Pregnane X Receptor Activation Attenuates Inflammation-Associated Intestinal Epithelial Barrier Dysfunction by Inhibiting Cytokine-Induced Myosin Light-Chain Kinase Expression and c-Jun N-Terminal Kinase 1/2 Activation

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ABSTRACT

The inflammatory bowel diseases (IBDs) are chronic inflammatory disorders with a complex etiology. IBD is thought to arise in genetically susceptible individuals in the context of aberrant interactions with the intestinal microbiota and other environmental risk factors. Recently, the pregnane X receptor (PXR) was identified as a sensor for microbial metabolites, whose activation can regulate the intestinal epithelial barrier. Mutations in PXR deletion leads to barrier dysfunction. In the current study, we sought to assess the mechanism(s) through which the PXR regulates barrier function during inflammation. In Caco-2 intestinal epithelial cell monolayers, tumor necrosis factor-α/interferon-γ exposure disrupted the barrier and triggered zonula occludens-1 relocalization, increased expression of myosin light-chain kinase (MLCK), and activation of c-Jun N-terminal kinase 1/2 (JNK1/2). Activation of the PXR [rifaximin and [(3,5-Bis(1,1-dimethyllethyl)-4-hydroxyphenyl]ethenylidene]bis-phosphonic acid tetraethyl ester (SR12813); 10 μM] protected the barrier, an effect that was associated with attenuated MLCK expression and JNK1/2 activation. In vivo, activation of the PXR [pregnenolone 16α-carbonitrile (PCN)] attenuated barrier disruption induced by toll-like receptor 4 activation in wild-type, but not Pxr−/−, mice. Furthermore, PCN treatment protected the barrier in the dextran-sulfate sodium model of experimental colitis, an effect that was associated with reduced expression of mucosal MLCK and phosphorylated JNK1/2. Together, our data suggest that the PXR regulates the intestinal epithelial barrier during inflammation by modulating cytokine-induced MLCK expression and JNK1/2 activation. Thus, targeting the PXR may prove beneficial for the treatment of inflammation-associated barrier disruption in the context of IBD.

Introduction

Although the etiology of the inflammatory bowel diseases (IBDs) has yet to be completely elucidated, the current paradigm suggests that these diseases are triggered by the integration of multiple factors, including specific genetic variants that govern host processes, the environment, the microbiota, and dysregulation of immune responses (Xavier and Podolsky, 2007). The intestinal mucosa, particularly the epithelium, provides a physical barrier that prevents noxious compounds and the microbiota from entering the internal tissues and inappropriately activating the mucosal immune system (Turner, 2009; Iacucci and Ghosh, 2011). Although increased permeability is observed in patients with IBD, it is well established that inflammatory processes can drive barrier dysfunction, thus it is difficult to establish causation (Teshima et al., 2012). However, data from a variety of studies suggest that compromised intestinal barrier function may

Abbreviations: ATCC, American Type Culture Collection; DSS, dextran sulfate sodium; FBS, fetal bovine serum; FITC-dextran, fluorescein isothiocyanate-dextran; GADD45γ, growth arrest and DNA damage-inducible 45γ; IBD, inflammatory bowel diseases; IEC, intestinal epithelial cell; IFNγ, interferon-γ; JNK1/2, c-Jun N-terminal kinase 1/2; KDO2, d[3-deoxy-α-manno-octulosonyl]-lipid A; LPS, lipopolysaccharide; MLCK, myosin light-chain kinase; MPO, myeloperoxidase; NFκB, nuclear factor κ-light-chain-enhancer of activated B cells; PBS, phosphate-buffered saline; PCN, pregnenolone 16α-carbonitrile; PXR, pregnane X receptor; SEAP, secreted embryonic alkaline phosphatase; SP600125, anthracyl-9-cyano-pyrazol-6(2H)-one; SR12813, [(3,5-Bis(1,1-dimethyllethyl)-4-hydroxyphenyl]ethenylidene]bis-phosphonic acid tetraethyl ester; TLR4, toll-like receptor 4; TNFa, tumor necrosis factor-α; UC, ulcerative colitis; WT, wild type; XREM, XXX; ZO-1, zonula occludens-1.
contribute to the pathogenesis of IBD (Hollander et al., 1986; Hollander, 1988; Katz et al., 1989; Clayburgh et al., 2004). Although insufficient to cause disease on its own (Cunningham and Turner, 2012), barrier dysfunction is associated with relapse and recurrence in IBD patients (D’Inca et al., 1999; Tibble et al., 2000). Thus, therapies designed to re-establish or preserve barrier function, through the direct modulation of intestinal epithelial cell (IEC) function or damping of mucosal immune responses, would prove beneficial in the treatment of IBD.

We, and others, have reported that the pregnane X receptor (PXR), a ligand-activated nuclear receptor that senses and responds to a variety of chemical stimuli, including intestinal microbial metabolites (Venkatesh et al., 2014), plays a key role in the intestinal epithelium, regulating inflammatory responses (Ma et al., 2007; Mencarelli et al., 2010, 2011), enhancing wound healing (Terc et al., 2014), and maintaining barrier function under basal (Venkatesh et al., 2014) and inflammatory conditions (Terc et al., 2014). Interestingly, genetic studies have identified single-nucleotide polymorphisms in NR1I2, the gene that encodes the PXR, which are associated with increased susceptibility for developing Crohn’s disease and ulcerative colitis (Langmann et al., 2004; Dring et al., 2006; Martinez et al., 2007; Glas et al., 2011). Furthermore, others have reported that selective activation of the PXR attenuates nuclear factor κ-light-chain-enhancer of activated B cells (NFκB)-dependent inflammatory signaling in the intestinal epithelium (Shah et al., 2007; Mencarelli et al., 2010, 2011), an effect associated with protection in experimental models of colitis (Ma et al., 2007; Shah et al., 2007; Dou et al., 2012, 2013; Terc et al., 2014). In the current study, we sought to assess whether stimulation of the PXR in IECs regulates the intracellular signaling pathways that modulate barrier function in the context of inflammation. We hypothesized that the PXR protects the epithelial barrier through its purported ability to regulate NFκB and c-Jun N-terminal kinase 1/2 (JNK1/2) signaling, key pathways that regulate tight-junction function in the context of cellular stress and inflammation.

Herein, we report that stimulation of the PXR in Caco-2 IECs prevented tumor necrosis factor-α (TNFα)/Interferon-γ (IFNγ)-induced barrier disruption, an effect that was associated with a preservation of tight junctions. PXR stimulation attenuated NFκB signaling and reduced TNFα/IFNγ-induced expression of myosin light-chain kinase (MLCK) and activation of JNK1/2. In vivo, using two distinct mouse models, we found that activation of the PXR blocked the barrier disruption evoked by toll-like receptor 4 (TLR4) activation and during dextran sulfate sodium (DSS)-induced experimental colitis, an effect that was associated with an attenuation of the expression of MLCK and activation of JNK1/2 in the intestinal mucosa.

Materials and Methods

Reagents. The selective human PXR agonists rifaximin (Ma et al., 2007) and [13,5,8-tris(1,1-dimethylallyl)-4-hydroxyphenyl-ethenylidene]bis-phosphonic acid tetraethyl ester (SR2813) (Watkins et al., 2001) (Sigma-Aldrich, St. Louis, MO) were dissolved in sterile dimethylsulfoxide (DMSO) and added to culture media to reach the appropriate experimental concentration (10 μM) (Ma et al., 2007; Lau et al., 2012; Sharma et al., 2013; Terc et al., 2014). The concentration of SR2813 (10 μM) is a maximal PXR-activating concentration (Jones et al., 2000), whereas the concentration of rifaximin (10 μM) is an effective concentration in activating the PXR (Ma et al., 2007); for comparative purposes, equimolar concentrations were used throughout our studies. Rifaximin was chosen based on its potential clinical efficacy in IBD (Prantera et al., 2006, 2012; Isaacs et al., 2007), whereas SR2813 was chosen due to its extensive characterization as a selective human PXR agonist (Jones et al., 2000; Watkins et al., 2001). Matching volumes of sterile DMSO were added to culture media for experimental vehicle controls. Hexahydro-1-[(5-iodo-1-naphthalenyl)sulfonyl]-1H,1,4-diazepine hydrochloride (ML-7, to inhibit MLCK; Sigma-Aldrich) and anethra[1,9-cd]pyrazol-6(2H)-one (SP600125, to inhibit JNK1/2; Sigma-Aldrich) were dissolved in sterile DMSO. Volumes of stock solutions were added to culture media to reach the appropriate experimental concentration (1 μM). Inhibitors were added 1 hour prior to the addition of TNFα/IFNγ (TNFα, 10 ng/ml; IFNγ, 20 ng/ml; R&D Solutions, Minneapolis, MN). TNFα and IFNγ concentrations and exposure time (16 hours) were based on values/ranges reported previously (Zolotarevsky et al., 2002; Bruewer et al., 2003; Wang et al., 2005, 2006). Ultra-pure lipopolysaccharide (LPS; InvivoGen, San Diego, CA) was dissolved in sterile LPS-free water and added to culture media to achieve the appropriate experimental concentration (100 ng/ml).

To activate the mouse PXR in our in vivo experiments, we used the rodent selective PXR agonist pregnenolone 16a-carbonitride (PCN; Sigma-Aldrich), which was dissolved in sterile corn oil. Each mouse was administered PCN via i.p. injection at a dose of 25 mg/kg, as we have published previously (Terc et al., 2014). Control mice were administered matching volumes of sterile corn oil via i.p. injection.

Cell Culture. Caco-2 cells (adult human colonic epithelial cell line derived from a colorectal adenocarcinoma; American Type Culture Collection (ATCC), Manassas, VA) were cultured in Dulbecco’s modified Eagles medium supplemented with 20% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (100 μg/ml; Invitrogen, Carlsbad, CA). All experiments were performed on cell passages 15–25.

THP-1-Blue NFκB cells which express an NFκB-directed secreted embryonic alkaline phosphatase (SEAP) reporter gene (InvivoGen) were propagated in RPMI 1640 media supplemented with 10% FBS, 1% penicillin-streptomycin, 1% sodium pyruvate, and β-mercaptoethanol (to a final concentration of 0.05 mM). Prior to exposure to LPS, THP-1-Blue NFκB cells were plated at a density of 100,000 cells/well (96-well plate) and differentiated into macrophages by exposing them to phorbol-12-myristate-13-acetate (50 ng/ml; 24 hours). All experiments were performed in OptiMEM media (Invitrogen) supplemented with 5% FBS.

HepG2 (human hepatocellular carcinoma cells; ATCC) and LS180 cells (human colon adenocarcinoma cells; ATCC) were cultured in minimum essential medium supplemented with 2 mM l-glutamine, 1% penicillin-streptomycin, and 10% FBS, as described previously (Lau and Chang, 2015).

Fluorescein Isothiocyanate-Dextran Flux Barrier Studies. To assess barrier function in vitro, fluorescein isothiocyanate-dextran (FITC-dextran) flux permeability assays were performed as previously described (Hansen et al., 2013; Schenck et al., 2013). In brief, Caco-2 IECs were plated on Transwell permeable inserts (Corning Inc., Corning, NY) and given fresh medium every other day. Monolayers were grown up to 14 days postconfluence to allow for polarization and then used in our experiments. To assess permeability, FITC-dextran (0.5 mg/ml; Sigma-Aldrich) was added to the apical compartment of the Transwell insert. Cells were pretreated with PXR agonists (rifaximin or SR2813) or inhibitors (ML-7 or SP600125) 1 hour prior to exposure to TNFα/IFNγ (TNFα, 10 ng/ml; IFNγ, 20 ng/ml) (Zolotarevsky et al., 2002; Bruewer et al., 2003; Wang et al., 2005, 2006) and media sampled from the basolateral compartment at 16 hours post-TNFα/IFNγ stimulation (Hansen et al., 2013; Schenck et al., 2013). The movement of FITC-dextran from the apical to the basolateral compartment was quantified by a fluorescence plate reader (excitation 490 nm, emission 520 nm).
PXR Regulates the Epithelial Barrier during Inflammation

Zonula Ocludens-1 Immunofluorescence Staining. Caco-2 IECs were plated at 1.2 × 10⁶ cells/ml on 8-well chamber slides (Thermo Fisher Scientific, Mississauga, Canada). Cells were grown for 14 days postconfluence and then used for our experiments. Cells were pretreated with PXR agonists (rifaximin or SR12813) 1 hour prior to exposure to TNFα/INFγ (TNFα, 10 ng/ml; INFγ, 20 ng/ml) for 16 hours (Zolotarevsky et al., 2002; Bruwer et al., 2003; Wang et al., 2005, 2006). At the end of the experiment, the cells were stained, as we have described previously (Hansen et al., 2013; Schenck et al., 2013). In brief, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and then fixed with ice-cold methanol for 30 minutes at 4°C. Following fixation, the cells were blocked with normal donkey serum (15 minutes at room temperature) and then incubated with rabbit anti–zonula occludens-1 (anti–ZO-1) primary antibody (1:100 dilution, 1 hour at 37°C; Invitrogen). Cells were then rinsed with PBS twice, incubated with Cy5-conjugated secondary goat anti-rabbit IgG (1:500 dilution, 1 hour at 37°C; Jackson ImmunoResearch, West Grove, PA), rinsed three times with PBS, and affixed on coverslips using Fluorosave Reagent (Calbiochem, San Diego, CA). Cells were viewed by fluorescence microscopy, and images were captured with a digital DS-Fi1 camera (Nikon, Tokyo, Japan).

Western Blots. To isolate cellular lysates, culture medium was removed, and the cells were washed with ice-cold PBS. Cell lysis buffer was added (150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease inhibitor cocktail; phosphatase inhibitor cocktail: Complete Minitab; Complete PhosStop (Laval, Canada), Roche). Total protein was quantified using the Precision Red Advanced Protein Assay (Cytoskeleton, Denver, CO) and sample protein concentration equalized. Samples were resolved and transferred to nitrocellulose membranes (0.2-μm pores; Bio-Rad, Hercules, CA) and blotted with the following antibodies: PXR (TA312120; OriGene, Rockville, MD), MLCK (SAV1300116; Sigma-Aldrich), JNK1/2 (9258; Cell Signaling, Danvers, MA), phosphorylated JNK1/2 (4668; Cell Signaling), and β-actin (A2066; Sigma-Aldrich). Blots were imaged (SAV1300116; Sigma-Aldrich), JNK1/2 (9258; Cell Signaling, Danvers, MA), phosphorylated JNK1/2 (4668; Cell Signaling). Blots were imaged using a ChemiDoc XS (Bio-Rad) and band intensity quantified using ImageJ (National Institutes of Health, Bethesda, MD).

PXR-Dependent Reporter Gene Assay. HepG2 and LS180 cells were transfected as described previously (Lau and Chang, 2015). In brief, cells were transfected within 6 hours of seeding by treating them with FuGENE 6 transfection reagent (Promega). Cells were transfected as described previously (Lau and Chang, 2015). In brief, cells were transfected within 6 hours of seeding by treating them with FuGENE 6 transfection reagent (Promega). Cells were transfected as described previously (Lau and Chang, 2015). In brief, cells were transfected within 6 hours of seeding by treating them with FuGENE 6 transfection reagent (Promega). Cells were transfected as described previously (Lau and Chang, 2015). In brief, cells were transfected within 6 hours of seeding by treating them with FuGENE 6 transfection reagent (Promega).

NFxB Reporter Assays. Phorbol-12-myristate-13-acetate–differentiated THP-1-Blue NFxB cells were pretreated with rifaximin 1 hour prior to exposure to LPS (100 ng/ml). NFxB activity was assessed by quantifying NFxB-dependent SEAP production, released into the culture supernatant following addition of SEAP detection reagent (InvivoGen). Following addition of SEAP, activity was assessed by quantifying NFxB activity following addition of SEAP detection reagent (InvivoGen).

Results

To model the intestinal epithelium experimentally, we plated Caco-2 IECs on Transwell inserts and cultured them for 14 days postconfluence. Caco-2 monolayers were then exposed to TNFα (10 ng/ml) and IFNγ (20 ng/ml) to model key

Experimental Colitis. All experiments outlined in this section were approved by the Health Sciences Animal Care Committee, University of Calgary, and conform to the guidelines set by the Canadian Council on Animal Care (protocol #AC12-0146). Male C57Bl/6 were used between 8 and 10 weeks of age. Colitis was induced by the addition of DSS (2.5% w/v); molecular weight, 40,000; ICN Biomedicals, Irvine, CA) to the drinking water as described previously (Hirotta et al., 2011; Terc et al., 2014). Animals were assessed and body weights were recorded daily. Mice were given a daily administration of PCN (mouse-specific PXR agonist; 25 mg/kg, i.p.) throughout the 7-day course of DSS, starting 1 day prior to commencement. At the end of the experiment, intestinal permeability was assessed by FITC-dextran flux assay (as described in the previous section). Colonic tissues were harvested, and a macroscopic damage score was given to each animal. Colons were opened longitudinally and placed on a flat surface. The mucosal and serosal surfaces of each colon were assessed for the presence of any of the following gross lesions: erythema, hemorrhage, edema, fecal blood, fecal mucous, diarrhea, strictures, ulcers, and adhesions. Each lesion was given a score of 1, and lesions were summed to produce an overall macroscopic score. In addition to this analysis, colonic sections were isolated and tissue myeloperoxidase (MPO) levels quantified, and the mucosal expression of MLCK and activation (phosphorylation) of JNK1/2 were assessed by immunohistochemical staining.

Tissue Myeloperoxidase Assay. Colonic tissue MPO activity, an inflammatory index of granulocyte infiltration, was determined following a published protocol (Diaz-Granados et al., 2000). MPO activity was reported as units per milligram of tissue, where one unit of MPO was defined as the amount of enzyme needed to degrade 1 µmol of H₂O₂ per minute at room temperature.

Immunohistochemical Staining. Freshly dissected colonic tissue was fixed overnight in 4% formaldehyde in phosphate-buffered saline and embedded in paraffin as a “Swiss roll.” MLCK and phosphorylated/active JNK1/2 were detected by immunohistochemical staining. In brief, tissue sections were deparaffinized/rehydrated and antigen retrieval performed (Tris/EDTA pH 9.0 in a vegetable steamer at 95–100°C for 20 minutes). Sections were then blocked (Serum Blocking Reagent, Avidin Blocking Reagent, Biotin Blocking Reagent; R&D Systems, Minneapolis, MN) and then incubated with primary antibodies (MLCK: #Ab76092, 1:100 (Abcam, Cambridge, UK); phospho-JNK1/2: #AF2305, 1:100 (R&D Systems)) diluted in incubation buffer (1% bovine serum albumin, 1% normal donkey serum, 0.3% Triton X-100, 0.01% sodium azide in PBS). Sections were then incubated with biotin-conjugated secondary antibodies, high sensitivity streptavidin-horseradish peroxidase, and DAB Chromogen (R&D Systems). Following the chromogen reaction, sections were counterstained with Gill-2 hematoxylin and dehydrated, and coverslips were mounted with xylene-based mounting media.

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aspects of the inflammatory milieu of the intestinal mucosa thought to contribute to the barrier dysfunction observed in patients with IBD (Turner, 2009). To assess barrier function, we quantified the movement of FITC-dextran (4 kDa) from the apical to the basolateral compartment of the Transwell insert, as we have done previously (Hansen et al., 2013; Schenck et al., 2013). TNFα/IFNγ exposure (16 hours) significantly increased FITC-dextran flux across the intestinal epithelial monolayer (Fig. 1A), indicating barrier dysfunction, as has been reported previously (Ma et al., 2005; Ye et al., 2006). TNFα/IFNγ had no effect on the expression of the PXR in these polarized monolayers (both the PXR.1 and PXR.2

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**Fig. 1.** Selective activation of the PXR preserves barrier function in Caco-2 IEC monolayers. Caco-2 monolayers grown for 14 days postconfluency were pretreated with selective PXR agonists [rifaximin (Rifx) or SR12813 (SR), each at 10 μM] for 1 hour prior to exposure to TNFα (10 ng/ml) and IFNγ (20 ng/ml) for 16 hours. (A) The barrier function of each monolayer was quantified by assessing FITC-dextran flux (4 kDa). ***P < 0.001 compared with NoRx (vehicle control); #P < 0.05 compared with TNFα/IFNγ (n = 6–8). Rifaximin and SR12813 are selective PXR agonists. Overexpression of the human PXR (hPXR) in LS180 IECs and HepG2 cells enhances the activation of PXR-responsive CYP3A4-XREM–driven luciferase when exposed to rifaximin and SR12813. (B) The PXR is expressed in Caco-2 IECs grown for 14 days postconfluence on Transwell inserts (blots are representative of four separate experiments). LS180 (C) and HepG2 cells (D) transfected with the hPXR and treated with rifaximin (10 μM) and SR12813 (10 μM) exhibit enhanced CYP3A4-XREM–driven luciferase activity compared with the vehicle control (DMSO). *P < 0.05 compared with the group not transfected with the hPXR construct (n = 3). A.F.U., arbitrary fluorescence units.
isoforms; Fig. 1B). Pretreatment with the selective human PXR agonists rifaximin (10 μM) or SR12813 (10 μM) significantly reduced the FITC-dextran flux induced by TNFα/IFNγ exposure (Fig. 1A), suggesting that PXR stimulation can afford barrier protection by exerting a direct effect on the intestinal epithelium.

Whereas rifaximin (Ma et al., 2007; Cheng et al., 2010; Mencarelli et al., 2010, 2011) and SR12813 (Ekins and Erickson, 2002; Lemaire et al., 2007) have been widely reported to act as selective agonists of the PXR, we confirmed their ability to elicit PXR-dependent effects. Rifaximin and SR12813 did not significantly activate an XREM-containing PXR-responsive reporter gene construct in LS180 and HepG2 cells (Fig. 1, C and D). However, overexpression of the human PXR significantly increased rifaximin- and SR12813-induced XREM-containing reporter activation (Fig. 1, B and C), suggesting that these compounds activate PXR-driven processes.

Next, we sought to determine the mechanism through which PXR stimulation preserves barrier function in intestinal epithelial monolayers exposed to inflammatory mediators. We have previously visualized ZO-1 and assessed its localization at cell-cell contacts as a measure of tight-junction integrity (Hansen et al., 2013; Schenck et al., 2013). Caco-2 monolayers treated with TNFα/IFNγ (TNFα, 10 ng/ml; IFNγ, 20 ng/ml; 16 hours) exhibited ruffling of the peripheral ZO-1 staining (Fig. 2A), an observation that was quantified by expressing the measured distance of the ruffling as a percentage of total cell-cell contact (Fig. 2B). When quantified, this ratio was significantly increased in TNFα/IFNγ-treated monolayers when compared with cell monolayers treated with vehicle or the selective PXR agonist rifaximin (10 μM) alone (Fig. 2B). Interestingly, pretreatment with rifaximin attenuated the ZO-1 ruffling (Fig. 2A) and blocked the increased ruffling/cell-cell contact ratio triggered by TNFα/IFNγ treatment (Fig. 2B), suggesting that PXR activation can directly

![Fig. 2. Selective activation of the PXR on Caco-2 IECs maintains tight-junction integrity in epithelial monolayers. (A) Caco-2 monolayers grown for 14 days postconfluence were pre-treated with rifaximin (Rifx; 10 μM), a selective PXR agonist, for 1 hour prior to exposure to TNFα (10 ng/ml) and IFNγ (20 ng/ml) for 16 hours. (B) The degree of ZO-1 mislocalization, an indicator of tight-junction disruption, was quantified by using ImageJ to measure the length of ZO-1 staining and expressing it as a ratio of the length of total cell-cell contact. A value of 100 indicates ZO-1 staining is maintained over the entire cell-cell interface. A value >100 indicates ZO-1 staining is disrupted over the cell-cell interface. **P < 0.005 compared with control; ##P < 0.005 compared with TNFα/IFNγ group (n = 5–9 replicates from four separate experiments).](image-url)
modulate the response of IECs to inflammatory stress and preserve tight-junction integrity.

The disrupted localization of ZO-1 that is associated with intestinal epithelial barrier dysfunction has been linked to a variety of intracellular signaling pathways. Some have reported that TNFα-induced activation of NFκB and subsequent upregulation of MLCK triggers cytoskeletal activation, disruption of tight junctions, and enhanced permeability (Ma et al., 2005; Ye et al., 2006; Al-Sadi et al., 2016). Others have reported that ZO-1 relocalization can be driven by the activation of JNK1/2 signaling (Samak et al., 2014, 2015; Zhang et al., 2015). Interestingly, activation of the PXR has been reported to interact with both of these pathways (Zhou et al., 2006; Wang et al., 2010). Furthermore, selective deletion of the PXR in IECs leads to a defect in intestinal epithelial barrier function in mice (Venkatesh et al., 2014). Thus, we sought to determine whether activation of the PXR affords barrier protection by inhibiting these two intracellular signaling pathways. First, we confirmed that MLCK and JNK1/2 activity drives TNFα/IFNγ-induced barrier dysfunction in Caco-2 monolayers. Indeed, inhibition of MLCK (with ML-7) and JNK1/2 signaling (with SP600125) attenuated the barrier dysfunction triggered by TNFα/IFNγ exposure (Fig. 3). As reported previously (Ma et al., 2005; Ye et al., 2006), TNFα/IFNγ exposure increased the expression of MLCK in Caco-2 IECs (Fig. 4A). However, pretreatment of the monolayers with rifaximin attenuated this response (Fig. 4A).

It is well established that selective activation of the PXR inhibits NFκB signaling in a variety of cell types, including IECs (Mencarelli et al., 2010, 2011). Using a THP-1–based TLR4/NFκB-reporter system, we then sought to confirm that activation of the PXR, with rifaximin, could modulate NFκB signaling. In our THP-1–based reporter system, rifaximin inhibited LPS-induced NFκB signaling (Fig. 4A), an observation that is in agreement with previous reports characterizing the anti-inflammatory properties of rifaximin and other PXR agonists (Ma et al., 2007; Shah et al., 2007; Mencarelli et al., 2010, 2011).

Whereas NFκB-driven MLCK expression contributed to the barrier disruption in our study, inhibition of JNK1/2 signaling also protected against TNFα/IFNγ exposure (Fig. 3). Activation of the PXR has been reported to increase the expression of growth arrest and DNA damage–inducible 45β (GADD45β) (Kodama and Negishi, 2011), a protein that can block JNK1/2 activation by inhibiting its upstream kinase MKK7 (De Smaele et al., 2001; Papa et al., 2004; Larsen et al., 2006). Thus, we assessed whether PXR activation could modulate TNFα/IFNγ-induced JNK1/2 activation in IEC monolayers. Selective activation of the PXR in IEC monolayers attenuated TNFα/IFNγ-induced JNK1/2 activation (Fig. 5A). This was associated with a significant increase in GADD45β transcript expression (Fig. 5B). Taken together, our in vitro data suggest that the selective activation of the PXR affords intestinal epithelial barrier protection by preserving tight-junction integrity by attenuating TNFα/IFNγ-induced upregulation of MLCK and inhibiting JNK1/2 signaling.

In the final set of experiments, we sought to confirm our in vitro findings using two distinct mouse models of barrier disruption. First, we assessed the role of the PXR in TLR4-induced small intestinal barrier disruption, using a model we have used previously (Venkatesh et al., 2014). Jejunal administration of KDO2, a selective TLR4 agonist, triggers the expression of mucosal Tnfα and disrupts the small intestinal mucosal barrier (Venkatesh et al., 2014). In wild-type (WT) and Pxr−/− mice, administration of KDO2 triggered a significant increase in permeability, as evidenced by increased FITC-dextran detected in the serum (Fig. 6, A and B). Pretreating mice with PCN (25 mg/kg, i.p.), a rodent-specific PXR agonist, attenuated KDO2-induced barrier disruption in WT mice, but not Pxr−/− mice, highlighting the selectivity of this agonist in our system. These data indicate that activation of the PXR can afford barrier protection in a model system where barrier disruption is associated with increased TNFα expression (Venkatesh et al., 2014).

In the next set of experiments, we used a mouse model of experimental colitis to assess the role of the PXR in regulating barrier function. We reported previously that stimulation of the PXR, with PCN, reduced barrier dysfunction in the acute and recovery phase of experimental colitis (Terc et al., 2014). In the latter case, this was independent of tissue inflammation. Thus, we hypothesized that the barrier protection afforded by activation of the PXR could be due to its ability to dampen inflammation-associated expression of MLCK and activation of JNK1/2 in the intestinal mucosa. Pretreating mice with PCN (25 mg/kg, i.p., daily) significantly reduced the macroscopic damage, colonic thickening, and barrier dysfunction triggered during DSS-induced colitis (2.5% (w/v) in drinking water, 7 days; Fig. 7, A–C), a finding that supports our previously published work (Terc et al., 2014). The preservation of barrier function by selective activation of the mouse PXR was associated with reduced colonic mucosal expression of MLCK (Fig. 7D) and the levels of phosphorylated/active JNK1/2, as assessed by immunohistochemical staining (Fig. 7E).
Taken together, our in vitro and in vivo data suggest that PXR activation can preserve intestinal epithelial barrier function in the context of inflammation by directly modulating intracellular signaling within IECs, ultimately maintaining tight-junction integrity.

**Discussion**

In the current study, we sought to assess the mechanism(s) through which the PXR regulates the intestinal epithelium, protecting against intestinal inflammation and tissue damage during colitis. We, and others, have reported previously that the PXR plays an important role in maintaining intestinal mucosal homeostasis (Zhou et al., 2006; Terc et al., 2014; Venkatesh et al., 2014), and its activation dampens inflammation and mucosal damage in experimental colitis (Ma et al., 2007; Shah et al., 2007; Dou et al., 2012, 2013, 2014; Terc et al., 2014). In our previous report, we found that treating mice with PCN attenuated colonic mucosal neutrophil infiltration and afforded barrier protection (Terc et al., 2014). However, we were unable to delineate whether the protection observed was driven by a direct effect of PXR signaling within the intestinal epithelium, or was due to an overall reduction in the inflammatory burden in the intestinal mucosa. We reported recently that selective deletion of the PXR in the intestinal epithelium increases mucosal permeability, suggesting that this receptor regulates the biology of the intestinal epithelial barrier (Venkatesh et al., 2014). Compromised barrier function has been associated with IBD; however, it is still unclear whether it plays a direct role in driving/initiating the disease or is secondary to tissue inflammation. Increased intestinal permeability is associated with IBD relapse and recurrence (Hollander et al., 1986; Katz et al., 1989; Wyatt et al., 1993; D’Inca et al., 1999; Tibble et al., 2000; Teshima et al., 2012), and inflammatory mediators, such as TNFα and IFNγ, which are elevated in the tissues of IBD patients (Turner, 2009), can directly disrupt the intestinal epithelial barrier (Turner, 2009; Cunningham and Turner, 2012). Thus, in the current study, we began with an in vitro approach to test whether selective activation of the PXR could regulate signaling pathways that contribute to intestinal epithelial barrier dysfunction in conditions that model aspects of the mucosal inflammation found in IBD. We found that stimulation of the PXR preserved the barrier function of IEC monolayers exposed to TNFα/IFNγ. This effect was associated with the preservation of tight-junction
integrity, attenuated TNFα/IFNγ-induced expression of MLCK, and inhibition of JNK1/2 activity. These data were supported by our in vivo findings, where selective activation of the PXR significantly reduced inflammation-induced intestinal barrier dysfunction evoked by TLR4 activation or DSS exposure, the latter associated with a reduction in MLCK expression and activation of mucosal JNK1/2.

Highly expressed in the liver and intestinal epithelium, the PXR has been previously characterized as a master regulator of xenobiotic metabolism and clearance (Watkins et al., 2002; Staudinger et al., 2006; Shukla et al., 2011; Dou et al., 2013; Pondugula and Mani, 2013). Acting as a sensor of exogenous chemicals, the PXR’s activation leads to the expression of a variety of target genes related to detoxification and efflux pathways. In addition to enhancing xenobiotic gene transcription, PXR activation has been reported to prevent NFκB-DNA interactions and inhibit the release of proinflammatory mediators (Zhou et al., 2006; Shah et al., 2007; Mencarelli et al., 2010, 2011). In agreement with these reports, we found that pretreating cells with rifaximin, a selective human PXR agonist, attenuated LPS-induced NFκB reporter activity. In our IEC monolayers, we observed the selective activation of the PXR with SR12813 and rifaximin protected against cytokine-induced barrier disruption. TNFα and IFNγ decrease transepithelial resistance, in part, by inducing NFκB-dependent expression of MLCK, activation of the cytoskeleton, ZO-1 redistribution, and disruption of cell-cell contacts (Ma et al., 2005; Ye et al., 2006). We found that the barrier protection afforded by PXR activation was associated with attenuated TNFα/IFNγ-induced MLCK expression and a preservation of tight-junction integrity. Interestingly, in their initial report, Ma et al. (2005) used curcumin to selectively inhibit NFκB, an approach that abrogated TNFα-induced MLCK expression and barrier disruption. Curcumin has been reported to activate the PXR, an effect thought to contribute to its anti-inflammatory activity in the gastrointestinal tract (Kluth et al., 2007; Nones et al., 2009). Taken together, these data suggest that the PXR can afford barrier protection, in part, by attenuating NFκB-dependent cytokine-induced expression of MLCK.

In addition to interacting with NFκB, the PXR has been reported to modulate the function of other signaling pathways. Previously, we reported that activation of the human PXR, with a variety of selective agonists, enhanced IEC migration...
and accelerated intestinal epithelial wound healing in a p38 mitogen-activated protein kinase–dependent fashion (Terc et al., 2014). Others have reported that PXR-dependent activation of p38 mitogen-activated protein kinase signaling is driven by increased expression of GADD45b, an early response gene that regulates a variety of intracellular signaling cascades (Kodama and Negishi, 2011). Relevant to our current study, Papa et al. (2007) reported GADD45b inhibits JNK1/2 activation by directly interacting with its upstream kinase MKK7, reducing its catalytic function. Others have reported that GADD45b contributes to anti-inflammatory signaling by inhibiting TNFα-induced JNK1/2 signaling (Papa et al., 2004; Bortoff et al., 2010). We found that activation of the PXR in IECs inhibited TNFα/IFNγ-induced JNK1/2 signaling, an effect that was associated increased expression of GADD45b.

To translate our in vitro findings, we sought to assess whether selective activation of the PXR in mice could protect the intestinal mucosal barrier in two different models of inflammation-driven barrier disruption. In the first set of experiments, we found that WT mice, but not Pxr−/− mice, pretreated with PCN (a selective rodent PXR agonist) were protected against KDO2-induced TLR4-driven intestinal barrier dysfunction, supporting our in vitro data and reinforcing PCN’s selectivity. In the second set of experiments, we sought to recapitulate key findings from our in vitro experiments. Specifically, we sought to determine whether PXR activation could afford barrier protection in the context of inflammation.

**Fig. 7.** Activation of the PXR reduces the clinical severity of colitis and attenuates intestinal damage and barrier dysfunction in mice exposed to DSS [2.5% (w/v) in the drinking water, 7 days], an effect that is associated with reduced mucosal expression of MLCK and activation of JNK1/2, as assessed by detecting phosphorylated JNK1/2. Treating male mice with PCN (25 mg/kg, i.p., daily) reduces colitis-associated body weight loss (A), macroscopic colonic tissue damage (B), colonic MPO levels (C), mucosal thickening, (D) and attenuates inflammation-associated barrier dysfunction, as assessed by FITC-dextran flux assay (E). (A–E) P < 0.05 compared with DSS+vehicle group; #P < 0.05 compared with naive and PCN-treated mice; *P < 0.05 compared with DSS+vehicle group (n = 5–10). PXR activation reduces colonic mucosal MLCK expression (scale bar: 100 μm) (F) and JNK1/2 phosphorylation (P-JNK; scale bar: 50 μm) (G) induced by DSS exposure. Veh., vehicle.
by regulating MLCK expression and JNK1/2 activity. DSS-exposed mice exhibited increased intestinal permeability, an effect that was attenuated when they were pretreated with PCN. Furthermore, in agreement with our in vitro findings, the barrier protection afforded by PXR activation was associated with reduced expression of mucosal MLCK and reduced JNK1/2 activity. Although the role of intestinal epithelial MLCK in regulating mucosal permeability has been characterized extensively (Cunningham and Turner, 2012), participation of JNK1/2 in this process is less clear. Some have reported that deletion of JNK1 or JNK2 (or both) increases the susceptibility of mice in experimental models of colitis (Chromik et al., 2007; Kersting et al., 2013b); however, we have reported that selective inhibition of JNK1/2 is protective, reducing IEC apoptosis and modulating mucosal inflammatory mediator expression (Assi et al., 2006; Mitsuyama et al., 2006; Kersting et al., 2013a). Interestingly, in Crohn’s disease patients, the expression of activated JNK is increased in the intestinal epithelium and mucosal immune cells (Bantel et al., 2002; Mitsuyama et al., 2006), and its selective inhibition reduces the expression of inflammatory mediators in colonic mucosal biopsies (Mitsuyama et al., 2006).

The findings that PXR stimulation protects the intestinal barrier during inflammation are in line with previous studies highlighting its role in regulating intestinal mucosal homeostasis. Deletion of the PXR leads to the development of mild spontaneous intestinal inflammation (Zhou et al., 2006) and decreased barrier function (Venkatesh et al., 2014). Furthermore, selective activation of the PXR reduces intestinal inflammation and tissue damage (Shah et al., 2007). Although the endogenous ligand(s) for the PXR in the gastrointestinal tract has yet to be completely elucidated, members of our team reported recently that the PXR could be activated by indole-propionic acid, a microbial metabolite, suggesting that this receptor may play a role in host-microbe interactions at the intestinal epithelium (Venkatesh et al., 2014).

Our study may provide insight into how PXR dysfunction may contribute to IBD susceptibility (Langmann et al., 2004; Dring et al., 2006; Martinez et al., 2007; Glas et al., 2011). Whereas others have characterized the anti-inflammatory effects of PXR signaling in the gut, in vitro and in vivo data suggest that selective activation of the PXR favors protection of the intestinal epithelial barrier in the context of inflammation. In addition, our findings also provide insight into the potential anti-antimicrobial mechanism(s) of action by which rifaximin elicits its clinical efficacy in the treatment of enteric infections and IBD, both of which are associated with disruption of the intestinal epithelial barrier. Rifaximin is a luminal antibiotic and selective human PXR agonist (Ma et al., 2007), which has shown promise in the treatment of IBD (Prantera et al., 2012). Future studies should be designed to assess the therapeutic efficacy of PXR agonists in a variety of preclinical models, assessing the anti-inflammatory and probiotic barrier effects after the induction of chronic inflammation, to better model the clinical course of human IBD.

Although the pathogenesis of IBD has yet to be completely elucidated, barrier dysfunction and increased epithelial permeability are strongly associated with disease relapse and recurrence. Thus, our data suggest that targeting the PXR may prove effective in the treatment of IBD by targeting the intestinal epithelium and enhancing its barrier function in the context of inflammation.
PXR Regulates the Epithelial barrier during Inflammation


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