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A Novel Role for the Rho-Associated Kinase, ROCK, in IL-1-stimulated Intestinal Epithelial Cell Responses

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Abstract

Intestinal epithelial cells (IEC) play a role in mucosal inflammation by producing pro-inflammatory chemokines that may initiate or amplify local responses. IL-1 is a potent activator of IEC and its receptor localizes to focal adhesions. Since the Rho-associated kinase, ROCK, also localizes to focal adhesions, we examined the role of ROCK in IL-1-induced chemokine responses in IEC cell lines. Suppressing ROCK with the Y27632 inhibitor suppressed IL-1-stimulated Caco-2 cell CXCL8/IL-8 and IEC-6 cell CCL2/MCP-1 secretion and mRNA levels. ROCK inhibition also suppressed IL-1-induced JNK phosphorylation in both cell lines, but high levels of the inhibitor had no significant effect on IL-1-stimulated Caco-2 I κ B α phosphorylation and degradation or IKK phosphorylation and kinase activity. Therefore, ROCK may exert an effect on IL-1-stimulated JNK signaling to AP-1 activation, with little effect on IKK/I κ B α signaling, defining a potentially important mechanism for regulating IL-1 signaling in IEC that may be essential for optimal cytokine responses.

Keywords

IL-1; intestinal epithelial; CXCL8; JNK; CCL2; ROCK

1. Introduction

IL-1 is a potent pro-inflammatory cytokine with a known role in mucosal infections and inflammatory bowel disease (IBD). This cytokine has been shown to activate hundreds of genes including inflammatory cytokines, growth factors, receptors, proteases, signaling proteins and transcription factors [1, 2]. The intestinal epithelial cells (IEC) that line the surface of the intestine and colon are responsive to IL-1 and can produce several important immunoregulatory cytokines and chemokines, such as IL-6, CXCL8 (IL-8) and CCL2 (MCP-1) [3–6], to amplify or sustain the inflammatory response. This suggests that IEC have a role in intestinal inflammation and IBD by their inflammatory cytokine production.

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IL-1 exerts its effect through a signaling pathway that results in the activation of the transcription factors, NF- κ B and AP-1 [2]. IL-1 stimulation of human colonic epithelial cells and cell lines induces nuclear accumulation of NF- κ B and CXCL8 secretion [7]. This results from the activation of the upstream serine kinases, I κ B kinase (IKK) α and β , which are responsible for phosphorylating I κ B α and initiating the activation of NF- κ B [8]. IL-1 can also activate an intracellular signaling pathway that results in phosphorylation of the c-Jun NH₂-Terminal Kinase, JNK [2]. Active JNK then phosphorylates c-Jun, which along with the Fos protein creates the archetypical AP-1 transcription factor [9]. High levels of phosphorylated JNK staining have been shown to localize to both leukocytes and epithelial cells in the inflamed mucosa of patients with IBD, as compared to normal controls [10]. These and other studies have suggested an important role for JNK signaling in IBD [11].

IEC attach to the extracellular matrix through cell surface integrins [12]. Integrin signaling can activate the Rho family of small GTPases, which are known to control the formation of actin stress fibers and actomyosin contractility in cell movement [13]. A major downstream effector for Rho is the Rho-associated protein kinase, ROCK, a serine/threonine kinase which phosphorylates myosin light chain phosphatase involved in actomyosin contractility [13]. ROCK exists as two isoforms, ROCK1 and ROCK2, which are highly homologous [14].

A recent study using rats with trinitrobenzene sulfonic acid induced colitis found that inhibiting the activity of ROCK with the chemical inhibitor, Y27632, reduced the symptoms of colitis suggesting that ROCK was involved in inflammatory bowel disease [15]. Then using peripheral blood mononuclear cells from Crohn's disease patients or a macrophage cell line, they found that inhibiting ROCK could inhibit LPS induced IL-1 and TNF production, and IL-1 signaling through IKK/I κ B α to NF- κ B. These results suggested that IL-1 and LPS stimulation of macrophages and peripheral blood mononuclear cells resulted in the activation of ROCK, which had an important positive regulatory effect on cytokine responses and IL-1 induced intracellular signaling events. Therefore ROCK may play an important role in mucosal inflammatory responses mediated by IL-1. However, the effect of Y27632 treatment on IEC was not considered, despite the role that IEC play in the mucosal inflammatory response. Also it is not clear whether all cell types share the effect of ROCK seen above. In cervical stromal cells, Y27632 was found to block LPS, but not IL-1, induced CXCL8 production [16], while in astrocytes, IL-1 deactivates Rho-ROCK signaling to alter astrocyte function [17].

IEC activation by IL-1 and subsequent cytokine and pro-inflammatory responses may be very important in the early stages of infection or inflammatory disease as initiators of inflammation and may help to sustain or amplify ongoing inflammation. Yet cell migration and wound healing events may also alter IEC function through the activation of ROCK by integrin signaling pathways [18]. Therefore, it is essential that the relationship between IL-1 and ROCK activation in IEC be well understood. We have determined the role of ROCK in IEC cytokine responses to IL-1 stimulation and provide evidence that ROCK may play an important role in IL-1 signaling in these cells. However, unlike with peripheral blood mononuclear cells and macrophages [15], inhibiting ROCK was found to suppress IL-1 induced CXCL8 secretion and mRNA levels through an inhibition of JNK, but not in IKK/I κ B α signaling in IEC. This novel finding provides important insights into the mechanisms that regulate IL-1 signaling in IEC.

2. Materials and Methods

2.1 Antibodies

Rabbit polyclonal antibodies against human phospho-I κ B α (Ser 32 and 36), phospho-IKK (IKK α Ser 180/IKK β Ser 181), or JNK; mouse monoclonal antibodies against human I κ B α or phospho-JNK (Thr 183/Tyr 185); HRP-conjugated anti-rabbit and HRP-conjugated anti-mouse detection antibodies were obtained from Cell Signaling Technologies (Beverly, MA). The mouse monoclonal antibodies against human tubulin along with rabbit polyclonal antibody against human IKK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Cell Culture Conditions

The human colonic Caco-2 cell line (ATCC HTB37; American Type Culture Collection, Manassas, VA) was cultured in RPMI-1640 (Mediatech, Herndon, VA) with 10% fetal bovine serum (FCS; Hyclone Laboratories, Logan, UT), 2g/L sodium bicarbonate, 2mM L-glutamine (Lonza, Walkersville, MD), non-essential amino acids (Lonza), 25 IU penicillin and 25 μ g/ml streptomycin (Mediatech), referred to as % FCS-RPMI. The rat IEC-6 small intestine epithelial cell line (ATCC CRL1592) was cultured in DMEM (Hyclone) with 5% FCS (Hyclone), 2g/L sodium bicarbonate, 70 USP units bovine insulin (Sigma, St. Louis, MO), 2mM L-glutamine (Lonza), 25 IU penicillin and 25 μ g/ml streptomycin (Mediatech), referred to as 5% FCS-DMEM. All cell lines were maintained at 37°C in a 90% air-10% CO₂ humid atmosphere.

The cells were removed from tissue culture flasks by brief treatment with a trypsin and EDTA solution (Sigma) and were resuspended in 5% FCS-DMEM. Wells of tissue culture plates (BD Labware, Franklin Lakes, NJ) were coated with 20 μ g/ml bovine plasma fibronectin (Sigma) in PBS (e.g. 200 μ l per well for 12-well plates) and incubated for 60 minutes at 37°C. The wells were then aspirated and allowed to dry under ultraviolet light for 30 minutes.

For experiments using the ROCK inhibitor, Caco-2 or IEC-6 cells were plated in FN coated wells and incubated for one hour at 37°C and 10% CO₂. The culture supernatants were then removed and the cells treated with serum-free DMEM containing insulin, transferrin, and selenium (ITS; BD Biosciences, Bedford, MA) with or without the ROCK inhibitor, Y-27632 (Sigma), for a final concentration of 1–100 μ M. Cells were incubated for another two hours at 37°C and 10% CO₂. At three hours after plating, the cells were treated with rhIL-1 (R&D Systems, Minneapolis, MN) at 1ng/ml (Caco-2) or 0.5ng/ml (IEC-6) as these were found to be optimal doses for CXCL8 or CCL2 secretion, respectively (data not shown).

2.3 Determining cytokine levels in culture supernatants

Caco-2 (1 $\times$ 10⁵ cells/well in 96-well plates) and IEC-6 (2.5 $\times$ 10⁵ cells/well in 24-well plates) cells were cultured and treated with the ROCK inhibitor and IL-1 as described above. Culture supernatants were harvested 24 hours after IL-1 stimulation and stored at –80°C. The total cells per well were determined by treating the cells with trypsin and EDTA (Sigma) and counting the cells using a hemacytometer. The amount of human CXCL8 or rat CCL2 present in the culture supernatants was determined with the DuoSet ELISA kit for human CXCL8/IL-8 (R&D Systems, Minneapolis, MN) or the Cytoscreen ELISA kit for rat CCL2/MCP-1 (Biosource, Camarillo, CA). The absorbances of the samples were measured using a Bio-Tek EL312e microplate reader (Bio-Tek Instruments Inc., Winooski, VT).

2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR)

To determine the effect of the ROCK inhibitor on CXCL8 and CCL2 mRNA levels, Caco-2 and IEC-6 (1×10^5 cells/well) cells in 12 well plates were treated with the ROCK inhibitor (40 μ M and 20 μ M, respectively) and IL-1 as described above. Six hours after IL-1 stimulation, total RNA was harvested using TRIZOL reagent (Invitrogen) as per the manufacturer protocol. Samples were reverse transcribed using the ReactionReady First Strand cDNA Synthesis Kit (SuperArray, Frederick, MD) with annealing of random primers. PCR was performed using RT² PCR Primer Sets for human glyceraldehyde-3-phosphate dehydrogenase (GAPD), human CXCL8, rat GAPD, and rat CCL2 (SuperArray). The PCR reaction with human primers was carried out at 95°C for 10 minutes followed by 26 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds and a final extension at 72°C for 7 minutes using a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer, Waltham, MA). Amplification using rat primers were done as above for 29 (CCL2) or 32 (GAPD) cycles with an annealing temperature of 55°C for 45 seconds. The samples were then separated by agarose electrophoresis and stained with ethidium bromide. The images were captured using the Kodak 1D Image Analysis Software (Kodak, Memphis, TN) and band densities were quantified using the LabWorks 4.0 Image Analysis software (UVP Inc., Upland, CA).

2.5 Preparation of cell extracts

The adherent Caco-2 or IEC-6 cells were washed once in ice-cold PBS before isolation of cytoplasmic extracts as previously described by Li, *et al.* [19]. The cells were lysed in a cytoplasmic extract buffer with Calbiochem Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA) for 10 minutes at 4°C. The extracts were centrifuged for 5 minutes at 13,000 $\times$ g to remove the nuclei and debris. The protein concentrations were then determined using the Bio-Rad DC protein assay kit (Bio-Rad, Melville, NY) and the samples were stored at -80°C until further use.

2.6 Immunoblot analysis

Cytoplasmic extracts with equal amounts of protein or equal volumes of immunoprecipitates were separated with 8–15% polyacrylamide resolving gels and transferred to polyvinylidene difluoride membranes using the Mini Trans-blot system (Bio-Rad). The blots were blocked with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS) with 0.1% Tween-20 (BSA-TBST) for 1 hour at room temperature and incubated overnight at 4°C with the primary antibody in BSA-TBST. The blots were washed with TBS-T followed by incubation with a secondary antibody in BSA-TBST of either an HRP-conjugated anti-rabbit or anti-mouse antibody (Cell Signaling Technologies, Danvers, MA). The blots were then washed three times with TBS-T and the protein bands were visualized using the Lumiglo detection kit (Cell Signaling Technologies) and exposure to x-ray film and scanning or images were collected using a Bio-Rad ChemiDoc System. The band densities of interest were then quantified using the LabWorks 4.0 Image Analysis software.

Blots were reprobbed by washing twice with TBS-T followed by incubating with a stripping buffer of 62.5mM Tris-HCl, 2% SDS, and 100mM 2-mercaptoethanol at 50°C for 12–15 minutes. The blots were then washed four times with TBS-T and were reblocked with 5% BSA-TBST for one hour at room temperature with ROCK1ng before reprobbed with the next primary antibody.

2.7 IKK kinase activity assay

A non-radioactive kinase assay was performed for IKK α/β activity as described in Li, *et al.* [19]. Cytoplasmic extracts with 200 μ g of protein in 0.5ml total volume were incubated with

1 μ g of anti-IKK α / β antibody overnight at 4°C with rocking. The samples were then incubated with 20 μ l of Protein A/G agarose (Santa Cruz Biotechnology) with rocking at 4°C before centrifuging at 5,000 rpm for 5 minutes and washing with kinase buffer. The immunoprecipitates of IKK α / β were suspended in the kinase buffer with ATP and 2 μ g of GST-I κ B α target (Santa Cruz Biotechnology) for 15 minutes at 30°C. The reaction was stopped by adding 20 μ l of 2 $\times$ Laemmli buffer and heating at 95–100°C for 5 minutes. Samples were separated on 4–20% Tris-HCl gradient gels (BioRad) and Western blotted with an anti-phospho-I κ B α antibody. The blots were stripped and reprobed for IKK α / β as described above. Band density of phospho-I κ B α was normalized with the corresponding band density of the IKK α / β .

2.9 Statistics

Results from three or more experiments were analyzed for significant differences using the Student's t Test for comparison of two samples or ANOVA and Fisher's protected least significant difference test with a level of significance set at $p < 0.05$ for comparing three or more samples. Analyses were done using Statview 4.01 (SAS Institute).

3. Results

3.1 Effect of inhibiting ROCK on IL-1 induced CXCL8 and CCL2 responses

As ROCK may have a potential role in regulating IL-1 responses in IEC and we have found that IEC cell lines express protein for both isoforms of ROCK (data not shown), the effect of inhibiting ROCK was determined on two IEC cell lines, the human Caco-2 and the rat IEC-6 cell lines. The cells were plated onto FN coated wells and incubated for one hour to allow cell attachment before treating the cells for two hours with different doses of the Y27632 ROCK inhibitor. The Y27632 ROCK inhibitor (RI) is a competitive inhibitor of ATP binding and inhibits equally both ROCK1 and ROCK2 isoforms [20, 21]. This inhibitor is specific for suppressing the ROCK kinase activity by up to 87% while showing little or no effect on other 23 other kinases [21]. Y27632 also has an affinity for ROCK that is 20 to 30 times higher than for the other Rho effector kinases like the citron kinase and PKN (also known as PRK) [20]. Although 20 μ M of Y27632 is commonly used for studies inhibiting ROCK [20], higher levels up to 100mM and 300mM have been used to show some effects such as G₁ to S phase progression in cell cycle [20] and bronchial smooth muscle contraction studies [22]. After two hours, the cells were treated with IL-1 β and incubated for 24 hours. CCL2 was chosen as a representative chemokine for the rat cell lines as rats do not produce CXCL8. Treatment of the Caco-2 or IEC-6 cells with the ROCK inhibitor had no significant effect at any of the concentrations used on the total number of cells counted at 24 hours at the end of the experiments ($p > 0.16$; data not shown). Treatment of the IEC cell lines with the ROCK inhibitor alone had no effect on cytokine secretion by either cell line (Fig. 1). However, inhibiting ROCK resulted in a significant 70% suppression of IL-1-stimulated Caco-2 CXCL8 secretion (Fig. 1A; 100 μ M) and an 84% (with 100 μ M) or 60% (with 10 μ M) suppression of IEC-6 CCL2 secretion. Of note, the IEC-6 cells were found to be somewhat more sensitive to ROCK inhibition than the Caco-2 cells.

Next, the effect of inhibiting ROCK on IL-1 induced cytokine mRNA levels was determined. Pre-treating the cells with the 40 μ M ROCK inhibitor as above and culturing with IL-1 stimulation for 6 hours resulted in a significant 57% suppression of IL-1 induced CXCL8 mRNA levels in the Caco-2 cells, as compared to that of cells stimulated with IL-1 alone (Fig. 2A). A similar 62% suppression of IL-1 induced CCL2 mRNA levels with ROCK inhibition was seen with the IEC-6 cells (Fig. 2B) confirming the effect. Taken together, these experiments suggest that ROCK may play a significant role in the IL-1 activation of IEC to produce important pro-inflammatory cytokines.

3.2 Effect of inhibiting ROCK on IL-1 signaling events through I κ B α /NF- κ B

A major downstream signaling pathway from IL-1 stimulation is the activation of the IKK complex, which results in the phosphorylation and eventual degradation of I κ B α to release active NF- κ B [2]. Therefore, the effect of inhibiting ROCK with the Y27632 inhibitor was first determined on the phosphorylation and degradation of I κ B α . Treatment of the Caco-2 cells with a high level of the ROCK inhibitor (100 μ M) resulted in no significant effect on I κ B α phosphorylation or degradation at any time point ($p = 0.1$ to 0.98) after IL-1 stimulation as compared to cells treated with IL-1 alone (Fig. 3). Although the IL-1 induced I κ B α phosphorylation levels at 15 minutes were lower with the ROCK inhibitor treated cells, this level was not significantly different from the IL-1 only treated cells ($p=0.1$).

Since the phosphorylation of I κ B α is dependent upon the phosphorylation and activation of the upstream kinase, IKK α/β , the phosphorylation status and kinase activity of IKK was determined. As shown in Fig. 4, inhibiting ROCK had no significant effect on the IL-1 induced phosphorylation of IKK α/β at any time-point after stimulation ($p = 0.21$ to 0.88). In addition, inhibiting ROCK had no significant effect on the IL-1 induced specific activity of immunoprecipitated IKK toward an I κ B α target (Fig. 4B; $p = 0.26$ to 0.94). These results with IKK activation confirm the findings that the ROCK inhibitor had little effect on I κ B α phosphorylation and degradation and suggest that ROCK may play no role in the IKK/I κ B α /NF- κ B signaling in IEC, even with relatively high levels of the Y27632 ROCK inhibitor (100 μ M).

3.3 Inhibiting ROCK results in an inhibition of IL-1 induced JNK phosphorylation

IL-1 signaling events downstream of the TAK1 kinase split into the IKK/I κ B α /NF- κ B signaling pathway and the JNK/c-Jun/AP-1 signaling pathway [2]. Therefore, the effect of inhibiting ROCK with the Y27632 inhibitor was determined on IL-1 induced JNK phosphorylation, a step necessary for the activation of this key kinase. Pre-treatment of the Caco-2 cells with 100 μ M Y27632 (Fig. 5A) resulted in a significant 87% suppression of p46 JNK phosphorylation at 30 minutes ($p<0.01$) and a significant 94% suppression of p54 JNK phosphorylation at 15 minutes ($p<0.05$). However, as this was a high dose of the Y27632, we also examined the effect of treating the Caco-2 cells with 40 μ M Y27632 ROCK inhibitor prior to IL-1 stimulation (Fig. 5B), which resulted in a significant 50% suppression of IL-1 induced p46 JNK phosphorylation at 30 minutes ($p<0.05$). Yet, treatment with this lower level of the ROCK inhibitor had no significant effect on IL-1 induced p54 JNK phosphorylation at any time point ($p = 0.15$ to 0.5). To confirm these results, the IEC-6 cells were treated with 20 μ M Y27632 inhibitor prior to IL-1 stimulation which yielded a significant 86% suppression of IL-1 induced p46 JNK phosphorylation at 15 minutes ($p = 0.02$) and 80% and 64% suppression of IL-1 induced p54 JNK phosphorylation at 15 ($p = 0.009$) and 30 minutes ($p = 0.02$), respectively (Fig. 5C). A lower level of Y27632 ROCK inhibitor was used with the IEC-6 cells as the results in Fig. 1 indicated that these cells were more sensitive to the inhibitor. These experiments suggest that ROCK may play a significant role in the IL-1 signaling pathway leading to JNK activation.

4. Discussion

IL-1 is an important component of the inflammatory response at mucosal surfaces and understanding the mechanism by which IL-1 activates cells at the intestinal mucosa, especially the IEC, is essential to understanding the inflammation and devising potential therapies to combat this inflammation. The results of this study indicate that the Rho-associated kinase, ROCK, plays a necessary role in IL-1 responses in IEC. Inhibiting ROCK resulted in a suppression of IL-1 induced pro-inflammatory cytokine responses by IEC, and this effect was mediated through an effect of ROCK on IL-1 induced JNK activation.

However, IKK/ $\text{I}\kappa\text{B}\alpha$ signaling was generally not affected. Therefore, ROCK, and an unknown upstream Rho small GTPase, must have a function that positively affects only the JNK signaling arm of IL-1 signaling. A role for ROCK in JNK signaling in angiotensin II induction of vascular smooth muscle cell migration [23] and epithelial tight junction remodeling [24] has been suggested, but the selective role of ROCK in IL-1 induced JNK signaling in IEC is novel. The impact of finding that ROCK may be involved in epithelial JNK, but not IKK/ $\text{I}\kappa\text{B}\alpha$ /NF- κB , signaling could then be expanded to include an effect on the many other IL-1 responsive genes, such as other cytokines, proteases, receptors, and signaling protein/transcription factor genes which are controlled by NF- κB and AP-1. As discussed below, inhibiting ROCK could provide a potential therapeutic avenue to selectively allow IL-1 induced NF- κB activation while suppressing AP-1 mediated responses, or possibly enhancing JNK responses by enhancing ROCK activity.

That inhibiting ROCK had little effect on the IKK/ $\text{I}\kappa\text{B}\alpha$ signaling arm of IL-1 responses was confirmed at the level of IKK phosphorylation and activation and with $\text{I}\kappa\text{B}\alpha$ phosphorylation and degradation providing strong evidence for the lack of an effect. This was in contrast to the findings of Segain and co-workers [15] who found that inhibiting ROCK lead to a suppression of IL-1-stimulated NF- κB reporter activity, $\text{I}\kappa\text{B}\alpha$ phosphorylation and degradation, and IKK activation in the THP-1 macrophage cell line. Inhibiting ROCK has also been shown to have no effect on IL-1 induced CXCL8 responses in cervical stromal cells [16]. These results strongly suggest that the effect of ROCK inhibition, and hence the role of ROCK in IL-1 signaling, may be very cell-type specific.

Of note, a complete inhibition of IL-1-stimulated CXCL8/CCL2 secretion or mRNA levels with the Y27632 ROCK inhibitor was not seen. The significant decrease was probably due to the inhibition of JNK signaling, but not IKK/ $\text{I}\kappa\text{B}\alpha$ signaling, the latter which could be responsible for the significant residual CXCL8 responses. CXCL8 and CCL2 gene transcription is known to be regulated by both NF- κB and AP-1, which can synergize to greatly enhance cytokine secretion [25, 26]. Inhibition of JNK induced AP-1 activity would reduce the IL-1 induced transcription of these cytokine genes, but would not completely inhibit the response.

Now that ROCK has been shown to have an important role in the JNK signaling arm of IL-1 responses, a direct link between these two signaling pathways must be investigated. ROCK may exert an essential effect on the upstream kinases of JNK, such as MKK4 or MKK7, or even the kinase further upstream, which for IL-1 signaling is presumably TAK1 [9]. A possible effect on TAK1 would be interesting as this kinase is the pivotal kinase in the branch to IKK/ $\text{I}\kappa\text{B}\alpha$ and JNK signaling arms of the IL-1 pathway [2]. Of note, the ROCK1 isomer is known to be essential for focal adhesion formation [27] and the IL-1 Receptor Type I has been shown to localize to focal adhesions [28], suggesting that ROCK1 could very well be involved in IL-1 signaling events. In addition, we have found that IL-1 stimulation can induce a significant activation of ROCK1 kinase activity (data not shown and experiments addressing this avenue of research are now underway) providing a second link of ROCK1 to IL-1 responses.

Several recent reports have investigated the importance of JNK signaling and NF- κB signaling in IBD and animal models. High levels of phosphorylated JNK were shown to be localized to both leukocytes and epithelial cells in the inflamed mucosa of patients with IBD, as compared to normal controls [10]. Furthermore, two recent reports have shown that treatment of dextran sodium sulfate-induced colitic rats or mice treated with the specific JNK inhibitor, SP600125, resulted in reduced inflammation and wasting [11, 29]. Also, JNK inhibition resulted in significantly reduced levels of epithelial apoptosis [29]. These studies strongly suggest a role for JNK in the pathology of IBD. However, genetic knock-out of

JNK1 or JNK2 did not prevent the development of colitis in dextran sodium sulfate-treated mice, and in fact the JNK2 knockout exacerbated the colitis [30]. This suggests that targeting only one JNK isoform may not be an appropriate therapy for IBD, but instead broader spectrum treatments may be needed.

On the other hand, impairment of the gene for NEMO (IKK γ), a regulatory component of the IKK complex, and hence impaired signaling to NF- κ B, in IEC of mice resulted in a rapid development of colitis and a compromised integrity of the epithelium [31]. Furthermore, genetic knockout or specific inhibition of IKK β in dextran sodium sulfate-treated mice resulted in delayed healing of the colitis, suggesting a role for NF- κ B in acute colitis [32]. Studies have also shown that NF- κ B is expressed by IEC in wounded monolayers and NF- κ B activation is essential for wound healing [33]. These studies emphasize the importance of epithelial cell NF- κ B responses in inflammation and maintaining normal mucosal homeostasis [34, 35].

Therefore, treatments that may blunt JNK mediated signaling, while allowing NF- κ B signaling, especially in IEC, may provide an important mechanism to treat acute inflammation at the level of the IEC. This becomes more significant when one considers the accessibility of oral treatments for targeting epithelial cells in the intestine and colon. To this end, treatment of trinitrobenzene sulfonic acid induced colitic rats with the Y27632 ROCK inhibitor has been shown to reduce inflammation in these rats [15], and although they suggested an effect of ROCK inhibition on peripheral blood mononuclear cells and macrophages, a protective effect could have occurred on IEC through inhibiting JNK mediated responses while allowing IKK/I κ B α /NF- κ B responses to occur. This, coupled with our findings that inhibiting ROCK suppresses IL-1 induced pro-inflammatory cytokine responses by inhibiting JNK, but not IKK/I κ B α signaling, may provide a mechanism for the treatment of the epithelial specific effects of acute colitis.

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Abbreviations

FN	fibronectin
GAPD	glyceraldehyde-3-phosphate dehydrogenase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IKK	I κ B kinase
IL	interleukin
ITS	insulin, transferrin, selenium
RI	Y27632 ROCK inhibitor

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Highlights

- Inhibiting ROCK suppressed IL-1-stimulated secretion and mRNA levels for Caco-2 cell CXCL8 and IEC-6 cell CCL2 responses.
- Suppressing ROCK inhibited IL-1-induced JNK phosphorylation, but had no significant effect on IKK or I κ B α responses.
- Suggests a role for ROCK in IL-1-stimulated JNK signaling, but not IKK/I κ B α signaling, in intestinal epithelial cells.

A. Caco-2 **B. IEC-6** **Fig. 1.**

The effect of the ROCK inhibition on IL-1 induced CXCL8 or CCL2 secretion. (A) Caco-2 cells or (B) IEC-6 cells were cultured in FN coated wells for 1 hour. Cells were then treated with the Y27632 ROCK inhibitor (RI) in ITS-RPMI as indicated and cultured for 2 hours. Cultures were then stimulated with IL-1 β for 24 hours. The culture supernatants were harvested and analyzed for cytokine levels by specific ELISA and the adherent cells were removed and counted. The data represents the mean $\pm$ SEM from 3 separate experiments. Asterisk indicates a significant difference when compared to the IL-1 only sample ($p < 0.05$).

A. Caco-2 **B. IEC-6** **Fig. 2.**

Treatment of cells with the ROCK inhibitor suppressed IL-1 induced CXCL8 or CCL2 mRNA production. (A) Caco-2 cells were cultured for 1 hour and then treated with the Y27632 ROCK inhibitor for 2 hours. Cultures then were stimulated with IL-1 β for 6 hours before isolating total RNA for reverse transcription and PCR amplification for CXCL8 mRNA. For PCR, (a) 0.5 μ l, (b) 0.25 μ l, or (c) 0.125 μ l of cDNA was used each sample. Graph shows a comparison of the means $\pm$ SEM of the 0.5 μ l cDNA samples as the fold-increase of the ratios of the CXCL8 to GAPD band densities from 4 separate experiments. (B) IEC-6 cells were cultured as described with the ROCK inhibitor and IL-1 β . RNA extracts were analyzed by RT-PCR for CCL2 mRNA. Graph shows a comparison of the

means $\pm$ SEM of the 0.25 μ l cDNA samples as the fold-increase of the ratios of the CXCL8 to GAPD band densities from 4 separate experiments. Asterisk indicates a significant difference when compared to the IL-1 only sample ($p < 0.05$).

Fig. 3.

Treatment with the Y27632 ROCK inhibitor does not affect IL-1 induced I κ B α phosphorylation or degradation in Caco-2 cells. Caco-2 cells were treated with the ROCK inhibitor (100 μ M) and IL-1 β as described. At the times indicated, cytoplasmic extracts were harvested and separated by SDS-PAGE for Western blotting. (A) A representative blot probed for phospho-I κ B α , total I κ B α , and tubulin. (B) A graph of the ratios of the phospho-I κ B α to total I κ B α band density values. (C) A graph of the ratios of the total I κ B α to tubulin band density values. The data represents the mean $\pm$ SEM of 3 separate experiments and no significant differences were seen between samples treated with or without the ROCK inhibitor.

Fig. 4.

Inhibition of ROCK does not affect IL-1-stimulated IKK phosphorylation or specific kinase activity in Caco-2 cells. Caco-2 cells were cultured, treated with the Y27632 ROCK inhibitor and IL-1, and cytoplasmic extracts were collected as described in Fig. 3. (A) Shown is representative blot and a graph of the ratios of the phospho-IKK α/β to total IKK α/β band density values. (B) Shown is a representative blot of immunoprecipitated IKK α/β subjected to a kinase assay with GST-I κ B α as the target. A graph of the ratios of the phospho-I κ B α to total IKK α/β band density values shows no effect of the ROCK inhibitor on IKK kinase activity. The data shown represent the mean $\pm$ SEM for 3 separate experiments.

Fig. 5.

Inhibition of ROCK significantly decreases IL-1-stimulated JNK phosphorylation. Caco-2 cells were cultured and treated with 100 μ M (A) or 40 μ M (B) of the Y27632 ROCK inhibitor and IL-1 similar to as in Fig. 3. Shown are representative blots of Caco-2 cytoplasmic extracts probed for phospho-JNK and total JNK and a graph of the means $\pm$ SEM of the ratios of the Caco-2 phospho-JNK to total JNK band density values for the p46 and p54 isoform bands from four (A) or three (B) separate experiments. (C) IEC-6 cells were cultured and treated with the ROCK inhibitor and IL-1 β to as in Fig. 3. Shown is a representative blot of IEC-6 cytoplasmic extracts probed for phospho-JNK and total JNK. The graph is the means $\pm$ SEM of the ratios of the IEC-6 phospho-JNK to total JNK band density values for the p46 and p54 isoform bands from three separate experiments. Asterisk indicates a significant difference compared to IL-1 treated samples at the corresponding time points ($p < 0.05$).