Original Article

Rebamipide suppresses TLR-TBK1 signaling pathway resulting in regulating IRF3/7 and IFN-α/β reduction

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TANK-binding kinase 1 (TBK1) regulates the interferon regulatory factor (IRF) 3 and IRF7 activation pathways by double strand RNA (dsRNA) via Toll-like receptor (TLR) 3 and by lipopolysaccharide (LPS) via TLR4. Rebamipide is useful for treating inflammatory bowel disease (IBD). Although IBD is associated with nuclear factor-κB (NF-κB), any association with the TBK1-IRF pathway remains unknown. How rebamipide affects the TBK1-IRF pathway is also unclear. We analyzed the relationship between IBD (particularly ulcerative colitis; UC) and the TLR-TBK1-IRF3/7 pathway using human colon tissue, a murine model of colitis and human colonic epithelial cells. Inflamed colonic mucosa over-expressed *TKB1*, *NAP1*, *IRF3*, and *IRF7* mRNA compared with normal mucosa. TBK1 was mainly expressed in inflammatory epithelial cells of UC patients. The expression of *TBK1*, *IRF3*, *IRF7*, *IFN-α* and *IFN-β* mRNA was suppressed in mice given oral dextran sulfate-sodium (DSS) and daily rectal rebamipide compared with mice given only DSS. Rebamipide reduced the expression of *TBK1*, *IRF3* and *IRF7* mRNA induced by LPS/dsRNA, but not of *NF-κB* mRNA in colonic epithelial cells. Rebamipide might suppress the TLR-TBK1 pathway, resulting in IRF3/7-induction of IFN-α/β and inflammatory factors. TBK1 is important in the induction of inflammation in patients with UC. If rebamipide represses the TLR-TBK1 pathway, then rectal administration should suppress inflammation of the colonic mucosa in patients with UC.

Key Words: **TANK-binding kinase 1, toll-like receptor 3/4, interferon regulatory factor 3/7, rebamipide, inflammatory bowel disease**

The pathogenesis of inflammatory bowel disease (IBD) remains unclear. The immune response to viral or bacterial infection is thought to be one etiology of intestinal inflammation. Toll-like receptors (TLR) induce innate immune responses by recognizing invading microbial pathogens that cause the activation of adaptive immune responses.[1,2] In TLRs, TLR3 and TLR4 impart ligand-specific recognition of double-stranded RNA (dsRNA) of viruses and of bacterial lipopolysaccharide (LPS), respectively.[3,4] The LPS- or polyinosine:polycytidine (poly(I:C))-induced activation of the Toll/IL-1R domain-containing adaptor inducing interferon (IFN)-β (TRIF; TICAM-1), which is an adaptor that functions independently of MyD88, leads to the delayed activation of nuclear factor-κB (NF-κB).[5,6] TRIF also induces activation of the transcriptional regulator, IFN regulatory factor (IRF) 3, and the expression of IFN-β and of IFN-inducible genes through the activation of TANK-binding kinase 1 (TBK1) and inhibitor of kappaB kinase ε (IKKε).[7,8] TLR3 activates primarily the TRIF pathway, whereas TLR4 activates both MyD88- and TRIF-dependent pathways—Both IKKε[9,10] and TBK1[11–13] are key regulators of the IRF3 and IRF7 activation pathways in cells that have been exposed to viruses and/or activated by dsRNA via TLR3.[7,14] NF-κB is induced by poly(I:C)/LPS through TLR3/4,[15] and NF-κB activation is required for the release of proinflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF).[16]

The initiation and perpetuation of the inflammatory intestinal responses in IBD might result from an exaggerated host defense reaction of the intestinal epithelium to endogenous luminal bacterial flora and viruses via TLR3/4-signaling. TLR3 is significantly down-regulated in intestinal epithelial cells from patients with active Crohn's disease (CD) but not in those with ulcerative colitis (UC). In contrast, TLR4 is obviously up-regulated in both UC and CD.[17] Moreover, a relationship has been identified between IBD and NF-κB,[18] but not between IBD and the TBK1-IRF pathway.

Rebamipide is widely used in Japan to treat gastric ulcers[19] and gastric injury.[20] Recently, there was a report of administered rebamipide enemas to a patient with IBD complicated by proctitis.[21] The therapeutic efficacy of rebamipide has been independently confirmed by others using a model of colitis induced by acetic acid[22] or dextran sulfate-sodium (DSS).[23,24] However, the relationship between rebamipide and TBK1 has not been described.

Here, we analyzed the relationship between IBD (in particular, UC) and the TLR-TBK1-IRF3/7 pathway. We then evaluated the effect of rebamipide on the TBK1-IRF3/7-IFN-α/β pathway and on the NF-κB activation pathway using colonic epithelial cells and mice with colitis induced by DSS.

Material and Methods

Human samples and tissue collection. Biopsy specimens of normal and moderately or severely inflammatory mucosa obtained during colonoscopy of 10 patients with UC at Nagoya City University Hospital between 2005 and 2007 were stored at −80°C for mRNA detection.

The Ethics Committee of Nagoya City University Graduate School of Medical Sciences granted approval for this study and written informed consent was obtained from all patients to participate in all procedures associated with the study.

Immunohistochemistry of UC patients. Immunohistochemical staining of colon tissues was performed with antibodies

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Table 1. Human primer sets

	Upper primer	Lower primer
IRF3	tcttccagcagaccatctcc	tgcctcacgtagctcatcac
IRF7	cagatccagtcccaaccaag	gtctctactgcccacccgta
TBK1	agcggcagagttaggtgaaa	ccagtgatccacctggagat
NAP1	tgggaggtggaaaagttgag	ccgtttgtttggctttctgt
NF-κB	tctgcttccaggtgacagtg	atcttgagctcggcagtgtt
ARP	cgaagccacgctgctgaacatgctcaac	gctgccattgtcgaacacctgctggatg

Table 2. Mouse primer sets

	Upper primer	Lower primer
TBK1	gagagctggaggacgatgag	acggtagccccgtacttctt
IRF3	gatggagaggtccacaagga	gagtgtagcgtggggagtgt
IRF7	cctcttgcttcaggttctgc	gctgcatagggttcctcgta
IFN-α	agtgagctgacccagcagat	agacagccttgcaggtcatt
IFN-β	ccctatggagatgacggaga	acccagtgctggagaaattg
β-Actin	gatctggcaccacaccttct	ggggtgttgaaggtctcaaa

against TBK1 (EP611Y, abcam, Tokyo, Japan; dilution 1:50). The procedure was performed with the appropriate positive and negative controls. Briefly, 3-μm-thick sections were deparaffinized and hydrated through a graded series of alcohols. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2/methanol solution, antigen retrieval was achieved by heating the samples in 10 mM citrate buffer (pH 6.0) using a microwave oven for 10 min at 98°C. Then, sections were incubated with the primary antibody. After thorough washing in PBS, the samples were incubated with biotinylated the secondary antibody and then with avidin-biotin horseradish peroxidase complexes (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA). Finally, immune complexes were visualized by incubation with 0.01% H_2O_2 and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB).

Experimental procedures of DSS mice. Eight-week-old female C57BL/6 mice were housed under conventional conditions in a temperature-controlled room with a 12 h light/dark cycle. Colitis was induced in the mice by orally administering 3% dextran sulfate sodium (DSS; MP Biomedicals Inc., Morgan, CA) in distilled water daily for 5 days. The mice were assigned to groups as follows: Control (no DSS and no rebamipide), DSS (oral DSS alone), DSS + rebamipide (oral DSS plus daily rectal administration of 50 mg/kg/day of rebamipide dissolved in 100 μl of 0.5% carboxymethylcellulose (CMC; Wako Pure Chemical Industries Ltd., Osaka, Japan). All mice were sacrificed 5 days after DSS administration was started. The Animal Care Committee of Nagoya City University approved the study protocol.

Histopathology of DDS mice colon tissues. All specimens were routinely processed and stained with hematoxylin and eosin for histological examination.

Cell culture. Human colonic cancer cells (CaCo2; ATCC number, HTB-37) were seeded in 6-cm dishes at a density of 2×10^6/dish and cultured for 48 h with RPMI1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum, 1% ampicillin and streptomycin in 5% CO_2.

Reagents. Lipopolysaccharide (LPS) from Escherichia coli O55:B5 and poly(I:C) were purchased from Sigma Chemical Co. and Amersham Biosciences (Pittsburgh, PA), respectively. Rebamipide was provided by Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan).

Experimental procedures of human colonic epithelial cells. Sub-confluent CaCo2 cells were incubated with Poly(I:C) or LPS at 37°C for 24 h to examine the TLR3 and TLR4 signaling pathways, respectively, in the presence or absence of rebamipide. Thereafter mRNA and protein expression were examined in the cells as described below.

Real-time RT-PCR. Total RNA was isolated from CaCo2 cells using TRIzol (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using Superscript II (Invitrogen) according to the manufacturer's instructions. Primers for human TLR3, TLR4 and control human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems (CA; TLR3, Hs00152933 m1; TLR4, Hs01061963 m1; GAPDH, 4310884E). The primers for human TBK1, NAP1, IRF3, IRF7, NF-κB and control human acid ribosomal phosphoproteins (ARP) are listed in Table 1 and those for mouse TBK1, IRF3, IRF7, IFN-α, IFN-β and control mouse β-actin are listed in Table 2. Real-time RT-PCR for *TLR3* and *TLR4* proceeded using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Real-time RT-PCR proceeded in a 20-μl volume containing 18 μl of TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 1 μl cDNA and 1 μl primers. Reactions for *TBK1*, *NAP1*, *IRF3*, *IRF7*, *IFN-α*, *IFN-β*, control human *ARP* and control mouse *β-actin* proceeded in a 20-μl volume containing 18 μl of Power SYBR Green PCR Master Mix (Applied Biosystems), 1 μl cDNA and 20 μM primers. Uniform amplification of the products was reconfirmed by analyzing the melting curves of the amplified products. All reactions proceeded in triplicate to assess reproducibility.

Western blotting. CaCo2 cells were washed with PBS (−) and subsequently dissolved in 1× lysis buffer (Cell Signaling Technology) containing 20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l Na_2EDTA, 1 mmol/l EGTA, 1% Triton, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, and 1 μg/ml leupeptin. Cells were disrupted using a Bio-ruptor sonicator (Cosmo Bio, Tokyo, Japan) for 15 s, and then lysates were centrifuged at 15,000 rpm for 10 min at 4°C. All samples were normalized to an equal protein concentration using a protein assay kit (Bio-Rad Laboratories, CA). An equal quantity of 2× SDS-PAGE sample buffer (0.5 mol/l Tris-HCl (pH 7.2), 1% SDS, 100 mmol/l β-mercaptoethanol, and 0.01% bromophenol blue) was added to the samples, and then the mixtures were boiled for 5 min at 100°C. Portions of boiled samples were fractioned on 7.5%, 10% or 12.5% SDS-PAGE gels and then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Non-specific binding on the membranes was blocked with 5% skimmed milk in PBS (−) for 1 h at room temperature. The membranes were incubated with TBK1 monoclonal antibody (clone: 108A429, Gene Tex, Inc., Irvine, CA) overnight at 4°C

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followed by three washes with 0.05% Tween 20 in PBS (–) at 5-min intervals. The membranes were incubated with an appropriate secondary antibody for 1 h at room temperature, followed by three washes with 0.05% Tween 20 in PBS (–) at 5-min intervals. Immunoreactive proteins were visualized using the ECL Plus Western blotting Detection system (Amersham Biosciences). Filters were stripped and reprobed using monoclonal β-actin antibody (Abcam Plc., Cambridge, England) as an internal control.

Immunofluorescence microscopy. CaCo2 cells in a sub-confluent state were incubated with Poly(I:C) or LPS at 37°C for 24 h respectively, in the presence or absence of rebamipide. Thereafter TBK1 was analyzed by immunofluorescence study. Cells were fixed with ethanol and acetone. Incubation with primary antibody of TBK1 was performed in a solution of PBS containing 0.1% milk at room temperature. Then, sections were incubated with the appropriate secondary antibody and all sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Kirkegaard and Perry Laboratories). Images were obtained with an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

Results

Inflammatory mucosa of UC over-expressed TBK1-IRF3/7 signaling pathway. We first performed real-time RT-PCR of *TBK1*, *NAP1*, *IRF3*, and *IRF7* to confirm the relationship between the mRNA expression of these genes and UC in 10 patients. The mRNA expression of all these genes was higher in atypical, than in normal mucosa from the patients (Fig. 1). These results indicated that the inflammation associated with UC is related to the TBK1-IRF3/7 signaling pathway.

Immunohistochemical analysis of TBK1 in UC. We performed immunohistochemical staining of TBK1 in UC tissues from humans. TBK1 was mainly expressed in obviously inflammatory colon epithelial cells of crypts (Fig. 2 A and B). On the other hand, TBK1 was hardly expressed in colon epithelial cells with weak inflammation (Fig. 2 C and D).

Histological findings in the colons of DDS mice. Crypts were diffusely absent and considerable numbers of inflammatory cells had infiltrated colon specimens from mice in the DSS group (Fig. 3B), whereas few crypts had disappeared and inflammatory cell infiltration was minimal in the DSS + rebamipide group (Fig. 3C).

Rebamipide suppressed TBK1-IRF3/7-IFN-α/β signaling pathway in DSS mice. To determine the effect of rebamipide on the TLR-TBK1 signaling pathway in colitis, we performed real-time RT-PCR of *TBK1*, *IRF3*, *IRF7*, *IFN-α* and *IFN-β* on colon specimens from DSS and from DSS + rebamipide groups. The mRNA expression of all these genes was increased due to inflammation in the colon of DSS mice, whereas such elevation was suppressed in that of the DSS + rebamipide group (Fig. 4). These results indicated that rebamipide suppresses the TBK1-IRF3/7-IFN-α/β signaling pathway in DDS mice.

Rebamipide suppressed TLR-TBK1 signaling pathway in human colonic epithelial cells. To clarify the mechanism of rebamipide on the TLR-TBK1 signaling pathway in colitis, we performed both real-time RT-PCR of *TBK1*, *NAP1*, *IRF3* and *IRF7*, and Western blotting of TBK1 using human colonic epithelial cells. Poly(I:C) (a TLR3 ligand) was added to CaCo2 colonic epithelial cells with or without rebamipide. Poly(I:C) alone increased the mRNA expression of all of these genes in

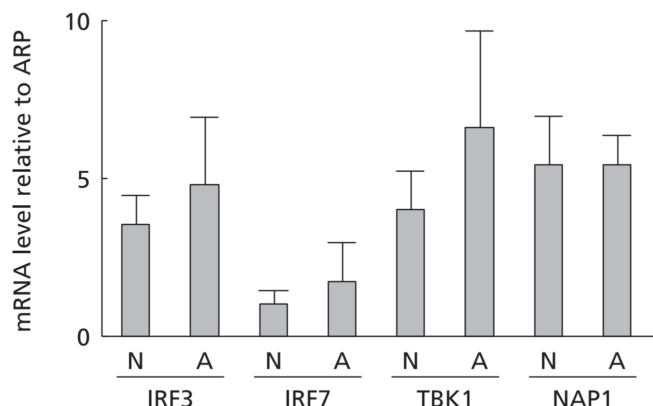

Fig. 1. Inflammatory mucosa of UC patients over-expressed TBK1-IRF3/7 signaling pathway in human biopsy tissues. Real-time RT-PCR of *TBK1*, *NAP1*, *IRF3*, and *IRF7* confirmed the relationship between mRNA expression of these genes and ulcerative colitis in 10 patients. More *TBK1*, *NAP1*, *IRF3*, and *IRF7* mRNA was expressed in atypical, than in the normal mucosa of patients with UC.

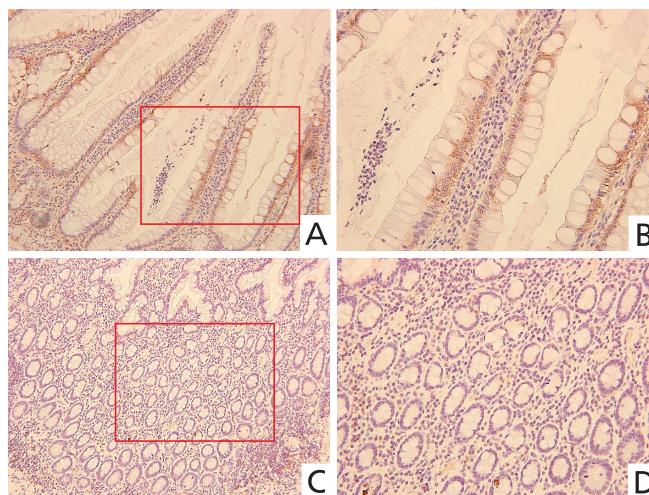

Fig. 2. Immunohistochemical analysis of TBK1 in UC. TBK1 was mainly expressed in inflammatory colon epithelial cells of crypts (A), but hardly expressed in colon epithelial cells with weak inflammation (C). Higher magnification of A (B). Higher magnification of C (D). (Original magnification: A, C ×200; B, D ×400).

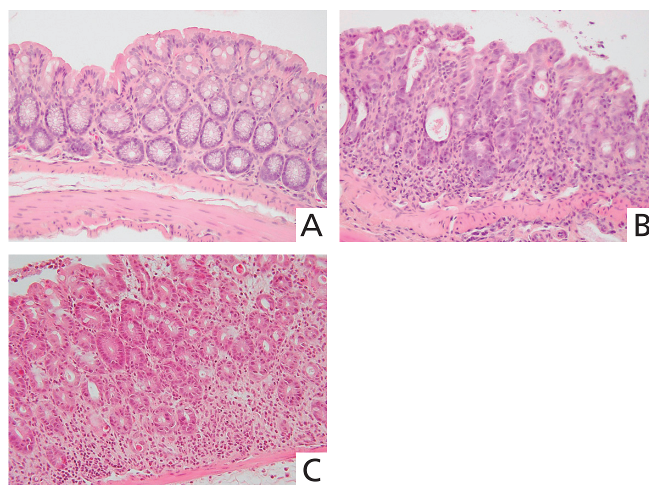

Fig. 3. Histological findings in the colon of DDS mice. Normal colon mucosa of untreated mouse (A). Colon specimen from DSS mouse shows diffuse crypt disappearance and inflammatory cell infiltration (B) indicating severe colitis. Colon specimen from mouse given oral DSS and daily rectal rebamipide, shows minimal crypt disappearance and inflammatory cell infiltration (C).

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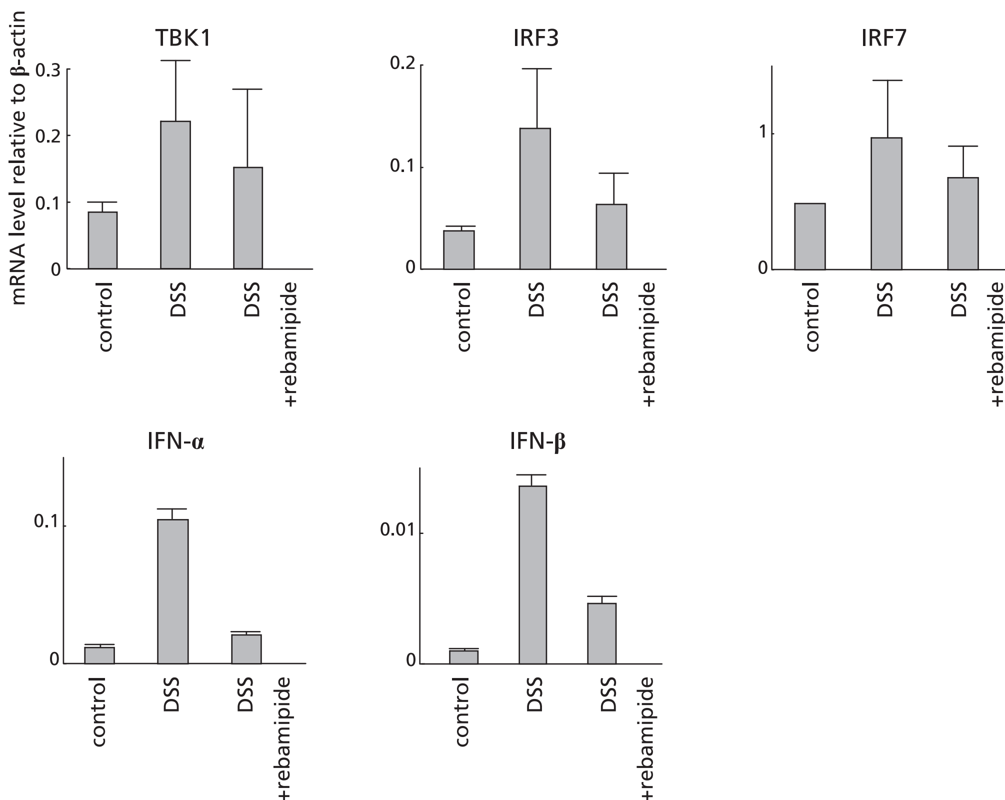

Fig. 4. Rebamipide suppressed TBK1-IRF3/7-IFN-α/β signaling pathway in DSS mice. Effect of rebamipide on TLR-TBK1 signaling pathway in colon specimens from mice given oral DDS examined by real-time RT-PCR of *TBK1*, *IRF3*, *IRF7*, *IFN-α* and *IFN-β*. Messenger RNA expression of these genes was increased because of colonic inflammation in DSS mice, but this increase was suppressed in DSS mice given daily rectal rebamipide.

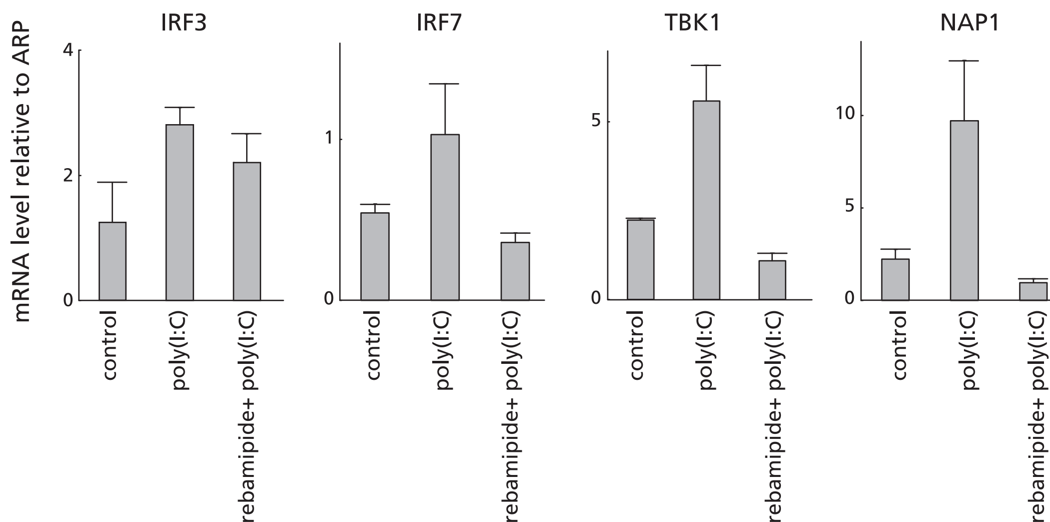

Fig. 5. Rebamipide suppressed TLR3-TBK1 signaling pathway in human colonic epithelial cells. Effect of rebamipide on TLR3-TBK1 signaling pathway in human colonic epithelial cells examined by both real-time RT-PCR of *TBK1*, *IRF3* and *IRF7* and by Western blotting of TBK1. Poly(I:C) (TLR3 ligand) was added to colonic epithelial cell line, CaCo2 with or without rebamipide. Poly(I:C) alone increased, whereas poly(I:C) plus rebamipide suppressed the mRNA expression of all of these genes to control levels.

the cells. However, the mRNA expression of these genes was suppressed to control levels when both poly(I:C) and rebamipide were added to the cells (Fig. 5). LPS, which is a ligand for TLR4, increased the mRNA expression of all these genes in CaCo2 cells, whereas rebamipide suppressed these LPS-induced increases to control levels (Fig. 6). Western blotting showed that rebamipide with either poly(I:C) or LPS suppressed TBK1 protein expression (Fig. 7). Immunofluorescence image of TBK1 indicated that rebamipide with LPS suppressed TBK1 protein expression (Fig. 8). Rebamipide also suppressed the expression of TBK-1 with poly(I:C) (data not shown). These results indicated that rebamipide suppresses the TLR3/4-TBK1 signaling pathway in

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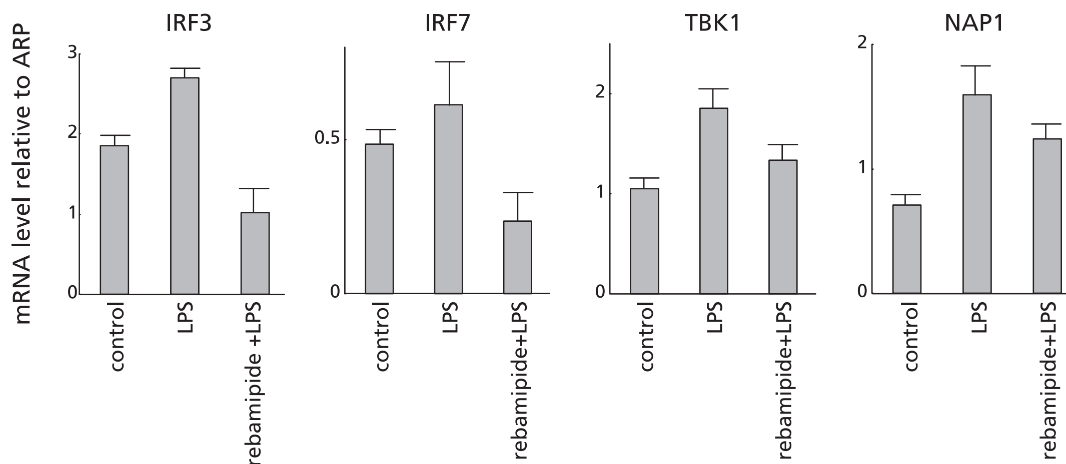
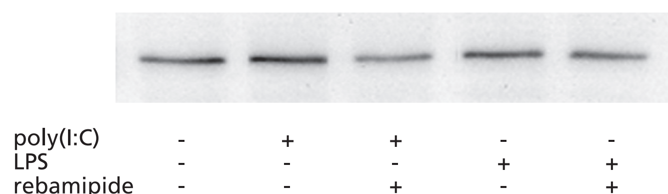


Fig. 6. Rebamipide suppressed TLR4-TBK1 signaling pathway in human colonic epithelial cells. Lipopolysaccharide (TLR4 ligand) was added to CaCo2 colonic epithelial cells with or without rebamipide. LPS alone increased, whereas LPS plus rebamipide suppressed mRNA expression of all of these genes to control levels in CaCo2 cells.



poly(I:C)	-	+	+	-	-
LPS	-	-	-	+	+
rebamipide	-	-	+	-	+

Fig. 7. Effect of rebamipide on protein expression level of TBK1. Western blots show that rebamipide suppressed TBK1 protein expression in human colonic epithelial cells induced by either poly(I:C) or LPS. These results indicate that rebamipide suppresses TLR3/4-TBK1 signaling in these cells.

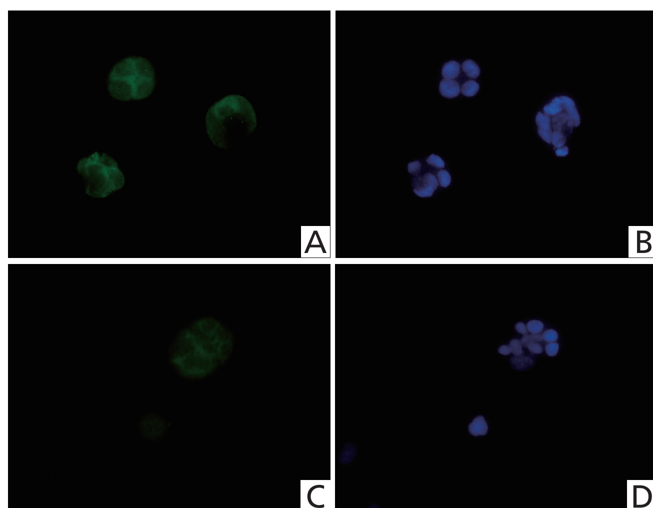


Fig. 8. Immunofluorescent image of TBK1. TBK1 expression (green) were visualized by immunofluorescent microscopy (A, C). Nuclei were stained blue by DAPI (B, D). TBK1 was clearly expressed in the cytoplasm of CaCo2 cells treated with LPS (A). On the other hand, TBK1 expression was reduced in the cytoplasm of CaCo2 cells treated with LPS and rebamipide (C).

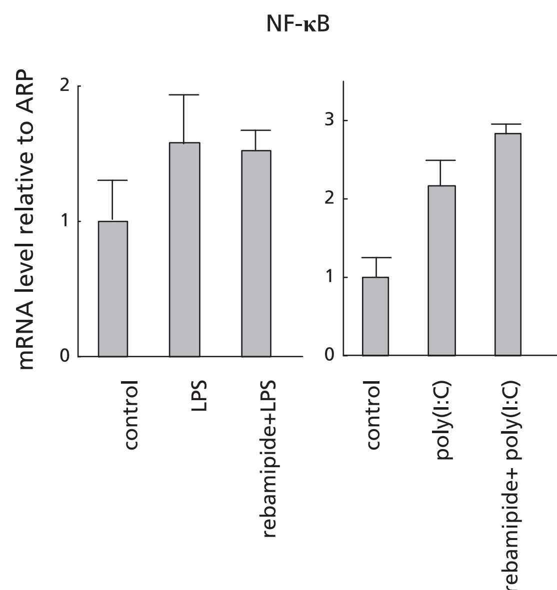


Fig. 9. Effect of rebamipide on NF-κB signaling pathway. Real-time RT-PCR of *NF-κB* in human colonic epithelial cells shows that LPS and poly(I:C) each increased *NF-κB* gene mRNA expression in colonic epithelial cells, and that adding rebamipide did not suppress either of these increases.

human colonic epithelial and serves as an important factor in anti-inflammation via this pathway.

To determine the effect of rebamipide on the NF-κB signaling pathway, we performed real-time RT-PCR of *NF-κB* in CaCo2 cells using the same procedure as described above. We found that LPS increased the expression of *NF-κB* mRNA in CaCo2 cells (Fig. 9), whereas LPS/poly(I:C) plus rebamipide did not suppress the expression and the level was similar to that in the presence of LPS/poly(I:C) (Fig. 9). These results indicated that rebamipide does not exert anti-inflammatory effects via the NF-κB signaling pathway.

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Discussion

The TLRs are the best-characterized family of innate immune receptors and they recognize conserved microbial motifs including dsRNA and LPS. Several intestinal cell types including epithelial cells, dendritic cells, monocyte/macrophages, granulocytes and lymphocytes express TLRs.[25–27] Studies using mice deficient in the MyD88 gene, which is the major signaling adaptor of the TLR family, suggest that the predominant role of TLR/MyD88 signal transduction is to prevent intestinal inflammation.[28,29] However, TLR/MyD88 signaling also promotes intestinal inflammation,[30–32] and the inhibition of NF-κB, a major target of this pathway, can ameliorate murine colitis.[33] On the other hand, few studies have investigated TRIF signaling in IBD.[34] Here, we found that the over-expression of TBK1, NAP1 and IRF3/7 activated TRIF signaling in a murine model of UC induced by DSS. These findings suggest that TRIF signaling plays an important role in the etiology of colitis.

Rebamipide is widely used to treat gastric ulcers in Japan[19] and the first successful case study of rebamipide administered rectally to a patient with IBD complicated by proctitis has recently been published.[21] The encouraging outcome of that study indicated a need for a prospective study to assess the safety and efficacy of rebamipide enemas in a cohort of patients with active UC. Rebamipide is a mucosal protective and ulcer-healing agent that is used to treat patients with acute and chronic gastritis and to heal gastric ulcers in Asian countries including Japan.[35–37] Clinical and experimental data indicate that rebamipide has anti-inflammatory properties; it scavenges free radicals,[38] and suppresses the production of pro-inflammatory mediators[39,40] as well as the migration and adherence of inflammatory cells.[41,42] Rebamipide also has mucosal protective and healing actions through promoting prostaglandin biosynthesis,[35,43,44] mucus production and release,[45] mucosal cell turnover,[46] and cell proliferation.[47] Because rebamipide is a radical scavenger, it has been investigated as an alternative treatment for chronic inflammatory diseases including colitis.[22–24,48] Anal administration of rebamipide has protective anti-colitis effects in the colitis model induced by tri-nitrobenzene sulfonic acid, in which colonic epithelial cells produce low levels of antioxidants.[48] The established DSS-induced mouse model of colitis has often been used to detect inflammatory factors including cytokines in the colon.[49] The therapeutic value of rebamipide has been independently confirmed by others using models of colitis induced by acetic acid[22] or DSS.[23,24] To our knowledge, an association between rebamipide and TBK1-IRF3/7 signaling in colitis has not been reported. Here, we demonstrated that rebamipide inhibited the TBK1-IRF3/7 pathway and IFN-α and IFN-β in mice with colitis induced by DSS. We also investigated how rebamipide causes such inhibition in colonic epithelial cells. Our data demonstrated that rebamipide inhibited the TLRs-TBK1-IRF3/7 pathway in these cells independently of NF-κB activation, indicating that rebamipide directly suppresses TBK1 activation.

The type I interferons, IFN-α and IFN-β, have been evaluated as therapy for active UC in pilot clinical trials.[50,51] IFN-β has induced the remission of ulcerative colitis in a Japanese patient with type C chronic hepatitis.[52] Our findings seem to contradict these reports. Whether IFN-β had a positive or a negative effect upon the mucosa was unclear. However, a systematic review concluded that type I IFNs cannot be recommended as treatment for active UC.[53] The contradictory effect of IFN-β might be dose-related.

In conclusion, the TLR-TBK1-IRF3/7 pathway might be one etiology of IBD and rebamipide directly thwarts this pathway. However, further studies are required to clarify the mechanism of action.

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