, MRP-2 and BCRP are the two principal apically located efflux transporters in intestinal epithelial cells. In cell lines not subjected to drug selection, Rh123 transport correlates well with P-gp activity in a variety of cell lines; however, there is concern that it may be transported by other proteins (Lee et al., 1994). The wild-type form of BCRP contains an arginine at amino acid position 482 and is not able to transport Rh123 (Ozvegy et al., 2002). Drug-selection of cell lines, however, can lead to mutations producing either a glycine or threonine at this position. These changes result in gain of function and the ability to transport Rh123 (Honjo et al., 2001). Therefore, we sought to exclude the possibility that changes in BCRP activity resulted in altered Rh123 uptake in LPS-treated SW480 cells. Sequence analysis of SW480 cells revealed that these cells were wild type at position 482 and, thus, unable to transport Rh123. The other possibility for the Rh123 data would be that MRP-2 activity was altered. No change in total MRP-2 protein levels or distribution on the plasma membrane was detected. In addition, functional assays using the fluorescent MRP-2 substrate CFDA were conducted and revealed no change with LPS treatment. Taking into consideration the ABCG2 sequence data, MRP-2 studies, and TER results, we are confident that changes in Rh123 uptake with LPS were due to diminished P-gp activity.

The exact mechanism for decreased P-gp activity in cells subjected to an inflammatory stimulus such as LPS is unclear. The ability to transport substrate would be diminished if endocytic trafficking defects of P-gp occurred, ultimately affecting localization of the protein on the plasma membrane (Kim et al., 1997; Fu et al., 2004). Efllerink and colleagues found that treatment of human liver slices with LPS virtually abolished the presence of the ABC-transporters ABCB11 (BSEP) and ABCC2 (MRP2) in the canalicular membrane (Efllerink et al., 2004). We, however, found the opposite in our system, with increased localization of P-gp on the plasma membrane of SW480 cells with total cellular levels unchanged. Reductions in P-gp activity occurred despite increased P-gp localization on the cell surface. One possibility for these findings is that LPS may have adversely affected protein folding or led to mislocalization of P-gp on the plasma membrane, leading to a reduced capacity to transport substrate. Even though there was an increase of P-gp in the plasma membrane in LPS-treated cells, defects in the way it anchored to the cytoskeleton may have led to impairment in function (Liang et al., 2003). A second potential explanation relates to the ATP-dependent nature of transport for P-gp. Substrate binding to key regions in the transmembrane domains and subsequent hydrolysis of ATP ultimately lead to drug efflux (Schinkel and Jonker, 2003). Interference with ATP-hydrolysis results in impairment of transport capacity (Batrakova et al., 2001). One could postulate that the signal transduction initiated by TLR4 activation over a 24-h period led to impairment of this key process.

A final potential mechanism for our findings may relate to inflammatory mediators as endothelin-1 (ET-1) released in response to TLR4 activation. Hartz and colleagues eloquently demonstrated in isolated rat brain capillaries that LPS-mediated activation of TLR4 led to the release of tumor necrosis factor-α, which bound to the tumor necrosis factor-R1 receptor, leading to the release of ET-1 (Hartz et al., 2006). Binding of ET-1 to the ETα receptor was believed to initiate a signal transduction cascade resulting in the activation of nitric oxide synthase and protein kinase C, ultimately leading to a loss of P-gp function. Similar to our study, no change in total P-gp protein levels was documented. Given the multitude of mediators released in response to LPS, this certainly may help to explain the decreased P-gp function in SW480 cells.

In summary, the present work indicates that the activation of TLR4 by LPS in intestinal epithelial cells induces a mobilization of P-gp to the plasma membrane. Despite an increased number of transporters, P-gp exhibited decreased transport ability. Under normal conditions, the intestinal epithelium is constantly exposed to LPS from commensal bacteria. Signaling through TLRs may represent one mechanism by which the intestinal microflora regulate the expression and function of P-gp.

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References


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