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The Effect of ROCK on TNF- α -Induced CXCL8 Secretion by Intestinal Epithelial Cell Lines is Mediated Through MKK4 and JNK Signaling

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Abstract

Intestinal epithelial cells (IEC) play a role in mucosal inflammatory responses by producing important chemokines like CXCL8 when stimulated by TNF- α . Previously, we found that IEC cell lines required the Rho-associated kinase, ROCK, for CXCL8 responses after IL-1 stimulation. This study extends these findings by showing that inhibiting ROCK suppressed TNF- α -induced CXCL8 secretion by Caco-2 and DLD1 colonic epithelial cell lines and CXCL8 mRNA levels in Caco-2 cells. RNAi knockdown experiments indicated that the inhibitory effect was mediated by ROCK2, and not ROCK1. Inhibiting ROCK had no effect on TNF-stimulated I κ B α phosphorylation and degradation or p38 MAPK phosphorylation indicating that ROCK plays no role in these signaling pathways. However, inhibiting ROCK suppressed TNF-induced phosphorylation of the p54 JNK isoform and phosphorylation of the upstream MKK4 kinase. These results suggest that ROCK is required for CXCL8 responses by TNF-stimulated IEC by affecting intracellular signaling through MKK4 and JNK.

Keywords

CXCL8; intestinal epithelial; JNK; MKK4; ROCK; TNF

1. Introduction

The pro-inflammatory cytokine tumor necrosis factor- α (TNF- α) plays a central role in Inflammatory Bowel Disease (IBD) [1]. Elevated levels of TNF- α have been detected in tissues and serum of patients affected with IBD [2-4] and some current IBD treatments focus on specifically inhibiting the effects of TNF- α [1]. Intestinal epithelial cells (IEC) also play a role in mucosal inflammatory responses by secreting a variety of pro-inflammatory cytokines and chemokines [5], including the neutrophil chemoattractant CXCL8 [6]. Indeed,

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TNF- α has been found to stimulate chemokine secretion in several IEC cell lines [7-9]. Because of their large numbers, IEC are probably important local producers of pro-inflammatory chemokines.

Transduction of the TNF- α signal is highly complex and entails simultaneous activation of multiple signaling pathways leading to the activation of two main transcription factors: NF- κ B and AP-1 [10]. An essential step in the activation of NF- κ B is the phosphorylation and subsequent proteasomal degradation of I κ B α , which frees NF- κ B to allow its translocation into the nucleus [11]. The AP-1 family of transcription factors are composed of combinations of Jun, Fos, and ATF proteins, with the c-Jun/c-Fos form being the prototypical member of this family [12]. Furthermore, NF- κ B and AP-1 are known to synergize in the enhancement of CXCL8 gene transcription [13]. An important regulator of AP-1 is the c-Jun N-terminal kinase (JNK), which activates AP-1 by phosphorylating c-Jun in response to many different stimuli, including TNF- α [12]. JNK proteins exist as three isoforms resulting from alternative splicing of JNK genes to yield proteins of approximately 46 and 54 kDa. Two upstream kinases, MKK4 and MKK7, can phosphorylate and activate JNK. Another signaling kinase involved in TNF-induced responses is the p38 MAPK, which plays a role in the stabilization of cytokine mRNAs to enhance cytokine responses [12].

IEC attachment to extracellular matrix proteins requires the binding of cell surface integrins, which can activate the Rho family of small GTPases [14]. The Rho-associated kinase, ROCK, is a downstream effector for Rho and this kinase is associated with cellular processes that involve acto-myosin contractility, cytoskeletal rearrangement, and cell adhesion, including motility, stress fiber formation, and membrane blebbing during apoptosis [15-17]. One study has described a link between ROCK and intestinal inflammation [18]. Inhibition of ROCK reduced intestinal inflammation in rats with TNBS-induced colitis and suppressed the production of TNF- α and TNF-induced activation of NF- κ B by lamina propria or peripheral blood mononuclear cells isolated from inflamed tissues of TNBS-treated rats and from patients with Crohn's Disease. However, the effect of ROCK on cytokine responses and intracellular signaling in IEC was not addressed. We have recently found that inhibiting ROCK suppressed IL-1-stimulated CXCL8 secretion by the Caco-2 colonic epithelial cell line [19]. Yet ROCK was found to play no role in the IL-1 induced phosphorylation and degradation of I κ B α , or the phosphorylation and kinase activity of the upstream IKK suggesting that IL-1 signaling to NF- κ B was not affected. However, inhibiting ROCK did suppress the IL-1-induced phosphorylation of JNK in these experiments. This suggests that IL-1-induced CXCL8 responses by IEC may require ROCK activation for the activation of JNK signaling.

Because of the importance of TNF- α in mucosal inflammatory disease, we have extended our studies to include the effect of ROCK on TNF-induced intracellular signaling and CXCL8 responses in IEC. As IEC also respond to external cues through the integrins, which can result in the activation of ROCK [20], the activation of ROCK during epithelial migration in wound healing may greatly affect the potential for IEC to produce important chemokines like CXCL8 during mucosal inflammatory diseases. Similar to our previous findings with IL-1 β , inhibiting ROCK resulted in a significant reduction in the TNF- α -induced secretion of CXCL8. Furthermore, our results show a concomitant suppression of

TNF- α -induced phosphorylation of JNK and its upstream kinase MKK4, while ROCK inhibition had no effect on the TNF-stimulated activation of components of the NF- κ B and p38 pathways. The aim of this study was to explore the role of ROCK in TNF-induced intracellular signaling to CXCL8 secretion in IEC. Using the Caco-2 and DLD1 cell lines as models for IEC, we examined the effect of inhibiting ROCK on TNF-induced CXCL8 secretion, mRNA levels, and signaling pathways involved in IEC CXCL8 responses. The aim of this study was to explore the role of ROCK in TNF-induced intracellular signaling to CXCL8 secretion in IEC.

2. Materials and methods

2.1 Antibodies

All antibodies used in this study were obtained from Cell Signaling Technologies (Beverly, MA). These include rabbit polyclonal antibodies against JNK (recognizing both p46 and p54 isoforms), p38 MAPK, MKK4, phosphorylated MKK4 (Ser257/Thr261), ROCK2, and β -actin; rabbit monoclonal antibodies against phosphorylated p38 MAPK (Thr180/Tyr182), phosphorylated I κ B α (Ser32), and ROCK1, and mouse monoclonal antibodies against phosphorylated JNK (Thr183/Tyr185) and I κ B α . HRP-conjugated anti-mouse and anti-rabbit antibodies were used for detection in Western blots.

2.2 Cell culture

The Caco-2 and DLD-1 human colonic cell lines were obtained from the American Type Culture Collection (ATCC HTB-37 and CCL-221, respectively; Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Logan, UT) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY), 3.7 g/L sodium bicarbonate, 2 mM L-glutamine (Lonza, Walkersville, MD), non-essential amino acids (Lonza), and 25 IU penicillin with 25 μ g/mL streptomycin (Mediatech, Herndon, VA) referred to as FBS-DMEM. Cultures were maintained at 37 °C in a 10% CO₂ humid atmosphere.

2.3 Determination of CXCL8 secretion levels in culture supernatants

The cells were removed from flasks by treatment with a trypsin/EDTA solution (Sigma-Aldrich), suspended in FBS-DMEM, and added at 2×10^5 cells/well to wells of 24-well tissue culture plates previously coated with fibronectin (FN; Sigma-Aldrich St. Louis, MO) at 20 μ g/mL in PBS [19]. After a 1 hour incubation at 37 °C to allow for cell attachment, the medium was removed and the cells were treated with or without 40 μ M of the Y-27632 ROCK inhibitor (Enzo Life Sciences, Farmingdale, NY) in serum-free DMEM with insulin, transferrin, and selenium (ITS; BD Biosciences, Bedford, MA) and incubated for 2 hours. Then the cells were treated with rhTNF- α (R&D Systems, Minneapolis, MN) at 10 ng/mL for 24 hours. Culture supernatants were then collected and analyzed for CXCL8 content using a DuoSet ELISA kit (R&D Systems). Immediately after supernatant collection, trypsin/EDTA was added to each well, and the number of cells per well was determined by direct count using a hemacytometer. Color development in the ELISA plates was quantified using a Bio-Tek EL312e microplate reader (Bio-Tek Instruments Inc., Winooski, VT), and the resulting CXCL8 concentration values were normalized to 10^5 cells.

2.4 Real-time reverse transcription polymerase chain reaction (RT-PCR)

For examining mRNA levels, the Caco-2 cells were cultured at 1×10^6 cells/wells in 6-well plates as above. After incubating the cells with TNF- α for 6 hours, the culture medium was removed and the total RNA was extracted using the Qiagen RNeasy kit (Valencia, CA). The RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and a thermocycler for 10 min at 25 °C to allow for primer annealing, then 2 hours at 37 °C for the reverse transcription reaction, followed by 5 minutes at 85 °C to denature the enzyme.

The resulting cDNA samples were then analyzed by real-time PCR. Each 20 μ L reaction contained 2 μ L of cDNA sample, 10 μ L of iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA), 0.8 μ L of 10 μ M forward/reverse primer mix, and 7.2 μ L RNase-free water. Human CXCL8 and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer mixes were purchased from SABiosciences (Valencia, CA). Real-time PCR was performed using a BioRad MiniOpticon thermocycler running the following program: 95 °C for 3 minutes, then 40 two-step cycles of 95 °C for 15 seconds and 60 °C for 60 seconds, followed by a melt curve determination. Relative transcript levels were calculated using the 2^{-C_t} method, and single-product amplification was verified by examination of the melt curves.

2.5 RNA interference

For the knockdown of ROCK1 or ROCK2, Caco-2 cells in antibiotic-free FBS-DMEM at 5×10^4 cells/well were added to FN-coated 24-well culture plates and were cultured for 24 hours. On the day of the transfection, 25 pmoles of siRNA/well for human ROCK1 or ROCK2, or of a Control siRNA that does not activate the RISC complex (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted in equal parts of Transfection Reagent (Santa Cruz Biotechnology) and then this mixture was diluted 1/10 in Transfection Medium (Santa Cruz Biotechnology). After incubating 45 minutes at room temperature, the siRNAs were further diluted 1/5 in Transfection Medium. The culture supernatants were then replaced with 250 mL of the mixture containing the diluted siRNAs. The cultures were incubated for 6 hours before adding 250 mL of 20% FBS-RPMI 1640 (Cellgro) and incubating for 18 hours. At this time, the cells in some cultures were lysed as described in the next section and the cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies for ROCK1, ROCK2 or β -actin to determine the percent knockdown in each experiment. For the remaining wells, the supernatants were replaced with FBS-RPMI with or without 10 ng/mL TNF- α and the culture was incubated for 24 hours before collecting culture supernatants for determination of CXCL8 content and counting cells as above.

2.6 Western blot analysis

To determine levels of intracellular proteins, Western blot analysis was performed on cytoplasmic extracts. Briefly, the Caco-2 cells were seeded in 12-well plates at 1×10^6 cells/well, treated with the ROCK inhibitor as indicated above, and then incubated for 0 to 45 minutes with or without 10 ng/mL rhTNF- α . The cells were then washed with cold PBS and scraped from culture plates in the presence of 100 μ L/well of cell lysis buffer as previously described [19]. The suspensions were then incubated on ice for 10 minutes before

centrifuging at 13,000g for 5 minutes to remove nuclei and debris. Protein concentrations were determined with the Bio-Rad DC protein assay kit and stored at -80°C .

Cytoplasmic extracts were diluted to equal protein amounts with Laemmli buffer and heated to 95°C for 5 minutes. The proteins were then separated by SDS-PAGE on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes with the BioRad Mini Trans-blot system as previously published [19]. The membranes were blocked in 5% bovine serum albumin (BSA; Fisher Scientific, Fair Lawn, NJ) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 hours at room temperature. To probe for specific proteins, the membranes were incubated with primary antibody in 5% BSA/TBST overnight at 4°C with constant rocking. The membranes were then washed and incubated at room temperature with HRP-conjugated secondary antibody in 2.5% BSA/TBST for 2 hours, then washed again with TBST. Protein bands were detected using either the LumiGLO Reagent (Cell Signaling Technologies) or SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and images were captured by exposure on x-ray film. For reprobing, membranes were washed twice in TBST, incubated in stripping buffer [19], and washed an additional 3 times in TBST before being reprocessed starting at the blocking step described above. Densitometry was performed by scanning films and analyzing images using the Bio-Rad Quantity One software.

2.7 Statistics

Experiments were run in 3 or more replicates and the statistical significance of any observed differences in treatment effects was determined using ANOVA with Fisher's protected least significant difference post-hoc test. Differences with $p < 0.05$ are reported as significant.

3. Results

3.1 The effect of inhibiting ROCK on TNF- α -induced CXCL8 responses

Our previous study showed that ROCK played an important role in CXCL8 secretion by IL-1 stimulated Caco-2 epithelial cells [19] suggesting that ROCK was important in the secretion of CXCL8 by IEC. We extended those studies by determining the effect of inhibiting ROCK activity on CXCL8 secretion by IEC stimulated with TNF- α , a potent pro-inflammatory cytokine also important in IBD. For these studies, the Y27632 ROCK inhibitor (RI) was once again used. This inhibitor inhibits both the ROCK1 and ROCK2 isoforms equally, but has little or no effect on 23 other kinases [21]. Furthermore, this inhibitor has an affinity for ROCK that is 20-30 times greater than for other similar kinases in the Rho effector group like PRK (PKN) and the citron kinase [22].

To investigate the role of ROCK in TNF- α -stimulated CXCL8 secretion, Caco-2 or DLD-1 colon epithelial cells were cultured in FN-coated wells and incubated for 1 hour to allow for attachment, followed by treatment with the Y-27632 inhibitor. After an additional 2 hours, the cells were treated with or without TNF- α and incubated for 24 hours. Analysis of the resulting culture supernatants showed that levels of CXCL8 secreted by cells treated with the ROCK inhibitor were similar to levels secreted by untreated cells for both cell lines (Fig. 1). However, Caco-2 cells treated with TNF- α in addition to the ROCK inhibitor yielded a

significant 46% suppression of CXCL8 secretion compared to cells treated with TNF- α alone (Fig. 1A). A similar 53% suppression in CXCL8 secretion was seen with DLD-1 cells (Fig. 1B) confirming the results.

To determine if the ROCK inhibitor was suppressing CXCL8 secretion by regulating mRNA levels, Caco-2 cells were treated with or without the ROCK inhibitor followed by a 6 hour incubation with 10 ng/mL TNF- α . Real-time RT-PCR analysis revealed that inhibiting ROCK had a significant 82% suppressive effect on TNF- α -induced CXCL8 mRNA levels (Fig. 2). Additionally, inhibiting ROCK also suppressed basal levels of CXCL8 mRNA by 90%. Taken together with the secretion experiments above, our findings suggest that ROCK may play an important role in regulating TNF-induced CXCL8 responses in IEC.

Although specific for ROCK1 and 2, the Y27632 ROCK inhibitor may have affected other Rho effector kinases (e.g. PRK1/PKN1 or PRK2) [23], which could have contributed to the inhibitory effect on TNF-induced CXCL8 responses. In addition, it is unclear if the effect was due to the ROCK1 or ROCK2 isoform. To address these issues, RNA interference (RNAi) was used to knockdown ROCK1 and ROCK2 levels in the Caco-2 cells 24 hours prior to addition of the TNF- α stimulus. Treatment of the Caco-2 cells with specific siRNAs resulted in a 22% knockdown of ROCK1 protein levels or a 29% knockdown of ROCK2 protein levels (Fig. 3) compared to cells treated with a random sequence Control siRNA that does not induce the RISC Complex (Santa Cruz Biotechnology). As shown in Figure 3, knockdown of ROCK1 did not significantly affect TNF-induced CXCL8 secretion. However, knockdown of ROCK2 resulted in a significant 42% suppression of TNF-stimulated CXCL8 secretion as compared to the Control siRNA treated cells. This confirms that suppression of ROCK2 can result in a suppression of CXCL8 responses by TNF-stimulated Caco-2 cells.

3.2 Effect of ROCK inhibition on TNF- α -induced phosphorylation of I κ B α and p38 MAPK

The activation of NF- κ B by TNF- α signaling plays a major role in inflammatory signal transduction. An important event in this pathway is the phosphorylation and subsequent proteasomal degradation of I κ B α , which, under unstimulated conditions, binds to NF- κ B and prevents its translocation to the nucleus [10]. Our previous study on the role of ROCK in IL-1-stimulated CXCL8 responses by IEC indicated that the phosphorylation and degradation of I κ B α was not affected by inhibiting ROCK, suggesting that intracellular signaling to NF- κ B was not affected. Similarly, pre-treatment of the Caco-2 cells with the ROCK inhibitor had no effect on the TNF-induced phosphorylation or degradation of I κ B α (Fig. 4A and B). This suggests that the NF- κ B pathway was activated by TNF- α and the inability of ROCK inhibition to suppress I κ B α phosphorylation and its subsequent degradation indicates that ROCK probably does not participate in signaling to NF- κ B activation.

The activation of p38 MAPK has also been shown to be important in the regulation of CXCL8 secretion in some cell types. One study has shown that inhibiting ROCK suppressed p38 MAPK kinase activity in human lung microvascular endothelial cells [24]. However, as shown in Fig. 4C, TNF- α stimulation of the Caco-2 cells resulted in an increased phosphorylation of p38 MAPK within 15 minutes, but inhibiting ROCK had no significant

effect on phosphorylated p38 MAPK levels at any time point. As p38 MAPK is a major signaling pathway leading to mRNA stability, we also examined the effect of inhibiting ROCK on TNF-induced CXCL8 mRNA stability after treatment with actinomycin D. Inhibiting ROCK had no effect on CXCL8 mRNA stability even after 120 minutes (data not shown), which supports our experiments on p38 MAPK phosphorylation.

3.3 The effect of inhibiting ROCK on the activation of JNK and MKK4 in TNF- α -treated Caco-2 cells

TNF- α stimulation also results in downstream signaling to JNK, resulting in the phosphorylation of c-Jun to form the AP-1 transcription factor [10]. Previously, we had found that inhibiting ROCK suppressed the IL-1-induced phosphorylation of JNK [19]. In the present study, inhibiting ROCK resulted in a significant 50% suppression of TNF-induced phosphorylation of the p54 JNK isoform at 15 minutes (Fig. 5A and B), yet inhibiting ROCK had no effect on the p46 JNK isoform, which was not activated by TNF- α in the Caco-2 cells. This indicates a significant variation from the effect of ROCK on IL-1 signaling noted previously.

The upstream kinases responsible for activating JNK are MKK4 and MKK7 [12]. Western blotting of Caco-2 cell extracts from TNF-stimulated cells showed that MKK4 was rapidly phosphorylated within two minutes and inhibiting ROCK suppressed the phosphorylation of MKK4 at this time point by 78% compared to cells treated with TNF- α only (Fig. 5C and D). However, treatment of the cells with TNF- α , with or without inhibiting ROCK, did not alter the phosphorylation of MKK7 (data not shown). This suggests that TNF- α signals through MKK4, but not MKK7, to activate the p54 JNK and ROCK plays a role in the signaling events leading to MKK4 and JNK activation.

4. Discussion

TNF- α plays a central role in IBD and is the target of current IBD treatments [1]. Despite the advanced nature of these treatments, there are still risks of potentially serious side-effects so studies investigating mechanisms to target the inflammatory effects of TNF- α are warranted. Expanding our knowledge of how TNF- α and other pro-inflammatory cytokines, such as IL-1, promote inflammation in the intestinal mucosa is essential if new therapies are to be developed. As important components of the intestinal mucosa, IEC can produce several immunoregulatory factors, including CXCL8 [7, 8], a major chemoattractant and activator of neutrophils. Elevated levels of CXCL8 that are associated with IBD may play an important role in the pathogenesis of this disease [1] and, therefore, examining the regulatory mechanisms of cytokine-stimulated CXCL8 production may provide clues in the search for new treatments.

In this study, we present evidence for the involvement of the Rho-associated kinase, ROCK, in the regulation of TNF-induced CXCL8 responses in IEC. Similar to previous experiments with IL-1 stimulated Caco-2 cells [19], inhibiting ROCK in Caco-2 and DLD1 cells resulted in a marked decrease in TNF-induced CXCL8 secretion and a decrease in CXCL8 mRNA levels in Caco-2 cells. Furthermore, the involvement of ROCK2, but not ROCK1, in the effect was confirmed by RNAi knockdown experiments. Previous studies have shown that

ROCK1 and 2 can have similar, as well as differential functions. ROCK1 has been shown to be involved in actin stress fiber and focal adhesion formation [25] while ROCK2 is important in phagocytosis [25], cell contractility [26], angiogenesis [27] and keratinocyte differentiation [28]. Although these studies imply that ROCK2 plays an important role in cell movement and differentiation, how ROCK2 is activated and affects intracellular signaling in our studies remains to be determined.

Investigating the effect of ROCK on CXCL8 responses by IEC, our previous study found that inhibiting ROCK had no effect on signaling to I κ B α phosphorylation and degradation in IL-1 stimulated cells [19]. Likewise, inhibiting ROCK had no effect on the TNF-induced I κ B α phosphorylation and degradation in the present study. The p38 MAPK has also been shown to regulate CXCL8 production, both at the transcriptional and post-transcriptional level [29, 30]. Our study shows that TNF- α induced an increase in p38 MAPK phosphorylation, yet this effect was unchanged by pre-treating the cells with ROCK inhibitor. These findings suggest that ROCK plays no role in TNF-stimulated p38 MAPK or I κ B α /NF- κ B intracellular signaling pathways in IEC.

In our previous study on the effect of ROCK on IL-1 stimulated CXCL8 responses [19], inhibiting ROCK suppressed phosphorylation of both the p46 and p54 isoforms of JNK. However, inhibiting ROCK affected TNF-stimulated phosphorylation of only the p54 isoform of JNK, as TNF-stimulation did not induce the phosphorylation of the p46 JNK in these cells. The implications of this preferential activation of p54 JNK in these cells are, as yet, unclear. However, due to differences in substrates among the JNK isoforms, this could provide a potential mechanism to regulate the activation of specific subsets of AP-1 proteins. Examining the upstream kinase that phosphorylates JNK, MKK4 (but not MKK7) was found to be phosphorylated after TNF- α stimulation, and inhibiting ROCK suppressed this phosphorylation. We have also found that the phosphorylation of MKK4 was suppressed by inhibiting ROCK in IL-1-stimulated Caco-2 cells as well (unpublished results). This suggests that MKK4 is probably the upstream kinase of JNK in these cells and that the effect of ROCK may be on this kinase or signaling components further upstream in the signaling pathway. We are now in the process of investigating the effect of inhibiting ROCK on potential kinases upstream of MKK4. Both TAK1 [10] and MEKK1 [31] have been shown to be upstream kinases of JNK activation after TNF- α stimulation and present possible candidates for the effect of ROCK on this pathway. Alternatively, ROCK could affect the scaffold protein (JIP1, 2, 3, etc.) that may be involved in TNF- α signaling to JNK. Regardless, these findings indicate that for IEC, the activation of ROCK is necessary for the activation of MKK4/JNK in response to TNF- α stimulation.

A role for ROCK in the activation of JNK in other systems has been noted. Inhibiting ROCK in lysophosphatidic acid (LPA)-treated human umbilical vein endothelial cells abrogated both JNK and c-Jun phosphorylation in addition to suppressing LPA-induced CXCL8 mRNA expression and secretion [32]. Yet, NF- κ B activation in these LPA-stimulated umbilical vein endothelial cells was also dependent upon ROCK, although CXCL8 production in these cells was shown to be regulated independently of NF- κ B. JNK has also been shown to be necessary for ROCK-mediated remodeling of epithelial tight junctions [33]. However, Segain *et al.* [18] demonstrated that ROCK can play a role in the

activation of the NF- κ B pathway in peripheral blood mononuclear cells and a monocyte cell line when stimulated with IL-1. These studies underscore the importance of ROCK in intracellular signaling, but suggest that the pathways affected by ROCK are highly dependent upon the cell type and condition. Yet for IEC, our results suggest that ROCK activation is necessary for MKK4/JNK signaling and the production of CXCL8 in response to IL-1 and TNF- α stimulation. However, ROCK could possibly have other effects on CXCL8 mRNA translation or protein trafficking and secretion, which were not addressed in this study.

Another point to note is that ROCK inhibition did not result in a complete suppression of TNF- α -induced CXCL8 secretion or mRNA expression. This is not surprising considering TNF- α activates signaling pathways leading to both NF- κ B and AP-1 [10], which are both known to regulate CXCL8 transcription [13]. The suppression of JNK signaling by the ROCK inhibitor probably accounted for the observed reduction in CXCL8 levels, while the inability of ROCK inhibitor treatment to affect I κ B α phosphorylation and degradation in our experiments suggests that the remaining CXCL8 production might be mediated by NF- κ B. It is also possible that the residual CXCL8 levels could be due to the action of p38 MAPK, which we also determined to be activated by TNF- α in a ROCK-independent manner.

ROCK is an important regulator of cell movement and cytoskeletal rearrangement [15, 17]. Because cell movement is essential for the wound healing process, it is likely that the edges of intestinal ulcerations in IBD lesions may be areas of high ROCK activity in IEC. Our results suggest that ROCK may also play a role in the cytokine responses of IEC. For our experiments, the Caco-2 or DLD1 cells were treated with TNF- α three hours after plating and were sub-confluent throughout the entire duration. During this period, the cells attached, spread, and began migrating on the FN matrix, processes that require the formation and turnover of focal adhesions and stress fibers and that are also known to involve ROCK activation [15, 17]. Similarly, during healing, IEC at the wound edge also spread and migrate to close the wound in a Rho/ROCK dependent manner [5, 34]. Therefore, the sub-confluent migrating cells in our experiments could be similar to IEC at a wound edge where ROCK is active in the migrating cells and would be present to affect TNF- α or IL-1-stimulated CXCL8 responses by the cells. In this scenario, the subsequent increase in CXCL8 near the wound edge could direct neutrophils to the wound opening where they could combat any invading microbes. In addition, CXCL8 has been shown to enhance epithelial cell migration in *in vitro* wounded monolayers [35] suggesting a second potential function for the secreted CXCL8. Therefore, ROCK may be an important and essential player in the overall response of IEC during intestinal wound closure as well as during inflammation. However, we must now determine if CXCL8 responses and intracellular signaling is different in these migrating cells as compared to non-migrating cells in confluent monolayers of cells.

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Abbreviations

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

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Highlights

- Inhibiting ROCK suppressed TNF-stimulated CXCL8 secretion
- Inhibiting ROCK suppressed TNF-stimulated CXCL8 mRNA levels
- Suppressing ROCK had no significant effect on TNF-induced I κ B α responses
- Suppressing ROCK inhibited TNF-induced JNK and MKK4 phosphorylation
- ROCK has a role in TNF-induced JNK signaling to CXCL8 responses in epithelial cells

Fig. 1.

The effect of inhibiting ROCK on TNF-induced CXCL8 secretion by Caco-2 and DLD1 colonic epithelial cells. Caco-2 (**A**) or DLD1 (**B**) cells were added to FN-coated plates and incubated for 1 hour before treatment with 40 μ M Y27632 ROCK inhibitor (RI) in ITS-DMEM. After 2 hours, TNF- α (10 ng/ml) was added and the cells were incubated for 24 hours. The culture supernatants were then collected for 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 TNF- α only sample ($p < 0.05$).

Fig. 2.

Inhibiting ROCK suppressed TNF-induced CXCL8 mRNA production. Caco-2 cells were cultured in FN-coated plates and treated with the Y27632 ROCK inhibitor as in Fig. 1. Cultures then were stimulated with TNF- α for 6 hours before isolating total RNA for reverse transcription and analysis by real-time PCR. CXCL8 transcript levels were normalized to GAPDH transcript levels and expressed as a value relative to the unstimulated controls. Shown are the means $\pm$ SEM of 3 independent experiments. Asterisk indicates a significant difference when compared to the TNF- α only sample ($p < 0.0001$)

Fig. 3. RNAi knockdown of ROCK2, but not ROCK1, significantly inhibits TNF-induced CXCL8 secretion by Caco-2 cells. Caco-2 cells were cultured in FN-coated wells for 24 h before adding Control or specific siRNAs into the cells. After 24 hours, some cells were collected for assessing the knockdown of ROCK1 and 2 by Western blot (**A**). The medium was then replaced in the remaining cultures with FCS-RPMI with or without TNF- α and the cells were cultured for 24 hours. The culture supernatants were then collected for CXCL8 ELISA and the cells counted to correct for cell numbers (**B**). Shown are the means $\pm$ SEM of 3 separate experiments. Asterisk indicates a significant difference when compared to the TNF- α only sample treated with the Control siRNA ($p < 0.01$).

Fig. 4.

Inhibiting ROCK had no effect on the phosphorylation of I κ B α or p38 MAPK in TNF- α -stimulated Caco-2 cells. Caco-2 cells were cultured in FN-coated wells with the ROCK inhibitor and TNF- α similar to as in Figure 1. After the times indicated, cytoplasmic extracts were prepared and examined by Western blot. (A) A representative blot of phosphorylated and total I κ B α . (B) Graph shows the means $\pm$ SEM of phosphorylated I κ B α band densities normalized to total I κ B α from three separate experiments. (C) A representative blot of

phosphorylated and total p38 MAPK. No statistically significant differences were found between cells pre-treated or not pre-treated with the ROCK inhibitor.

Fig. 5.

The effect of inhibiting ROCK on JNK and MKK4 phosphorylation in Caco-2 cells treated with TNF- α . Caco-2 cells were treated as in Fig. 4 with the ROCK inhibitor and TNF- α for the specified times. **(A)** A representative blot of phosphorylated and total JNK. **(B)** A graph of the ratio of phosphorylated p54 JNK band densities to total p54 JNK ($\pm$ SEM) from 3 separate experiments. **(C)** A representative blot for phosphorylated and total MKK4. **(D)** A graph of the ratio of phosphorylated MKK4 to total MKK4 from 3 separate experiments.

Asterisk indicates a significant difference between cells pre-treated or not pre-treated with the ROCK inhibitor ($p < 0.05$).