Multidrug Resistance-Associated Transporter 2 Regulates Mucosal Inflammation by Facilitating the Synthesis of Hepoxilin A3

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Multidrug Resistance-Associated Transporter 2 Regulates Mucosal Inflammation by Facilitating the Synthesis of Hepoxilin A3

Michael Pazos,* Dario Siccardi,† Karen L. Mumy,*† Jeffrey D. Bien,* Steve Louie,* Hai Ning Shi,* Karsten Gronert,‡ Randall J. Mrsny,*¶ and Beth A. McCormick2*†

Neutrophil transmigration across mucosal surfaces contributes to dysfunction of epithelial barrier properties, a characteristic underlying many mucosal inflammatory diseases. Thus, insight into the directional movement of neutrophils across epithelial barriers will provide important information relating to the mechanisms of such inflammatory disorders. The eicosanoid hepoxilin A3, an endogenous product of 12-lipoxygenase activity, is secreted from the apical surface of the epithelial barrier and establishes a chemotactic gradient to guide neutrophils from the submucosa across epithelia to the luminal site of an inflammatory stimulus, the final step in neutrophil recruitment. Currently, little is known regarding how hepoxilin A3 is secreted from the intestinal epithelium during an inflammatory insult. In this study, we reveal that hepoxilin A3 is a substrate for the apical efflux ATP-binding protein transporter multidrug resistance-associated protein 2 (MRP2). Moreover, using multiple in vitro and in vivo models, we show that induction of intestinal inflammation profoundly up-regulates apical expression of MRP2, and that interfering with hepoxilin A3 synthesis and/or inhibition of MRP2 function results in a marked reduction in inflammation and severity of disease. Lastly, examination of inflamed intestinal epithelia in human biopsies revealed up-regulation of MRP2. Thus, blocking hepoxilin A3 synthesis and/or inhibiting MRP2 may lead to the development of new therapeutic strategies for the treatment of epithelial-associated inflammatory conditions. The Journal of Immunology, 2008, 181: 8044–8052.

Transmigration of polymorphonuclear neutrophils (PMNs) across epithelial surfaces represents a shared phenomenon among a diverse array of inflammatory mucosal conditions. For example, salmonellosis, shigellosis, and pneumonia, as well as autoimmune/idiopathic states such as Crohn’s disease, ulcerative colitis, bronchitis, chronic obstructive pulmonary disease, and oral diseases such as periodontitis all culminate in the destructive breach of the protective outer epithelium by activated PMNs. Furthermore, the severity and clinical outcome of these inflammatory diseases correlate with the extent of PMN infiltration (see Refs. 8 and 12). An emerging concept over the past decade suggests an orchestrated movement of PMNs from the bloodstream to luminal sites of inflammatory stimuli. For example, in response to inflammatory stimuli within the intestinal lumen, IL-8, as well as other CXC chemokines, are secreted from the basolateral surface of enterocytes prompting PMN escape from the bloodstream and eventual accumulation within the submucosal space (1). In concert, the potent PMN chemoattractant hepoxilin A3 (HXA3) is secreted from the apical surface of the epithelial barrier where it establishes a chemotactic gradient to guide PMNs from the submucosa across the epithelium, a process constituting the final step in PMN recruitment to the mucosal lumen (2). HXA3 is a hydroxyepoxide metabolite formed from the intermediate 12S-hydroperoxyeicos-5Z,8Z,10Z,14Z-tetraenoic acid to HXA3 has been confirmed for human platelet 12-LOX, human epidermal LOX type 3, and murine and porcine 12/15-LOX (5, 6). Recently, a critical role for HXA3 in PMN-mediated events in both intestinal and pulmonary inflammation has been demonstrated (2, 7–9).

To perform its specific function of drawing PMNs into a mucosal lumen, HXA3 must be secreted from the apical surface of epithelial cells, thereby establishing a concentration gradient across epithelial tight junctions to incite paracellular PMN movement. HXA3 performs this function as a pure chemoattractant that does not induce neutrophil activation (10), leaving that event to occur in the presence of pathogens and other stimuli following arrival at the epithelial luminal surface (2). Despite the critical nature of PMN transmigration in pathogen elimination and disease pathophysiology, many of the details regarding the regulation of PMN migration across mucosal surfaces remain undefined. Herein, we describe a mechanism for the vectored secretion of HXA3 from

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the apical surface of epithelial cells involving the ATP-binding cassette (ABC) transporter multidrug resistance-associated protein 2 (MRP2). In general, the physiological role of ABC transporters covers a wide spectrum of functions varying from the transport of excretory compounds and toxins to the elimination of xenobiotics (11). Our findings reveal a previously unappreciated role of MRP2 in mediating the inflammatory response as we demonstrate that inflammatory signals up-regulated MRP2 expression in association with 12-LOX activity, a critical element in the synthesis of HXA₃. Furthermore, our studies not only provide vital insight into the contribution of ABC transporters in the pathogenesis of inflammatory disorders of the gastrointestinal tract with respect to mechanisms underlying PMN recruitment but also identify novel targets for the treatment of epithelial-associated inflammatory conditions such as inflammatory bowel disease (IBD).

Materials and Methods

Cell culture

T84 intestinal epithelial cells (passages 40–58) were grown as previously described (12). For transport experiments, T84 cells were seeded at 250,000/cm² on inverted carbonate membrane Transwell inserts (4.5-mm diameter) and cultured at 37°C, 90% relative humidity, and 5% CO₂. HCT-8 (at passages 30–40) were seeded on inverted Transwell inserts at a density of 40,000/cm² and cultured to days 7–10 postseeding for efflux experiments. The formation of restrictive monolayers (at least 800 ohm·cm²) was monitored for all cell types by measurement of transepithelial electrical resistance using an EVOM Voltmeter (World Precision Instruments).

Growth of bacteria for assays using cell culture inserts

Nonagitated microaerophilic bacterial cultures were prepared as previously described (12). Salmonella enterica serotype Typhimurium (S. typhimurium) SL1344 (wild type), EE633, and 750 M. Folowing pretreatment, the drug was removed by washing with HBSS (Cayman Chemical) as an internal standard and was brought to a pH of 3–4 (methanol:water:acetaet, 65:35:0.03, v/v/v) with a 0.35 ml/flow rate. MS/MS analyses were conducted in negative ion mode and fatty acids were identified and quantified by the multiple reaction monitoring mode using specific transitions for HXA₃ (335→27 m/z) and 12-HETE (319→179 m/z). Specific retention time for HXA₃ and 12-HETE was established using a synthetic standard (BIOMOL) and transition ions identified by MS/MS analyses using enhanced product ion mode with appropriate selection of the parent ion in quadruple 1. Calibration curves (1–1000 pg) for HXA₃, 12-HETE, and PGB₂ were established with synthetic standards.

Immunoprecipitation studies

T84- or HCT-8-polarized monolayers were apically infected with either wild-type S. typhimurium (SL1344), EE633, or the bacterial negative control commensal isolate E. coli F-18 (12). Immunoprecipitation was performed using the Seize X Mammalian Immunoprecipitation Kit (Pierce) according to the manufacturer’s instructions. The bead column was cross-linked with 70 μg of goat anti-MRP2 polyclonal or goat anti-multidrug resistance (MDR) polyclonal Abs (Santa Cruz Biotechnology). Whole cell lysates added to each column were normalized to 0.5 mg/column. Eluted proteins were electrophoresed on a nondenaturing gradient SDS-PAGE gel (8–16%) in Tris-glycine; Cambrex Biosciences). Proteins were transferred using a standard semidry transfer method onto nitrocellulose and probed with MRP2 or MDR polyclonal Abs (1:500) overnight at 4°C followed by washes and incubation with HRP-labeled donkey anti-goat Abs (1:1000; Santa Cruz Biotechnology) for 1 h. Bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Streptomycin model of acute inflammation

Based on a modification of Barthel et al. (16), female C57Bl/6 mice, 6–8 wk old (The Jackson Laboratory) were orally treated with 75 μl of streptomycin sulfate (stock 50 mg/ml; Sigma-Aldrich) in sterile distilled water following a 4-h starvation period of both food and water. After 24 h, the mice were starved for 4 h and orally gavaged with 5×10⁶ CFU of the appropriate bacterial strain in 500 μl of sterile HBSS, monitored for 48 h, and sacrificed.

Human intestinal biopsy material

The diagnosis of IBD was based on clinical, endoscopic, and histological criteria, and infectious colitis was ruled out by stool cultures. The collection of samples was approved by the Institutional Review Board at the University of Chicago. Intestinal biopsy specimens were obtained from four ulcerative colitis and four Crohn’s disease patients during routine endoscopy upon informed consent. Control colonic biopsy specimens were obtained from age- and sex-matched individuals undergoing surgery for cancer resection.

Slide preparation and hematoxylin staining

Murine intestinal sections were harvested from the proximal colon of mice at necropsy. Human tissue was obtained from inflamed or noninflamed biopsy samples of matched patient samples acquired through the Department of Pathology at the University of Chicago as a generous gift from Dr. J. R. Turner. All tissue sections were embedded in TissueTek OCT compound (Sakura Finetek USA), flash frozen in liquid nitrogen, and sectioned (5-μm thickness) on a 2800 Fricogut cryostat (Reichert-Jung). Hematoxylin and Eosin Y staining and scoring were performed as previously documented (17).

Immunohistochemistry

Murine and human colon sections were fixed in cold acetone (~20°C) for 10 min and air dried for 5 min. Blocked sections were incubated at 4°C overnight in goat anti-MRP2 polyclonal Ab (Santa Cruz Biotechnology) diluted 1/750 or 1/250 in TBS for mouse and human tissue, respectively. Sections were washed in TBS, followed by a 1-h incubation with FITC-conjugated donkey anti-goat Ab (Santa Cruz Biotechnology) diluted 1/1000 and 1/250 in TBS for mouse and human sections, respectively. Sections were mounted with M Tris-HCl (pH 8.0)/Vectashield (Vector Laboratories) mixture (1:1) and analyzed by immunofluorescent microscopy at ×20 oil magnification (Nikon Eclipse TE-2000S).

Determination of MRP2 activity in S9 cells

This assay was performed according to the manufacturer’s instructions using membrane product SB-MRP2-S9-ATPase (Solvo Biotechnology).
ATPase activity of the substrates probenecid (1 mM; BIOMOL) and HXA₃ (1, 10, 100 µM; BIOMOL) was measured with respect to baseline activity.

MRP2 functional studies

Cells were treated with 10 µM 5-chlormethylfluorescein diacetate (CMFDA; Molecular Probes) for 1 h to allow uptake and intracellular conversion to the fluorescent MRP substrate 5-chloromethylfluorescein (CMF) (18). CMF, actively transported by MRPs as the glutathione conjugate glutathione-methylfluorescein (GS-MF), was detected by measuring the appearance of fluorescence in the bathing solutions (excitation 495 nm and emission 535 nm; LS-5 spectrofluorometer; PerkinElmer Life and Analytical Sciences).

Permeability studies were conducted with T84 monolayers incubated in HBSS and used [³H]mannitol (0.088 µM, 0.147 MBq) as a control probe for low permeability paracellular transport. Radioactivity was determined by liquid scintillation counting, and coefficients of permeability for probe molecules were similar to those found in the literature for viable monolayers (19). Transport experiments in the apical to basal (A→B) direction were initiated by adding 200 µl of the drug solution (CMFDA) to the donor chamber. Transport experiments in the basal to apical (B→A) direction were initiated by adding 1 ml of CMFDA to the basal chamber. At predetermined times over the course of the experiment, 100-µl samples were taken from the respective chamber and replaced with fresh HBSS. Transport rates were conducted in the presence of the MRP inhibitor MK571 (100 µM with 0.2% DMSO) were conducted as above. The cumulative mass of the drug transported from donor to receiver chambers was assessed over the time course of the experiment (dM/dt) using linear regression analysis. The following equation was used to fit the data: dM/dt = pA/Co, where p is the permeability coefficient (cm/s), A is the surface area of the Transwell membrane (0.333 cm²), and Co the initial drug concentration in the donor chamber (µmol/cm²). In all experiments, the amount of drug transported from donor to receiver was ≤5% of the donor drug mass, ensuring the validity of Co as a constant in the equation.

Generation of small interfering RNA (siRNA) for suppression of MRP2 and P-gp expression

Plasmids used to generate siRNAs were constructed with the pSUPER vector (Oligogene) using the method described by Brummelkamp et al. (20). Briefly, two oligonucleotides were designed incorporating a 19-nt sequence (Oligoengine) using the method described by Brummelkamp et al. (20). For 12/15-LOX inhibition, T84 cell monolayers were incubated in the presence of baicalein (stock concentration at 1 mM in DMSO) in medium for 2 h at 37°C. Subsequently, S. typhimurium SL1344 was added to the apical surface of the T84 monolayers for 1 h at 37°C and the cells were then washed free of nonadherent bacteria and processed for the PMN transmigration assay. For 5-LOX inhibition, the T84 cells were incubated for 24 h in the presence of caffeic acid (stock concentration at 22 mM in DMSO). Both inhibitors were purchased from BIOMOL.

Statistical analysis

PMN isolation was limited to repetitive donations by 10 different donors over the course of these experiments. Due to variation in both PMNs and transepithelial resistance between monolayers (baseline resistance of ~1500 ohm·cm²), data were analyzed within an individual experiment and not between experiments. All results are expressed as the mean ± SD of an individual experiment performed in triplicate. Values of p values were calculated according to Student’s t test and values <0.05 were considered statistically significant.

Results

Blockade of MRP2 function inhibits PMN transepithelial migration

Elucidation of mechanisms governing HXA₃ synthesis and apical secretion could provide novel therapeutic strategies to impede and/or limit neutrophil involvement in deleterious events at epithelia associated with a number of chronic inflammatory conditions. HXA₃ must be secreted from the apical surface of epithelial cells to establish a chemoattractant gradient across tight junction complexes to incite the paracellular movement of PMNs (2). Given the structural characteristics of HXA₃ and its strict apical secretion requirement, we considered the involvement of ABC transporters as potential substrates for HXA₃. P-gp and MRP2 were promising candidates due to their localization on apical membranes of enterocytes (25–27). In vitro treatment of polarized human intestinal epithelial cell monolayers (T84) with inhibitors of either P-gp (cyclosporin A and verapamil) or MRP2 (probenecid and MK571) demonstrated a progressive, dose-dependent decrease in the ability of pathogenic S. enterica Typhimurium (S. typhimurium) to induce HXA₃-mediated PMN transepithelial migration (Fig. 1). Moreover, at the maximal doses tested, none of these drugs had an effect on PMN migration induced by imposed gradients of fMLP nor did they affect S. typhimurium internalization by T84 cell monolayers (data not shown). These results suggest that inhibitors of ABC transporters known to localize to the apical surface of intestinal epithelial cells block the recruitment of PMNs across epithelial cell monolayers induced by an inflammatory stimulus driven by HXA₃; in this case, apical infection with the enteric pathogenic S. typhimurium.

ABC transporter inhibitors are known to be poorly selective (28). To better define the relative roles played by P-gp and MRP2 in PMN transmigration, polarized epithelial cell monolayers were transfected with plasmids generating siRNA for one of each of these two transporters. Decreased expression of MRP2 significantly attenuated (>80% inhibition) S. typhimurium-induced transepithelial PMN migration while monolayers generating siRNA specific for MDR1 (encoding P-gp) showed PMN transmigration in response to S. typhimurium infection equivalent to control monolayers (Fig. 2a). Importantly, neither siRNA indirectly affected the ability of PMN to transmigrate as PMN migration in response to an imposed fMLP gradient was similar under all conditions (Fig. 2a). Modulation of MRP2 and P-gp expression by siRNA was verified by immunoblot analysis (Fig. 2b). Densitometric analysis showed a 75% decrease in MRP2 with siRNA in S. typhimurium-infected cells and a complete loss for P-gp (data not shown). Inhibition of transepithelial PMN migration induced by S. typhimurium was consistent with the extent of protein reduction for MRP2 but not for P-gp (Fig. 2, a and b), suggesting that MRP2, and not P-gp, is involved in PMN transepithelial migration.
During inflammatory events MRP2 is functionally active and is a substrate for HXA3

In polarized epithelial cells, the majority of MRP transporters localize to the basolateral membrane (18, 27). To examine functional MRP2 transporter expression related to inflammatory events, directional efflux of the MRP substrate CMFDA from polarized epithelial cell monolayers was studied (Fig. 2c). CMFDA passively diffuses into cells where upon hydrolysis of the acetate moieties by cytosolic esterases it becomes the fluorescent MRP substrate CMF; CMF is actively transported by MRPs as the glutathione conjugate

FIGURE 2. Demonstration of the functional importance of the apical ABC transporter MRP2 during PMN transepithelial migration and as a substrate for HXA3. a, Polarized intestinal cell monolayers silenced for the expression of MRP2 or P-gp (MDR1) were assessed for the ability to promote S. typhimurium-induced PMN transepithelial migration. Data are represented as mean ± SD of triplicate samples and are representative of three experiments.* p < 0.01. b, Immunoblot analysis of the modulation in protein expression of MRP2 and P-gp (MDR1) based on siRNA as described in Materials and Method. Results are representative of three independent experiments. c, MRP2 functional activity was assessed by measuring GS-MF efflux from polarized monolayers of T84 infected in the absence and presence of S. typhimurium. T84 cell monolayers were infected apically for 1 h with either SL1344 (wild-type S. typhimurium) or VV341 (an isogenic derivative of SL1344 which lacks the hilA gene and is invasion deficient). Control represents baseline transport in the presence of physiological buffer (HBSS). A→B represents transport in the apical to basolateral direction; B→A represents transport in the basolateral to apical direction; MK571 is an MRP2 inhibitor (100 μM containing 2% DMSO). Data are expressed as mean ± SD of triplicate determinations representative of at least three independent experiments; * p < 0.05.
GS-MF. In the baseline state, T84 cell monolayers have a ∼25-fold higher permeability coefficient in the apical to basolateral (A→B) direction relative to the B→A direction (20.3 ± 1.04, and 0.8 ± 0.03 × 10^{-6} cm/s, respectively). This relationship favors an overall basolateral secretion, or A→B transport, of GS-MF. Interestingly, T84 monolayers infected at their apical surface with wild-type *S. typhimurium* decreased the A→B:B→A permeability coefficient ratio to 1.8, with an 89% reduction of probe movement in the A→B direction, suggesting activation of an apical efflux pathway. Addition of the MR2 inhibitor MK571 to T84 monolayers infected with wild-type *S. typhimurium* restored the A→B transport of GS-MF to 43% compared with controls with no effect on transport in the opposite direction. As a negative control, non-pathogenic *S. typhimurium* (VV341) only modestly reduced GS-MF transport (15%; p < 0.05). In contrast, P-gp functional activity was not increased during *S. typhimurium* infection (21).

The above results indicate that inflammatory events can enhance apical MR2-specific efflux activity. We next sought to determine whether HXA3 serves as a substrate for MR2. Interestingly, MR2 exhibits preference for negatively charged, hydrophobic molecules such as the eicosanoid cysteinyl leukotriene (29–32). HXA3 shares these characteristics at neutral pH, suggesting it fits the profile of an MR2 substrate. Since ABC transporters hydrolyze ATP during active substrate transport, the ATPase activity of purified inside-out membrane vesicles isolated from *Spodoptera frugiperda* (Sf9) cells expressing the MR2 transporter were examined following HXA3 addition. MR2-containing membrane vesicles exhibited a dose-dependent increase in ATPase activity in the presence of HXA3 (Fig. 2d). Addition of the MR2 substrate inhibitor probenecid was used as a positive control to depict maximal ATPase activity. Such data provide direct evidence that HXA3 can be a substrate for MR2.

Expression of MR2 is up-regulated in acute and chronic models of intestinal inflammation

Previous studies have shown that the *S. typhimurium* effector protein SipA is required for the ability of *S. typhimurium* to induce PMN transepithelial migration (13, 16, 33). We examined the role of virulence factors, such as SipA, to incite functional expression of MR2 by comparing MR2 expression levels following infection with either wild-type *S. typhimurium* (SL1344) or the isogenic SipA mutant strain (EE633). Our results show that *S. typhimurium* stimulated MR2 expression in a SipA-dependent manner (Fig. 3, a and b); P-gp levels were not affected by *S. typhimurium* infection during a 1-h infection (Fig. 3a). To control for specificity of SipA, we performed this assay using a commensal *E. coli* strain lacking SipA (F18) as well as an isogenic strain of *S. typhimurium* mutated for an alternative effector protein, AvrA, that retains SipA expression. As anticipated, the AvrA mutant induces MR2 expression (Fig. 3b), whereas *E. coli* F18 fails to do so. Moreover, complementing with a *sipa*-expressing plasmid (AJK63) restores the ability of the SipA mutant (EE633) to induce MR2 protein expression (Fig. 3b). Thus, under these conditions *S. typhimurium* infection of T84 cell monolayers modulates MR2 expression with no effect on P-gp expression.

Using SipA as a prototypical virulence element, we could also show its involvement in the release of HXA3, since wild-type *S. typhimurium* elicited significantly more HXA3 release from T84 cell monolayers compared with the SipA mutant (EE633), and this was correlated, respectively, with the ability to facilitate PMN migration (*Materials and Methods: Method 1*) (Fig. 3c). These results suggest that induction of intestinal inflammatory events, in this case SipA-mediated induction of HXA3 release, stimulates MR2 expression to provide a potential apical secretion mechanism for HXA3. Furthermore, since 12-HETE is a biomarker for the HXA3

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**FIGURE 3.** MR2 is up-regulated by an inflammatory pathway activated by *S. typhimurium* that involves a SipA-dependent-mechanism. a, T84 cells were infected apically with SL1344 for 1 h, then lysed, and enriched for the Triton X-100-insoluble membrane fraction as described in *Materials and Methods*. Incubation with the physiological buffer HBSS served as the baseline control for MR2 expression. Twenty-five micrograms of protein was separated on a 10% PAGE gel and immunoblotted for MR2. b, To determine specificity of the response (i) T84 cell monolayers were apically infected with SL1344, the isogenic SipA mutant strain (EE633), and a normal intestinal human flora *E. coli* isolate, *E. coli* F-18, or left uninfected in the presence of HBSS (−) control). (ii) T84 cells were infected apically for 1 h in the presence of either SL1344 or its isogenic AvrA (*S. typhimurium* effector protein) mutant strain. −. The baseline buffer control. (iii) T84 cell monolayers were apically infected for 1 h in the presence of SL1344, EE633, or the sipa-complemented strain AJK63. This strain is derived from EE633 containing the pAK68C plasmid that encodes sipA. −. The baseline buffer control. For each immunoblot, after bacterial infection the cells were lysed and then enriched for the Triton X-100-insoluble membrane fraction. Twenty-five micrograms of protein was separated on a 10% PAGE gel and immunoblotted for MR2. The data represent a single experiment and are repetitive of at least three experiments performed. c, SipA affects the release of HXA3 from model intestinal epithelia. Monolayers of T84 epithelial cells were infected for 1 h with the SL1344 or EE633 *S. typhimurium* strain. PMN transepithelial cell migration (left) and HXA3 secretion (right) were measured. HXA3 was identified according to *Method 1* as described in *Methods and Materials*. Data are presented as the mean ± SD of assays performed in triplicate and are representative of three independent experiments; *p < 0.01.
biosynthetic pathway and is easily detected by LC/MS/MS, we next measured the amount of 12-HETE released from intestinal epithelial cells displaying reduced levels of MRP2 (via siRNA) during an infection with *S. typhimurium*. We found that the inhibition of 12-HETE from infected epithelial cells with reduced levels of MRP2 was at least 20% (*n* = 2) compared with infected siRNA vector control monolayers. Similarly, 12-HETE release by *S. typhimurium*-infected was also profoundly inhibited by pharmacological treatment with a MRP2 inhibitor (MK571 100 μM). To further substantiate a link between MRP2 and the inhibition of 12-HETE from infected epithelial cells with reduced levels of MRP2 was at least 20% (*n* = 2), we then therapeutically treated with baicalein (*b*–*f*).

Next we determined whether MRP2 expression is modulated during an infection with *S. typhimurium* in vivo (*g*–*c*). Mice infected with wild-type *S. typhimurium* exhibited severe intestinal pathology characterized by a predominant PMN infiltrate and extensive tissue damage, whereas mice infected with the sipA mutant demonstrated only a slight level of intestinal inflammation (Fig. 4, *a*–*c*). Apical localization of the ABC transporter MRP2 was uniformly increased at the villus tips during the acute phase of intestinal inflammation (Fig. 4, *d*–*f*), confirming MRP2 up-regulation by pathogenic *S. typhimurium*. In contrast, P-gp was not up-regulated (data not shown and Ref. 21). Collectively, these data are consistent with our in vitro findings suggesting MRP2 may play a role in acute salmonellosis. Although chronic inflammatory diseases have been correlated with excessive PMN recruitment and destruction of protective epithelial barriers, many aspects of events that initiate and perpetuate PMN transmigration are poorly understood. However, using a mouse leukocyte-type 12/15-LOX Ab, an earlier study by Shannon et al. (34) recognized a fundamental change in the pattern of 12/15-LOX expression in colonic tissue during the onset of IBD; inflamed colonic tissue expressed increased levels of 12-LOX with a corresponding increase in 12-LOX activity relative to healthy colonic tissue. However, there are four enzymes known to possess significant 12-LOX activity in humans: platelet-type 12-LOX (ALOX12), 12/15-LOX (ALOX12B), epidermis-type LOX (ALOXE3), and 15-LOX type 1 (ALOX15) (35–38). 15-LOX type 1 is the human homolog of murine leukocyte-type 12/15-LOX (39), and we have previously shown that PMN transepithelial migration in response to infection with *S. typhimurium* is dependent on 12/15-LOX activity (2, 9). Formation of 12-HpETE by these LOXs is the first step for the formation of HXA3 (40).

To delineate the in vivo relevance of the above findings during chronic states of inflammation, we sought to determine whether 12-LOX inhibition blocks inflammatory outcomes associated with an IBD model induced by transfer of CD4+CD45RB<sup>high</sup> T cell adoptive transfer model. Following disease induction, inhibition of the 12/15-LOX activity (2, 9). Formation of 12-HpETE by these LOXs is the first step for the formation of HXA3 (40).
We next posed the question of whether the 12/15-LOX activity and MRP2 expression might be coordinated as a regulated element of response to an inflammatory signal. In the presence of the 12/15-LOX inhibitor baicalein, *S. typhimurium*-infected monolayers in vitro showed significantly lower levels of MRP2 than those induced by this pathogen in the absence of the inhibitor (Fig. 5a). Similar treatment with caffeic acid, a 5-LOX inhibitor, failed to affect enhanced MRP2 expression induced by *S. typhimurium* infection (data not shown). Examination of intestinal tissues from CD45RB<sup>high</sup> T cell transfer mice revealed that therapeutic intervention with baicalein reduced apical membrane MRP2 expression (Fig. 5b), suggesting a link between the regulation of 12/15-LOX activity and MRP2 expression during both acute and chronic inflammatory events. Taken together, these results imply that the 12/15-LOX pathway is required for maximal induction of MRP2 expression associated with epithelial inflammation.

To determine the in vivo relevancy of the above findings with respect to nonpathogen-induced human disease, we assessed whether MRP2 is up-regulated in patients with IBD. Indeed, as shown in Fig. 5c, we provide direct evidence demonstrating that intestinal biopsy specimens obtained from IBD patients with either active Crohn’s disease or ulcerative colitis exhibit marked MRP2 up-regulation at the intestinal epithelial apical surface compared with healthy intestinal segments. One hundred percent of the ulcerative colitis patients (four of four) and 50% of the Crohn’s disease patients (two of four) examined in this study demonstrated an up-regulation of MRP2. None of the age- and sex-matched control biopsies showed any increase above baseline levels (Fig. 5c).

**Discussion**

Given that migration of PMNs across mucosal surfaces contributes to epithelial cell dysfunction in a host of mucosal diseases (2, 7, 8, 12), the data provided here not only identify a new mechanism underlying the vectored release of HXA<sub>3</sub> but also reveal interference with HXA<sub>3</sub> synthesis or apical secretion from epithelial cells as novel therapeutic strategies for the treatment of mucosal inflammatory disorders such as IBD. We used *S. typhimurium* as a tool to induce an acute intestinal inflammatory response and found that during active states of intestinal inflammation apical expression of the efflux transporter MRP2 is profoundly up-regulated, functionally active, and capable of using HXA<sub>3</sub> as a substrate. The molecular basis of this observation is consistent with the role of the *S. typhimurium* type III-secreted product SipA, as this effector protein has been found to be both necessary and sufficient for induction of PMN transepithelial migration across model intestinal epithelia (13). In keeping with its role as a virulence factor, our data now reveal that SipA mediates PMN transepithelial migration by directly affecting the release of HXA<sub>3</sub> via modulation of MRP2 expression. Thus, identification of the molecular mechanism by which SipA activates PMN transepithelial migration through MRP2 defines a novel proinflammatory signaling cascade essential for the pathogenesis of *S. typhimurium* that likely represents a paradigm of mucosal pathogen-elicited events.

Insights into pathogen-elicited active inflammation of the intestine may provide important information relating to mechanisms of disorders underlying IBD, which appear to be unrelated to pathogen colonization (43). Therefore, we hypothesize that although evolution of such responses most likely targeted pathogens such as *S. typhimurium*, aberrant activation of such pathways may lead to induction of mucosal inflammation associated with some chronic diseases of the intestine and airway. Consistent with this notion, we found a profound up-regulation of MRP2 at the apical surface of the colonic epithelium in murine models of chronic intestinal

**Table I. Quantification of intestinal inflammation**

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<th>Cellular Infiltration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tissue Damage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total Inflammation Score&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Normal control</td>
<td>0.25 ± 0.11</td>
<td>0.18 ± 0.05</td>
<td>0.43 ± 0.17</td>
</tr>
<tr>
<td>Untreated</td>
<td>3 ± 0.81</td>
<td>2.75 ± 0.85</td>
<td>5.75 ± 1.50</td>
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<tr>
<td>12-LOX inhibitor</td>
<td>1 ± 0.10</td>
<td>1.5 ± 0.70</td>
<td>2.5 ± 0.70</td>
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</tbody>
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<sup>a</sup> The combined score represents the total inflammatory score (maximum = 8). Five animals were used per experimental treatment condition and the experiment was performed three times.

**FIGURE 5.** 12/15-LOX activity and the expression of MRP2 are linked events in response to active states of intestinal inflammation. *a.* Immunoblot analysis of MRP2 expression during apical infection of SL1344 in the absence and presence of the 12-LOX inhibitor baicalein, as described in Materials and Methods. For each sample, 25 µg of protein was isolated from the membrane fraction and run on a 10% PAGE gel and immunoblotted for MRP2. The data represent a single experiment and are repetitive of at least three experiments performed. *b.* Immunohistochemistry depicting the localization of MRP2 to the apical epithelial surface of the proximal colon in mice treated in the absence and presence of 12-LOX inhibition using the CD4<sup>+</sup>CD45RB<sup>high</sup> T cell adoptive transfer model of IBD: *i* represents the healthy control that did not undergo the adoptive transfer of the CD4<sup>+</sup>CD45RB<sup>high</sup> T cells; *ii* represents mice induced for IBD; and *iii* represents mice induced for IBD and then therapeutically treated with baicalein; original magnification, ×10. *c.* Human intestinal biopsy specimens stained for MRP2: *i* , healthy colonic section; *ii* , colonic section from a patient with active Crohn’s disease; and *iii* , colonic section from a patient with active ulcerative colitis; original magnification, ×20. Data represent a single experiment that is representative of eight patient colonic biopsy specimens examined (four each for Crohn’s and ulcerative colitis patients).
inflammation as well as in intestinal biopsies from patients pre-
senting with active Crohn’s disease and ulcerative colitis. Under
normal healthy conditions, the most abundant constitutive expres-
sion of human and rat MRP2/MPR2 mRNA is found in the renal
proximal tubule brush border membrane and the hepatocyte can-
ciliar membrane (44). Significantly lower levels of MRP2 have
been found in the small intestine, with exclusive localization at the
apical brush border membrane of villi (44). Furthermore, MRP2
expression decreases in intensity from the villus tip to the crypts,
where no expression has been observed (44). At this location,
MRP2 is thought to play an important role in drug disposition. We
now describe an unanticipated function of MRP2 as it is uniformly
up-regulated at the villus tips of the apical surface of epithelial
cells during active states of inflammation and plays a pivotal role
in the inflammatory response.

Secreted HXA₃ then establishes a gradient through the tight
junction complex to provide a chemotactic gradient used by PMNs
to target the lumen of mucosal tissues at sites of inflammation (2).
The observation that HXA₃ can serve as a substrate for the apical
efflux transporter MRP2 fits well with the appreciation that other
eicosanoids such as the cysteinyl leukotriene C₄, a metabolite of the
5-LOX pathway, is one of the highest affinity MRP2 substrates
characterized to date (29–32). Detailed studies, however, are re-
quired to fully characterize and provide direct evidence for MRP2
transport of hepoxilin.

There are four enzymes that possess 12-LOX activity in humans
and the most abundant LOX expressed in epithelial cells is 12/15-
LOX. In humans, unlike mice, 12HpETE is not the major product of
However, 12/15-LOX is highly expressed in epithelial cells,
including intestinal epithelial cells (45, 46), and the synthesis of
12-HpETE through this pathway is likely significant. At present it
is unclear which 12-LOX enzymes specifically contribute to HXA₃
production in the human mucosa and thus the potential contribu-
tion of ALOX12, ALOX12B, or ALOX3 cannot be excluded.
Our studies, however, determined that pharmacological inhibition
of 12/15-LOX activity (both in vitro and in vivo) was found to not
only block HXA₃ synthesis but also the enhanced apical expres-
sion of MRP2 typically observed as part of the inflammatory
process.

Our observation that 12/15-LOX activity is coupled to MRP2
up-regulation and apical localization implies that either a metab-
olite of the 12/15-LOX pathway or an active form of the enzyme
itself participates in MRP2-related cellular changes associated
with inflammation. However, it is unclear at this point in our in-
vestigation at what level or through what mechanism this associa-
tion is achieved. Nevertheless, previous studies have demonstrat-
ed that cyclooxygenase 2 expression enhances the functional
activity of P-gp, providing a precedent of a causal link between
arachidonic acid metabolism and expression of an ABC transporter
(47). Furthermore, given that epithelial cells appear to regulate
12/15-LOX and MRP2 expression in a coupled fashion, several
novel therapeutic strategies are potentially available to treat in-
flammatory conditions, such as IBD, based upon regulation of 12/
15-LOX and MRP2. Underscoring such important clinical implica-
tions, we show that treatment with baicalein, a 12-LOX
inhibitor, which inhibits HXA₃ synthesis as well as the enhanced
apical expression of MRP2, resolved PMN transmigration/intesti-
nal inflammation in a murine model of IBD.

Although our studies provide the first description that MRP2 is
involved in the mechanisms that promote active states of intestinal
inflammation (i.e., PMN recruitment), other ABC transporters,
namely, P-gp (MDR1), have been found to play a role in the de-
nvelopment of colitis (48), albeit by a completely different mecha-
nism than MRP2. Consistent with this observation, we have re-
cently determined that prolonged apical colonization of intestinal
epithelial cells by wild-type S. typhimurium (~4 h) leads not only
to a profound functional decrease in P-gp but that the presence of
P-gp adversely influences the ability of S. typhimurium to invade
host cells (21). These results demonstrate that MRP2 and P-gp can
be differentially regulated within the same tissue during a disease
process. This observation supports the finding that during the pro-
gression of chronic renal failure MRP2 is up-regulated in both the
kidney and liver, whereas P-gp remains unaffected (49).

In summary, we are beginning to understand how the PMN che-
moattractant HXA₃ is secreted in a vectorial fashion through a
unique efflux transport system located at the apical surface of the
intestinal epithelium that involves the ABC transporter MRP2.
This study demonstrates a critical link between apically expressed
MRP2 and the HXA₃ biosynthetic pathway. Surprisingly, inhibi-
tion of an enzyme critical for the synthesis of HXA₃, 12/15-LOX, was
found to also suppress the apical expression of MRP2 induced by
inflammatory signals. This unanticipated coupling of 12/15-
LOX and MRP2 identified in an in vitro acute inflammatory model
initiated by a pathogen was recapitulated in an in vivo mouse model
of IBD and was consistent with observations made in bi-
opsy samples obtained from IBD patients. Since HXA₃ has also
been shown to be involved in pathogen-induced inflammation of
both intestinal (2, 9) and pulmonary (7) epithelial cells, it is likely
that our results describe a generalized mechanism governing
HXA₃ synthesis and its subsequent apical release that could pro-
vide novel therapeutic strategies to impede and/or limit PMN in-
volvement in deleterious events associated with a number of acute
and chronic inflammatory conditions.

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Disclosures

One of the authors (R.J.M.) is affiliated with a company focusing on the
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