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Protein kinase D1 mediates synergistic MMP-3 expression induced by TNF-α and bradykinin in human colonic myofibroblasts ☆

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ABSTRACT

Stromal myofibroblasts regulate extracellular matrix components through the secretion of matrix metalloproteinases such as MMP-3. Both myofibroblasts and MMP-3 have been implicated in colonic inflammation and cancer but the regulatory signaling mechanism(s) are unknown. Exposure of the human colonic myofibroblast cell line 18Co to TNF-α and bradykinin induced synergistic MMP-3 mRNA and protein expression, which were blocked by the preferential PKC inhibitors GF109203X and Go6983 and by the MEK inhibitor U0126. Transfection with siRNA targeting PKD1, a known downstream target of both bradykinin and PKC, completely inhibited MMP-3 mRNA and protein expression. Our results imply that TNF-α and bradykinin amplify MMP-3 expression at a transcriptional level through a signaling cascade involving PKC, PKD1, and MEK. PKD1 plays a critical role in the expression of MMP-3 in human colonic myofibroblasts, and may contribute to the pathophysiology underlying colitis-associated cancer.

1. Introduction

The regulation of gastrointestinal (GI) function involves a dynamic communication between the epithelium and its underlying mesenchymal elements. Myofibroblasts are an influential subpopulation of stromal cells located in the lamina propria just subjacent to the epithelial layer. They interact with neighboring cells in a paracrine fashion to regulate a number of important normal and abnormal cellular processes, including epithelial proliferation and differentiation along the crypt-villous axis, mucosal repair, fibrosis, inflammatory bowel disease and colorectal cancer.

TNF-α and bradykinin (BK) are two important pro-inflammatory mediators implicated in the pathophysiology underlying both inflammatory bowel disease [1–5] and colorectal cancer [6–8]. BK is an endogenous nonapeptide with potent pro-inflammatory and vasoactive properties produced through the kallikrein-kinin system [2,3]. BK is released in the setting of chronic inflammatory diseases such as rheumatoid arthritis and ulcerative colitis [2,3], and initiates signaling events via two distinct cell surface G protein-coupled receptors (GPCRs) that propagate signals through PLCβ-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) [9]. TNF-α is a 17-kDa pro-inflammatory cytokine that has been strongly implicated in the pathogenesis of both ulcerative colitis [1,10] and colitis-associated cancer [7,11,12]. Binding of TNF-α to its receptors, TNF-α receptor 1 (TNFR1) and TNF-α receptor 2 (TNFR2), triggers the formation of a multiprotein complex (TRADD, RIP, TRAF-2) that initiates downstream signaling via phosphorylation cascades that culminate in the activation of MAP kinases and the transcription factor NFκB [reviewed in [13]]. Myofibroblasts have recently been identified as an important mediator of TNF-α-associated colitis, but the precise mechanism(s) remains incompletely understood [1]. The ability of a targeted antibody against TNF-α to induce clinical remission in patients with Crohn's disease and ulcerative colitis suggests that TNF-α is a central regulator of multiple inflammatory signaling pathways. In fact, recent evidence suggests that myofibroblast function is regulated by crosstalk between TNF-α and other pro-inflammatory mediators such as BK, leading to exaggerated signaling and amplified physiologic responses [8,14].

Protein kinase D1, or PKD1, is one known downstream target of the crosstalk between TNF-α and GPCR-mediated signaling in colonic myofibroblasts [8,14]. PKD1 is a serine threonine kinase that is activated by PLC-mediated hydrolysis of PIP_2, serving as both a direct target of diacylglycerol (DAG), and also as a downstream target of protein kinase C (PKC) isoforms [15]. PKD1 has been implicated in a variety of biological responses [16], including inflammation [8,14,17], oxidative stress [18,19], cell proliferation [20,21], and carcinogenesis [14,22–24]. Amplification of PKD1

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signaling by treatment of myofibroblasts with TNF-α and BK has been previously shown to directly enhance the expression of cyclo-oxygenase-2 (COX-2) [8]. Myofibroblasts are a major source of matrix metalloproteinase-3 (MMP-3) [25], one member of a family of calcium-dependent and zinc-containing neutral endopeptidases involved in extra-cellular matrix (ECM) remodeling and mucosal immunity [26–28]. We hypothesized that augmented PKD1 signaling initiated by BK and TNF-α up-regulates the expression of MMPs in human colonic myofibroblasts.

Here, we show that crosstalk between TNF-α and BK-mediated signaling leads to the synergistic production of MMP-3 through a cascade involving PKC, PKD1 and the p42/44 MAPK (ERK) pathway in cultures of human colonic myofibroblasts (18Co). Knockdown of PKD1 in these cells with specific small interfering RNAs (siRNAs) prevented the enhanced production of MMP-3 mRNA and protein induced by BK and TNF-α, suggesting that PKD1 plays a critical role in MMP-3 expression in colonic myofibroblasts in the setting of inflammation.

2. Materials and methods

2.1. Cell culture

18Co cells (CRL-1459) were purchased from American Type Culture Collection (Rockville, MD). These cells share many of the structural and functional characteristics of *in situ* colonic subepithelial myofibroblasts, including a reversible stellate morphology, α-smooth actin expression and the presence of multiple cell surface receptors [29]. 18Co cells provide a model to elucidate physiological and pathophysiological functions of intestinal subepithelial myofibroblasts and accordingly, have been used extensively to study colonic myofibroblast function in a variety of settings [30–32]. 18Co cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO_2 and 90% air. For experimental purposes, cells were plated in 35 mm dishes (1×10^5 cells/dish) and grown in DMEM containing 10% fetal bovine serum for 5–7 days until confluent, and used from passages 8–14.

2.2. ELISA

The supernatant of serum-starved, confluent 18Co cells was collected following treatment conditions and MMP-3 levels were quantified according to EIA kit instructions (Human MMP-3 ELISA Kit, Ray Biotech Inc., Norcross, GA). Absorbance readings were set between 405 and 420 nm on a spectrophotometer.

2.3. PKD siRNA transfection

The SMART pool PKD siRNA duplexes were purchased from Dharmacon (Lafayette, CO). The PKD siRNA pool was designed to target against the mRNA of human PKD (NM_002742) and consists of four selected siRNA oligonucleotides. The sequences were as follows: oligo 1, CGGCAAAUGUAGUGUAUUAUU; oligo 2, GAACCAACUUGCACAGAGAUU; oligo 3, GGUCUGAAUUACCAUAAGAUU; oligo 4, GGAGAUAGCCAUCCAGCAUUU. siCONTROL Nontargeting siRNA#3 (D-001210-03-20) was used as the control. 18Co cells were plated at ~70–80% confluency in a 12 well plate with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic at 37 °C in a humified atmosphere containing 10% CO_2. After 24 h, each well was replaced with 400 μl DMEM + 10% FBS (no antibiotic). Added to this was a mixture containing the Mirus TKO-IT transfection agent and PKD siRNA or control nontargeting siRNA (total volume: 500 μl per well, total transfection agent: 4 μl per well, siRNA: 50 nM). After incubation for 72 h, cells were used for experiments and subsequently analyzed by Western Blot.

2.4. Real-time PCR

18Co cells, maintained as described above, were washed with 4 ml of PBS (GIBCO, Grand Island, NY) and harvested with 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted with 0.2 ml of chloroform, centrifuged at 12,000*g* for 15 min at 4 °C, and precipitated with 0.5 ml of 2-propanol at 12,000*g* for 10 min at 4 °C. The RNA pellet was washed with 75% ethanol at 7500*g* for 5 min at 4 °C, dissolved in 30 μl of RNA Storage Solution containing 1 mM sodium citrate, pH 6.4 (Ambion, Austin, TX), and stored at −20 °C for subsequent analysis. RNA concentration was quantified on a spectrophotometer (GeneQuant Pro, Amersham Biotechnology, Piscataway, NJ) reading dual wavelengths of 260 and 280 nm. After RNA extraction, total RNA samples (25 ng) were reverse transcribed and cDNAs were amplified with a TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Transcripts encoding human MMP-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were quantified by real-time PCR analysis with an ABI Prism 7700 Sequence Detection System (PE Biosystems, Foster City, CA). The human GAPDH primer and probe set were acquired from Applied Biosystems. Thermal cycling conditions for reverse transcription and amplification activation were set at 48 °C for 30 min and 95 °C for 10 min, respectively. PCR denaturing was set at 95 °C at 15 s and annealing/extending at 60 °C at 60 s for 40 cycles.

2.5. Materials

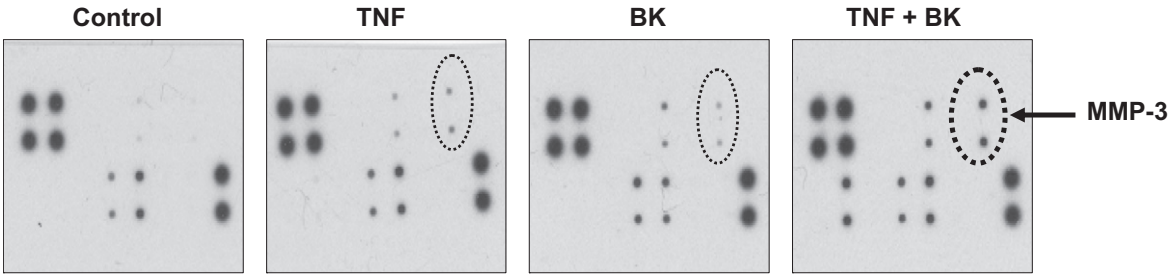
TNF-α was purchased from R&D Systems (Minneapolis, MN). Bradykinin, NS398, PGE_2, and the PKC inhibitor GF109203X were purchased from Sigma (St. Louis, MO). The PKC inhibitor Go6983 and U0126 were purchased from Calbiochem (La Jolla, CA). PKD siRNA was purchased from Dharmacon (Lafayette, CO). The human matrix metalloproteinase antibody array 1 and the MMP-3 ELISA kit were purchased from Ray Biotech Inc. (Norcross, GA).

3. Results and discussion

3.1. TNF-α and BK lead to increased production of MMP-3

As an initial approach for detecting the production of matrix metalloproteinases by myofibroblasts in the setting of inflammation, the human colonic myofibroblast cell line 18Co was exposed to either BK, TNF-α, or both for 24 h. Cell culture supernatant was collected and the expression of various MMPs was analyzed using a human matrix metalloproteinase antibody array, which detects both pro- and active MMP forms. As shown in Fig. 1, untreated 18Co cells were associated with a baseline production of TIMP-1 and TIMP-2, with no appreciable baseline production of the other MMPs analyzed by this array. The expression of TIMP-1 and TIMP-2 did not appear to be affected by treatment with TNF-α or BK, either alone or in combination. Exposure to either TNF-α or BK induced a small increase in the expression of MMP-3 in these cells. However, simultaneous treatment of 18Co cells with TNF-α and BK elicited a marked increase in the expression of MMP-3. MMP-3 has been linked to TNF-α-mediated intestinal injury [33,34] and tumor growth [35], and therefore may be an important link between pro-inflammatory cytokine signaling and the development of cancer [27,28, 36–38].

To verify the results of this initial experiment, MMP-3 levels were quantified by ELISA. 18Co cells were treated with either



POS	POS	NEG	NEG	MMP -1	MMP -2	MMP -3	MMP -8
POS	POS	NEG	NEG	MMP -1	MMP -2	MMP -3	MMP -8
MMP -9	MMP -10	MMP -13	TIMP -1	TIMP -2	TIMP -4	NEG	POS
MMP -9	MMP -10	MMP -13	TIMP -1	TIMP -2	TIMP -4	NEG	POS

Fig. 1. TNF-α and BK lead to increased production of MMP-3. Confluent 18Co cells were washed and equilibrated in serum-free media for 30 min, followed by treatment with 10 ng/ml TNF-α (TNF), 50 nM bradykinin (BK), or both (TNF + BK) for 24 h. Cell culture supernatant was collected and analyzed by a human matrix metalloproteinase antibody array, which detects the presence of a number of pro- and active MMPs and TIMPs that are listed in the corresponding array map.

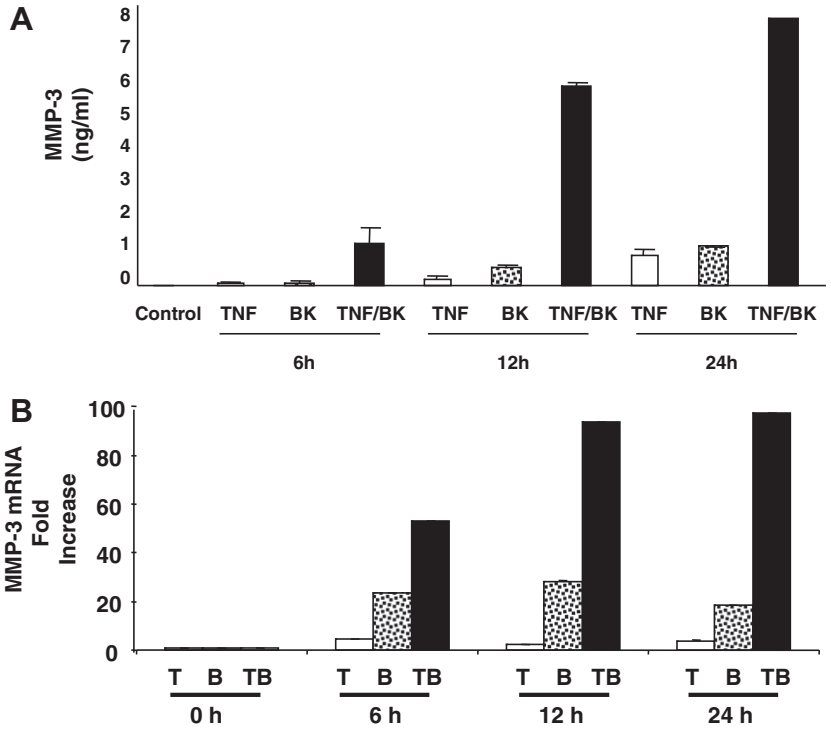


Fig. 2. TNF-α and BK lead to a parallel increase in MMP-3 mRNA and MMP-3 protein expression. Panel A: Cultures of confluent 18Co cells were incubated in serum-free medium with 50 nM bradykinin (BK, dotted bar), 10 ng/ml TNF-α (TNF, open bar), or both (TNF/BK, filled bar) for various times (6, 12, and 24 h, as indicated) with untreated cells serving as a control. MMP-3 released into the medium was quantified by ELISA. The results shown are the mean ± SE of three independent experiments. Panel B: Cultures of confluent 18Co cells were treated under identical conditions as outlined in Panel A. MMP-3 mRNA levels were quantified by RT-PCR, as described under Section 2, using GAPDH mRNA as an internal control. The results shown are the mean ± SE of two independent experiments and are expressed as fold increase of MMP-3 mRNA compared to GAPDH mRNA.

TNF-α, BK, or both, and cell culture supernatant was collected at various times for up to 24 h (Fig. 2A). Treatment with either BK or TNF-α alone led to low levels of MMP-3 production that increased in a time-dependent fashion over 24 h. However, simultaneous treatment of 18Co cells with both TNF-α and BK led to a synergistic increase in the expression of MMP-3 that was evident after 6 h and was sustained and markedly enhanced over 24 h.

As a first step to determine the mechanism for the up-regulation of MMP-3, 18Co cells were stimulated with either TNF-α, BK or both and levels of MMP-3 mRNA were determined by RT-PCR. As shown in Fig. 2B, the synergistic expression of MMP-3 following treatment with TNF-α and BK was associated with a parallel increase in MMP-3 mRNA, suggesting that the upregulation induced by TNF-α and BK was occurring at the transcriptional level.

3.2. The synergistic increase in MMP-3 induced by TNF-α and BK is PGE_2 insensitive

We have previously reported that interactions between TNF-α and BK-mediated signaling events leads to the synergistic expression of COX-2 and PGE_2 in colonic myofibroblasts [8]. PGE_2 is involved in a number of cellular responses through direct binding to one of four E prostanoid receptors (EP1-4). Given the known association between COX-2, PGE_2, and the expression of MMPs [39,40], we considered the possibility that PGE_2 or the other downstream products of COX-2 may be involved in the synergistic increase in MMP-3. To test this possibility, 18Co cells were treated with TNF-α and BK for 24 h in the presence and absence of NS398, a COX-2-specific inhibitor. Additionally, 18Co cells were treated with varying concentrations of PGE_2, and MMP-3 levels were quantified by ELISA. Pre-treatment with the COX-2-specific inhibitor NS398 had no effect on the production of MMP-3 (Fig. 3A) at baseline or after treatment with TNF-α and BK. Likewise, treatment with PGE_2 at various concentrations did not stimulate the production of MMP-3, confirming that the synergistic increases in COX-2/PGE_2 could be dissociated from the expression of MMP-3 in these cells.

3.3. TNF-α and BK-mediated MMP-3 production involves the PKC/PKD1 axis and p42/44 MAPK

Previous work in our laboratory identified the PKC/PKD1 axis and the p42/44 MAPK (ERK) pathway as downstream targets of crosstalk between TNF-α and G protein coupled receptor agonists in human colonic myofibroblasts [8,14]. To determine whether these signaling pathways were involved in the expression of MMP-3, 18Co cells were pre-treated with the PKC inhibitors Go6983 and GF-109203X, as well as with the P42/44 MAPK (ERK) inhibitor U0126 for 1 h prior to treatment with TNF-α and BK, and MMP-3 levels were quantified by ELISA. As shown in Fig. 3B, pre-treatment with Go6983, GF-109203X, and U0126 led to a statistically significant decrease in MMP-3 production following treatment with TNF-α and BK.

3.4. The synergistic production of MMP-3 induced by TNF-α and BK is mediated by PKD1

Having identified PKC as an important mediator of MMP-3 expression, we tested whether the downstream signaling target PKD1 plays a role in mediating this response. BK-induced PKD1 activation is augmented by pre-treatment with TNF-α (18), and this enhanced signaling could be responsible for the dramatic increase in MMP-3 expression. We have previously shown that PKD1 expression can be efficiently knocked down in 18Co cells transfected with PKD siRNA. We verified that transfection of 18Co cells with PKD1 siRNA led to the virtual loss of PKD1 protein expression in these cells (Fig. 4, inset). Following transfection with PKD1 siRNA or a non-targeting siRNA as control, cells were treated with TNF-α and BK for 24 h. MMP-3 mRNA levels were measured using RT-PCR, and corresponding MMP-3 levels were quantified by ELISA. Compared to cells transfected with a non-targeted siRNA, knockdown of PKD1 expression prevented the increase in MMP-3 mRNA and MMP-3 protein expression induced by BK and TNF-α (Fig. 4). The results identify PKD1 as a critical element in the pathway leading to MMP-3 expression in human colonic myofibroblasts challenged with the pro-inflammatory mediators BK and TNF-α.

4. Concluding remarks

MMP-3 degrades multiple ECM proteins such as proteoglycans, collagens type II, IV, IX, XI, laminin and fibronectin [41]. Both myo-

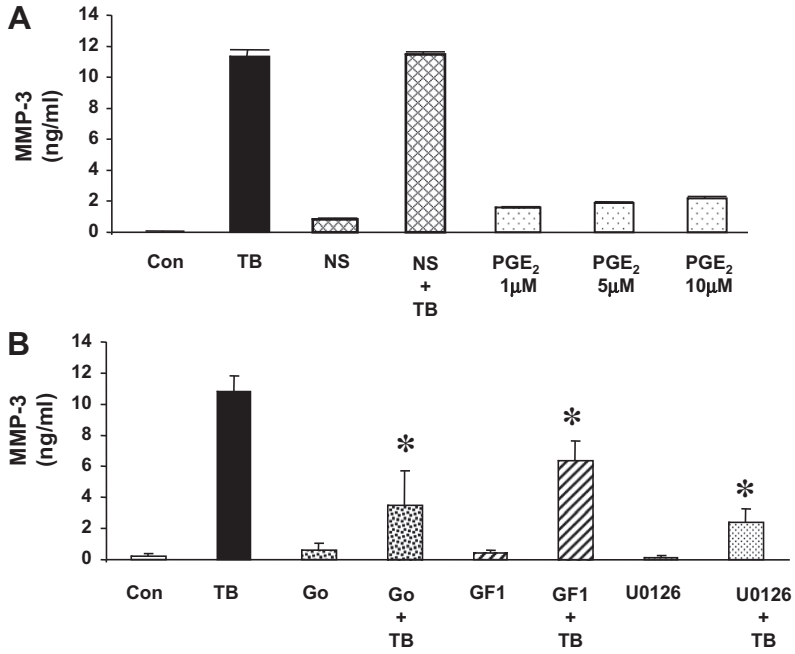


Fig. 3. TNF-α and BK-mediated MMP-3 production involves the PKC/PKD1 axis and p42/44 *MAPK*. Panel A: 18Co cells were incubated with TNF-α (10 ng/ml) and BK (50 nM) for 24 h (TB, filled bar), in the presence or absence of the COX-2-specific inhibitor NS398 (1 μM, NS, hashed bar). 18Co cells were also incubated with varying concentrations of PGE_2 for 24 h (dotted bars). Cell culture supernatant was collected and MMP-3 levels were quantified by ELISA. The results shown are the mean ± SE of six independent experiments. Panel B: 18Co cells were incubated with TNF-α (10 ng/ml) and BK (50 nM) for 24 h (TB, filled bar), in the presence or absence of a 1 h pre-incubation with either the preferential PKC inhibitors Go6983 (2.5 μM, Go, dotted bar) and GF109203X (2.5 μM, GF1, striped bar), and the MEK inhibitor U0126 (5 μM, dotted bar). Cell culture supernatant was collected and MMP-3 was quantified by ELISA. The results shown are the mean ± SE of three independent experiments. A * denotes statistical significance ($p < 0.05$).

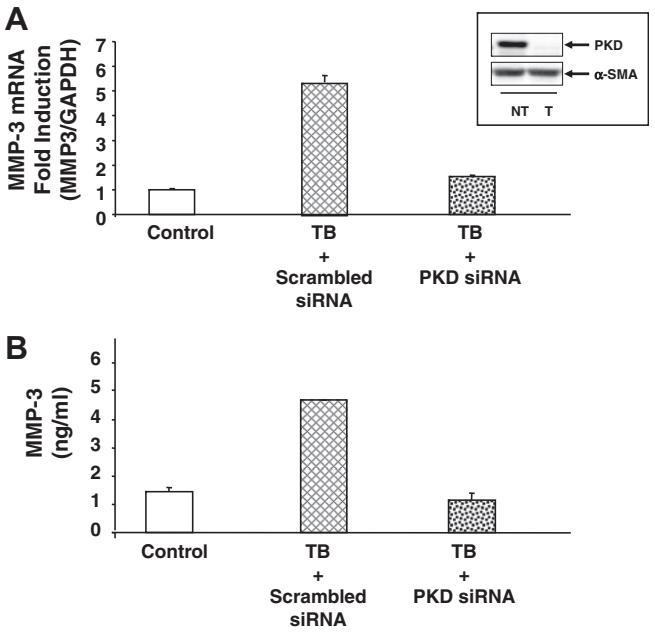


Fig. 4. Inset: 18Co cells were transfected with either non-targeting siRNA (NT) or with PKD siRNA (T) at 50 nM in the presence of 4 μl Mirus TKO-IT transfection agent for 72 h. The level of PKD protein was analyzed by Western blotting using the anti-PKD C-20 antibody. Antibody against α-SMA was used to verify equal protein loading. Shown here is a representative autoluminogram; similar results were obtained in three independent experiments. Panel A: 18Co cells were transfected with 50 nM siRNA targeting PKD or with a non-targeted sequence also at 50 nM, as described in A, followed by incubation with 10 ng/ml TNF-α and 50 nM BK for 24 h. MMP-3 mRNA was analyzed by RT-PCR, as previously described, using GAPDH mRNA as an internal control. The results shown are the mean ± SE of two independent experiments and are expressed as fold increase of MMP-3 mRNA compared to GAPDH mRNA. Panel B: Under the same experimental conditions outlined in Panel A, cell culture supernatant was collected and MMP-3 was quantified by ELISA. The results shown are the mean ± SE of three independent experiments.

fibroblasts and MMP-3 levels are upregulated in areas of intestinal inflammation [25,38,41,42] and neoplasia [27,28,36,43–45] suggesting that MMP-3 produced by myofibroblasts may play a role in the pathophysiology underlying both colitis and cancer. Alterations in the ECM may impair barrier defenses, exacerbating mucosal injury in inflammatory bowel disease or facilitating tumor invasion. Indeed, genetic ablation of MMP-3 resulted in mice with impaired colonic mucosal immunity in response to bacterial pathogens [26]. Consequently, elucidation of the signaling mechanisms that regulate the induction of MMP-3 expression in colonic myofibroblasts may be of importance for understanding the pathogenesis of inflammation-associated cancer in the gastrointestinal tract.

The results presented here demonstrate that prolonged exposure of 18Co cells, a model of human colonic myofibroblasts, to TNF-α and BK leads to the synergistic expression of MMP-3, an effect that was inhibited by the PKC inhibitors Go6983 and GF-109203X, by the P42/44 MAPK (ERK) inhibitor U0126, as well as by knockdown of PKD1 with specific siRNA. The results imply that PKD1 is a critical downstream target that links TNF-α and BK-mediated signaling to MMP-3 expression. PKD1 may play a critical role in the interaction between pro-inflammatory cytokine signaling, ECM degradation, and tumor progression, and may be a potential novel target for the prevention of colitis-associated cancer.

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