Protein kinase D mediates synergistic expression of COX-2 induced by TNF-α and bradykinin in human colonic myofibroblasts

James Yoo, Christine Chung, Lee Slice, James Sinnett-Smith, and Enrique Rozengurt

Departments of Surgery and Medicine, David Geffen School of Medicine, CURE: Digestive Diseases Research Center, and Molecular Biology Institute, University of California, Los Angeles, California

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Yoo J, Chung C, Slice L, Sinnett-Smith J, Rozengurt E. Protein kinase D mediates synergistic expression of COX-2 induced by TNF-α and bradykinin in human colonic myofibroblasts. Am J Physiol Cell Physiol 297: C1576–C1587, 2009. First published September 30, 2009; doi:10.1152/ajpcell.00184.2009.—Myofibroblasts have recently been identified as major mediators of tumor necrosis factor-α (TNF-α)-associated colitis, but the precise mechanism(s) involved remains incompletely understood. In particular, the possibility that TNF-α signaling cross talks with other proinflammatory mediators, including bradykinin (BK), has not been examined in these cells. Here we show that treatment of 18Co cells, a model of human colonic myofibroblasts, with BK and TNF-α induced striking synergistic COX-2 protein expression that was paralleled by increases in the levels of transcripts encoding COX-2 and microsomal prostaglandin E synthase 1 (mPGES-1) and by the production of PGE2. COX-2 expression in 18Co cells treated with BK and TNF-α was prevented by the B2 BK receptor antagonist HOE-140, the preferential protein kinase C (PKC) inhibitors Ro31-8220 and GF-109203X, and Go¨-6976, an inhibitor of conventional PKCs and protein kinase D (PKD). In a parallel fashion, TNF-α, while having no detectable effect on the activation of PKD when added alone, augmented PKD activation induced by BK, as measured by PKD phosphorylation at its activation loop (Ser²⁵⁰) and autoprophosphorylation site (Ser³⁶⁷). BK-induced PKD activation was also inhibited by HOE-140, Ro31-8220, and Go¨-6976. Transfection of 18Co cells with small interfering RNA targeting PKD completely inhibited the synergistic increase in COX-2 protein in response to BK and TNF-α, demonstrating, for the first time, a critical role of PKD in the pathways leading to synergistic expression of COX-2. Our results imply that cross talk between TNF-α and BK amplifies a PKD phosphorylation cascade that mediates synergistic COX-2 expression in colonic myofibroblasts. It is plausible that PKD increases COX-2 expression in colonic myofibroblasts to promote an inflammatory microenvironment that supports tumor growth.

18Co cells; prostaglandin E₂; protein kinase C; phosphorylation
colorectal cancer (36, 50a). BK is an endogenous kinin with potent proinflammatory, nociceptive, and vasoactive properties produced by the kallikrein-kinin system (12, 48). BK-initiated signaling events occur via two distinct cell surface G protein-coupled receptors (GPCRs), the constitutively present B2 receptor and the inducible B1 receptor, both of which act through Goq subunits to propagate signals via PLCβ-mediated hydrolysis of PIP2 (40). TNF-α is a 17-kDa proinflammatory cytokine that plays a pivotal role in regulating the inflammatory signaling cascades characteristic of ulcerative colitis (4) and has been strongly implicated in colitis-associated colon cancer (20, 36). Binding of TFN-α to its receptors, TNFα receptor 1 (TNFR1) and TNF-α receptor 2 (TNFR2), triggers the formation of a multiprotein complex [TNF receptor 1-associated death domain (TRADD), receptor-interacting protein (RIP), TNF-α receptor-associated factor 2 (TRAF-2)] that initiates downstream signaling via phosphorylation cascades that culminate in the activation of MAP kinases and the transcription factor NF-κB (reviewed in Ref. 53). Myofibroblasts have recently been identified as major mediators of TNF-α-associated colitis, but the precise mechanism(s) involved remains incompletely understood (4). In particular, the possibility that TNF-α signaling cross talks with other proinflammatory mediators, including BK, has not been examined in these cells.

In the present study, we show striking synergistic effects between BK and TNF-α in cultures of human colonic subepithelial myofibroblasts (18Co cells) leading to dramatic COX-2 expression and PKC/PKD axis activation. A salient feature of the results presented here is that knockdown of PKD in these cells via specific small interfering RNAs (siRNAs) prevented the synergistic increase in COX-2 expression induced by BK and TNF-α. Thus we show, for the first time, that PKD plays a critical role in mediating COX-2 expression in response to potent proinflammatory mediators in 18Co cells, a model of human colonic myofibroblasts.

MATERIALS AND METHODS

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 (52). 18Co cells provide a model to elucidate the physiological and pathophysiological functions of intestinal subepithelial myofibroblasts and, accordingly, have been used extensively to study colonic myofibroblast function in a variety of settings (24, 32, 44). 18Co cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and transfected with 0.2 ml of chloroform, centrifuged at 12,000 × g for 5–7 days were preincubated with TNF-α (8.3 ng/ml) for 4 h and then washed in Hanks’ balanced salt solution supplemented with 0.03% NaHCO3, 1.3 mM CaCl2, 0.5 mM MgCl2, 0.4 mM MgSO4, and 0.1% BSA (pH 7.4) (Hanks’ buffer). After washing, cells were incubated with 5 μM fura-2 tetra-acetoxyethyl ester (fura-2 AM) from a stock of 1 mM in dimethyl sulfoxide for 10 min in a 37°C incubator. Cells were then washed again with Hanks’ buffer and left at room temperature for an additional 5 min. The fura-loaded cells were introduced in a cuvette containing the incubation medium (Hanks’ buffer), and the cuvette was placed into a Hitachi (Tokyo, Japan) F-2000 fluorospectrophotometer. The incubation medium in the cuvette was continuously stirred at 37°C. The excitation wavelengths were set at 340 and 380 nm, and the emission wavelength was set at 510 nm. Maximum fluorescence was determined after membrane permeabilization by the addition of 37.5 μM digitonin. Minimum fluorescence was measured after the Ca2+ in the solution was chelated by the addition of EGTA at a final concentration of 25 mM. A Kd of 224 nM was used for the Ca2+ dissociation constant from fura 2 in the cells at 37°C. Intracellular Ca2+ concentration ([Ca2+]i) was determined automatically by the cation measurement software of the F-2000 fluorospectrophotometer. BK was added as indicated in the individual experiments.

In situ hybridization and detection of PKD and COX-2. Confluent 18Co cells, treated with different agonists, antagonists, inhibitors, and siRNA as indicated in the individual experiments, were lysed in 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (20 mM Tris-HCl, pH 6.8, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol) and boiled for 10 min. After SDS-PAGE, proteins were transferred to Immobilon-P membranes. The transfer was carried out at 100 V, 0.4 A at 4°C for 5 h with a Bio-Rad transfer apparatus. The transfer buffer consisted of 200 mM glycine, 25 mM Tris, 0.01% SDS, and 20% CH3OH. For detection of proteins, membranes were blocked with 5% nonfat dried milk in PBS (pH 7.2) and then incubated for 2 h with the desired antibodies diluted in PBS (pH 7.2) containing 3% nonfat dried milk. Primary antibodies bound to immunoreactive bands were visualized by enhanced chemiluminescence (ECL).

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 7,500 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 was quantified on a spectrophotometer (GeneQuant Pro, Amersham Biotechnology, Piscataway, NJ) reading dual wavelengths of 260 nm 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 COX-2, microsomal prostaglandin E synthase 1 (mPGES-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control were quantified by 10.22±0.33.5 on June 25, 2017 http://ajpcell.physiology.org/ Downloaded from dp.readcon2017.org by June 25, 2017
respectively. PCR denaturing was set at 95°C at 15 s and annealing/extension at 60°C at 60 s for 40 cycles.

Enzyme-linked immunosorbent assay. PGE2 was quantified from the supernatant of serum-starved, confluent 18Co cells after treatment conditions according to EIA kit instructions (Prostaglandin E2 EIA kit, Cayman Chemical, Ann Arbor, MI). The collected supernatant was centrifuged at 5,000 g for 5 min to remove cell debris. Absorbance readings were set between 405 and 420 nm on a spectrophotometer.

PKD siRNA transfection. SMART pool PKD siRNA duplexes were purchased from Dharmacon (Lafayette, CO). The PKD siRNA pool was designed to target the mRNA of human PKD (NM_002742) and consists of four selected siRNA oligonucleotides. The sequences were as follows: oligo 1, CGGCAAAUGUAGUGUAUUAUU; oligo 2, GAAACAAACUUGCACAGAGAUU; oligo 3, GGUCGUAUUACCAAUAAGAUU; oligo 4, GGAGAUAGCCAUCCAGCAUUU. siCONTROL nontargeting siRNA no. 3 (D-001210-03-20) was used as the control. 18Co cells were plated at ~70–80% confluence in a 12-well plate with DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic at 37°C in a humidified atmosphere containing 10% CO2. After 24 h, each well was replaced with 400 µl of 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/well; total transfection agent: 4 µl/well; siRNA: 50 nM). After incubation for 72 h, cells were used for experiments and subsequently analyzed by Western blot.

Materials. BK, HOE-140, and the PKC inhibitor GF-109203X were purchased from Sigma (St. Louis, MO). TNF-α was purchased from R&D Systems (Minneapolis, MN). COX-2 antibody was purchased from Cell Signaling Technology (Beverly, MA). The PKC inhibitors Ro31-8220 and Gö-6976 were purchased from Calbiochem (La Jolla, CA). PKD C-20 and total ERK2 polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-PKD polyclonal antibodies pSer916 (Millipore, Billerica, MA) and pSer744 (Cell Signaling Technology) detect PKD when it is phosphorylated on Ser916 or Ser744, respectively. ECL detection was performed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies obtained from GE Healthcare (Piscataway, NJ). [3H]BK (specific activity 80 Ci/mmol) was obtained from PerkinElmer (Waltham, MA).

RESULTS

Bradykinin and TNF-α lead to synergistic COX-2 expression in 18Co cells. To determine whether COX-2 expression in human colonic myofibroblasts is regulated by proinflammatory

Fig. 1. Bradykinin (BK) and tumor necrosis factor-α (TNF-α) induce synergistic cyclooxygenase (COX)-2 expression in 18Co myofibroblasts. A: 18Co cells were washed and equilibrated in serum-free medium for 30 min, followed by treatment with 8.3 ng/ml TNF-α, 100 nM BK, or both for various times (2, 4, 8, and 24 h, as indicated). Lysates of these cells were then analyzed by SDS-PAGE and Western blot using a polyclonal antibody that detects COX-2 protein. Shown is a representative autoradiogram; similar results were obtained in 3 independent experiments. Autoradiograms were quantified by densitometric scanning. Results shown are means ± SE (n = 3) and are expressed as % of maximum level of COX-2 expression. Protein loading was quantified by using an antibody that detects ERK2, and COX-2 expression was corrected to the density of the ERK2 bands. *Statistical significance (P < 0.05). B and C: 18Co cells were treated with 100 nM BK (gray bars), 8.3 ng/ml TNF-α (hatched bars), or both (filled bars) for 4 h, and mRNA encoding COX-2 (B) or microsomal prostaglandin E synthase 1 (mPGES-1, C) were quantified by RT-PCR, as described under MATERIALS AND METHODS, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. Results shown are means ± SE of 3 independent experiments and are expressed as fold induction of COX-2 or mPGES-1 mRNA compared with GAPDH mRNA. D: cultures of 18Co cells were incubated in serum-free medium with 100 nM BK (B, gray bar), 8.3 ng/ml TNF-α (T, hatched bar), or both (B + T, filled bar) for 4 h, with untreated cells (open bar) serving as a control (C). PGE2 released into the medium was quantified by ELISA per manufacturer’s instructions.
mediators, 18Co cells were treated with BK and TNF-α, either alone or in combination, and COX-2 protein was assessed by Western blot analysis. There was no detectable COX-2 protein in unstimulated 18Co cells (Fig. 1A). Treatment of these cells with 100 nM BK induced COX-2 expression that was evident after 2 h, with maximal expression at 4 h followed by a gradual decline over a 24-h time period. Treatment with TNF-α (8.3 ng/ml) stimulated low levels of COX-2 expression that were far lower than those seen in the BK-treated cells. The salient feature of the results shown in Fig. 1 is that combined treatment of myofibroblasts with BK and TNF-α led to a striking synergistic increase in COX-2 protein expression that was evident after 4 h and was dramatically augmented and sustained over a 24-h time period (Fig. 1A).

To characterize further the synergistic effects between BK and TNF-α on COX-2 expression in human colonic myofibroblasts, 18Co cells were treated with 100 nM BK, 8.3 ng/ml TNF-α, or both for 4 h and the level of mRNA encoding COX-2 was quantified by real-time PCR analysis. In line with the striking increase in COX-2 protein induced by BK and TNF-α (Fig. 1A), treatment with the combination of these proinflammatory mediators produced a prominent synergistic accumulation of COX-2 mRNA (Fig. 1B). These results show that the enhancement of COX-2 protein expression induced by BK and TNF-α is paralleled by upregulation of transcripts encoding COX-2.

COX-2 converts arachidonic acid to PGH2, leading to the production of PGs, including PGE2, which is implicated in colon cancer. mPGES-1 is the first committed step toward the synthesis of PGE2. To determine the effect of proinflammatory mediators on mPGES-1 mRNA levels, 18Co cells were treated with BK, TNF-α, or both for 4 h and transcripts encoding mPGES-1 were quantified by RT-PCR (Fig. 1C). Treatment with BK and TNF-α induced synergistic increase in the level of mPGES-1 mRNA, which was markedly higher than the level of mPGES-1 mRNA seen after treatment with either BK or TNF-α alone.

We also determined whether exposure to proinflammatory mediators stimulates PGE2 production from 18Co cells. As shown in Fig. 1D, treatment of these cells with BK stimulated PGE2 production, whereas TNF-α had only a small effect. Exposure to both TNF-α and BK induced synergistic production of PGE2, as quantified by ELISA. Thus the proinflammatory mediators BK and TNF-α induced synergistic COX-2 protein expression that was paralleled by increases in the levels of transcripts encoding COX-2 and mPGES-1 and by the production of PGE2 in human colonic myofibroblasts.

**TNF-α enhances BK-induced COX-2 expression leading to synergistic COX-2 protein accumulation.** We next determined whether the synergistic effect induced by BK and TNF-α on COX-2 protein expression required simultaneous exposure to both agonists or, alternatively, whether it could be resolved in stages, shown by sequential treatment with the proinflammatory agonists. To distinguish between these possibilities, 18Co cells were treated with TNF-α (8.3 ng/ml) for 1–3 h, washed extensively, and then treated with BK (100 nM for 3 h). The reverse experiment was performed by treating 18Co cells with BK for 1–3 h, followed by washing and treatment with TNF-α for 3 h. For comparison, 18Co cells were exposed to TNF-α and BK simultaneously for 3 h. As shown in Fig. 2, simultaneous treatment with TNF-α and BK led to synergistic COX-2 protein expression in colonic myofibroblasts, as described above. Treatment of 18Co cells with TNF-α followed by BK (Fig. 2A) also induced synergistic expression of COX-2 protein. In contrast, exposure of 18Co cells to BK followed by TNF-α failed to induce any detectable synergistic increase in COX-2 expression (Fig. 2B). These results, suggesting that TNF-α signaling augments BK-induced COX-2 expression, prompted us to identify elements in BK-induced signaling that play a key role in promoting COX-2 expression synergistically with TNF-α.
BK induces COX-2 expression via B2 receptor and PKC. BK-mediated signaling is initiated by its binding to one of two distinct cell surface GPCRs (inducible B1 and constitutively present B2). To determine whether inhibition of the B2 receptor would affect synergistic COX-2 expression, confluent 18Co cells were pretreated with the B2 receptor-specific antagonist HOE-140 (10 μM), followed by treatment with BK, TNF-α, or both for 6 h. As shown in Fig. 3A, pretreatment with HOE-140 prevented both BK-induced COX-2 expression and the synergistic expression of COX-2 in response to BK and TNF-α. HOE-140 had no effect on COX-2 expression induced by TNF-α. These results indicate that the B2 BK receptor mediates expression of COX-2 and synergistic interaction with TNF-α.

We next determined whether treatment of 18Co cells with TNF-α increases B2 BK receptors, a plausible mechanism leading to augmentation of BK-induced COX-2 expression by TNF-α. This possibility was assessed by means of binding studies using [3H]BK as the radioligand. Cultures of 18Co cells were treated with or without TNF-α (8.3 ng/ml) for either 4 or 18 h, washed extensively, and then incubated with [3H]BK at 4°C in the absence or presence of excess of unlabeled BK to determine the specific binding of [3H]BK to the cells. As can be seen in Fig. 3B, treatment of 18Co cells with TNF-α did not induce any significant change in specific binding of [3H]BK to the cells. We conclude that TNF-α signaling augments BK-induced COX-2 expression at a postreceptor locus. This conclusion was further substantiated by measuring the elevation of \([Ca^{2+}]_i\) induced by BK, one of the earliest events induced by the Gq-coupled B2 receptor. In line with the results indicating that treatment with TNF-α did not increase BK receptor expression, treatment of 18Co cells with TNF-α did not affect the
rapid increase in \([\text{Ca}^{2+}]_i\), induced by BK in these cells (Fig. 3, C and D).

Isoforms of the PKC family are major downstream targets of BK-mediated signaling via \(G_\alpha\)-coupled \(B_2\) receptors in other cell systems. We next determined the role of PKC in the synergistic expression of COX-2 in response to BK and TNF-\(\alpha\) in colonic myofibroblasts. Cultures of 18Co cells were pretreated for 60 min with the preferential PKC inhibitors Ro31-8220 at 2 \(\mu\)M or GF-109203X (also known as bisindolylmaleimide I) at 5 \(\mu\)M, before stimulation with TNF-\(\alpha\) and BK for 6 h. Cell treatment with either Ro31-8220 or GF-109203X inhibited the synergistic expression of COX-2 induced by BK and TNF-\(\alpha\) (Fig. 4, A and B). These results suggest that PKC isoforms play a fundamental role in the synergistic enhancement of COX-2 expression mediated by TNF-\(\alpha\) and BK.

However, the mechanism(s) by which PKC-mediated signals are propagated to downstream targets and contribute to synergistic COX-2 expression in colonic myofibroblasts is not known. The PKD family has emerged as major downstream targets of PKCs in a phosphorylation cascade that has been implicated in multiple biological responses (see Ref. 41 for review). In this context, we also found that Gö-6976 (10 \(\mu\)M), which inhibits the catalytic activity of classic PKC isoforms (but not novel or atypical PKCs) and PKD (see below), also prevented synergistic accumulation of COX-2 protein in response to BK and TNF-\(\alpha\) (Fig. 4C). Given the results shown in Fig. 4, we next determined whether PKD plays a role in mediating COX-2 expression induced by BK and TNF-\(\alpha\).

**BK induces PKD activation via \(B_2\) receptor and novel PKCs.** To evaluate a possible role of PKD in the synergistic expression of COX-2 in colonic myofibroblasts in response to BK and TNF-\(\alpha\), we first tested whether BK induces PKD activation in these cells. Confluent 18Co cells were treated with BK for various times and lysed. Activation of PKD was assessed by Western blot analysis of the cell lysates using site-specific primary antibodies that detect the phosphorylated state of Ser\(^{744}\) located in the activation loop of PKD (21, 55) and the autophosphorylated state of PKD on Ser\(^{916}\) (27). Treatment of 18Co cells with BK led to a rapid and sustained PKD activation, as judged by phosphorylation on Ser\(^{744}\) and Ser\(^{916}\) (Fig. 5, A and B). To determine the role of PKC in BK-induced PKD activation, 18Co cells were pretreated with the preferential PKC inhibitor Ro31-8220 (2 \(\mu\)M) for 60 min, followed by treatment with BK. Ro31-8220 completely inhibited BK-induced phosphorylation at the activation loop (Ser\(^{744}\)) and on the autophosphorylation site (Ser\(^{916}\)), in line with a critical role of PKC isoforms in mediating PKD activation (Fig. 5A). In contrast, treatment of 18Co cells with Gö-6976, an inhibitor of conventional but not novel PKCs, failed to inhibit PKD phosphorylation on Ser\(^{744}\) induced by BK (Fig. 5B), suggesting that novel PKC isoforms were responsible for phosphorylation at the activation loop of PKD. In line with a direct inhibitory effect of Gö-6976 on PKD catalytic activity (14), cell exposure to this compound markedly decreased PKD autophosphorylation on Ser\(^{916}\) induced by BK.

![Fig. 4. BK induces COX-2 expression via protein kinase C (PKC).](http://ajpcell.physiology.org/)

Cultures of 18Co cells were pretreated for 1 h with the PKC inhibitors Ro31-8220 (Ro, 2 \(\mu\)M; A) and GF-109203X (GF1, 5 \(\mu\)M; B) or the PKC/protein kinase D (PKD) inhibitor Gö-6976 (Go, 10 \(\mu\)M; C). Cultures were then challenged with TNF-\(\alpha\) (8.3 ng/ml), BK (100 nM), or both for 6 h and lysed. Cell extracts were analyzed by SDS-PAGE and Western blot using anti-COX-2 antibody. Results shown are representative autoluminograms; similar results were obtained in at least 3 independent experiments for each condition. COX-2 expression was quantified by densitometric scanning. Results shown are means \(\pm\ SE\) and are expressed as % of maximum level of COX-2 expression. Equal protein loading was verified with an antibody that detects ERK2.
To identify the BK receptor involved in the activation of PKD in colonic myofibroblasts, 18Co cells were pretreated with the B2 receptor antagonist HOE-140 at either 1 μM or 10 μM before stimulation with 100 nM BK for various times (0–240 min). Cell cultures were lysed with 2× SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting performed with antibodies that detect the phosphorylated state of Ser916 or Ser744. In addition, Western blot analysis with an antibody directed against the COOH-terminal region of PKD (PKD-C20) is also shown. Regarding the apparent difference in total PKD in some of the lanes, it should be pointed out that Ser916 (in COOH-terminal region of PKD) lies in the epitope recognized by the PKD-C20 antibody and that the phosphorylation of this residue (as induced by BK) was found to interfere with the binding of PKD-C20 antibody to its epitope. In line with this explanation, the results show that immunoreactivity with PKD-C20 was inversely related to Ser916 phosphorylation. Results shown are representative of at least 3 independent experiments. PKD phosphorylation at Ser916 and Ser744 either in the absence (open bars) or presence (filled bars) of R031-8220 or Go6976 was quantified by densitometric analysis and shown as ± SE, expressed as % of maximum level of phosphorylated Ser916 or Ser744. C: 18Co cells were pretreated without or with the B2 BK receptor antagonist HOE-140 for 60 min at 1 μM or 10 μM followed by treatment with 100 nM BK. PKD phosphorylation at Ser916 was analyzed by Western blot. Membranes were stripped and reprobed for ERK2 to verify equal protein loading. Result shown is a representative autoradiogram; similar results were obtained in at least 3 independent experiments. Ser916 phosphorylation was quantified by densitometric scanning. Results shown are means ± SE and are expressed as % of maximum level of phosphorylated Ser916. Equal protein loading was verified with an antibody that detects actin. *Statistical significance (P < 0.05).

To identify the BK receptor involved in the activation of PKD in colonic myofibroblasts, 18Co cells were pretreated with the B2 receptor antagonist HOE-140 at either 1 μM or 10 μM before stimulation with the agonist and PKD activation was assessed by autophosphorylation on Ser916. As shown in Fig. 5C, pretreatment with HOE-140 inhibited BK-induced PKD activation, with complete inhibition occurring at 10 μM. These results demonstrate that BK triggers PKD activation via the Gq-coupled B2 receptor in colonic myofibroblasts and raised the possibility that PKD could mediate cross talk between BK and TNF-α.

TNF-α augments BK-mediated PKD activation. As a first step to examine the role of PKD in the synergistic effects of proinflammatory mediators, we determined whether TNF-α enhanced the intensity and/or duration of BK-induced PKD activation in colonic myofibroblasts. Confluent 18Co cells were treated with TNF-α for 4–18 h, and PKD activation was assessed by Western blotting using site-specific antibodies that detect the phosphorylated state of Ser744 or Ser916. TNF-α added alone at various concentrations (8–25 ng/ml) did not induce any detectable increase in PKD phosphorylation in 18Co cells (data not shown). However, pretreatment of 18Co cells with TNF-α enhanced the intensity and duration of BK-mediated PKD activation (Fig. 6A). Pretreatment with the B2 receptor antagonist HOE-140 (10 μM) completely inhibited the enhanced PKD activation after treatment with BK and TNF-α (Fig. 6B). These experiments demonstrate that TNF-α augments BK B2 receptor-mediated PKD activation in 18Co cells, while having no detectable effects on PKD activation in these cells when added alone.

Synergistic COX-2 expression induced by BK and TNF-α is mediated by PKD. The stimulatory effects produced by TNF-α and BK on COX-2 expression and PKD activation and the inhibitory effects on both responses by preferential PKC/PKD...
inhibitors reinforced the hypothesis that PKD plays a critical role in mediating synergistic COX-2 expression in response to TNF-α and BK. To test this hypothesis directly, we determined whether siRNA-mediated knockdown of PKD protein in 18Co cells abrogates the synergistic expression of COX-2 in response to the proinflammatory mediators. After the optimal concentration of PKD siRNA (50 nM), conditions for transfection, and incubation time (72 h) in 18Co cells were determined, lysates of cells treated with targeting or nontargeting PKD siRNAs were analyzed by Western blotting to assess the level of PKD. As shown in Fig. 7A, siRNA targeting PKD produced a robust depletion of PKD protein in 18Co cells. Having demonstrated that PKD protein can be strikingly depleted in 18Co cells via transfection with PKD siRNA, we next determined the role of PKD in mediating synergistic COX-2 expression in response to BK and TNF-α. As shown in Fig. 7B, knockdown of PKD in 18Co cells completely prevented the synergistic increase in COX-2 protein induced by treatment with BK and TNF-α. In addition, PKD depletion drastically reduced COX-2 mRNA levels induced by BK and TNF-α (Fig. 7C). The results show that PKD plays a critical role in mediating synergistic expression of COX-2 protein and mRNA induced by BK and TNF-α in 18Co myofibroblasts.

In other cell types, including fibroblasts, one of the mechanisms by which PKD mediates GPCR-induced signaling is by increasing the duration of MEK/ERK/p90 ribosomal S6 kinase (p90RSK) activation (47), one of the major pathways leading to COX-2 transcriptional activation (28, 46, 59). It is known that TNF-α induces only weak stimulation of ERK signaling in most cells (53). However, we found that treatment of 18Co cells with TNF-α, under conditions that enhanced PKD activation, resulted in marked prolongation of BK-induced ERK signaling (data not shown), and consequently we hypothesized that the ERKs could play a role in relaying the PKD signal. To examine this possibility, 18Co cells were incubated for 1 h in the absence or presence of 5 μM U-0126, a selective inhibitor of MEK, and subsequently challenged with TNF-α, BK, or both. After 4 h, the cells were lysed and extracts were analyzed
by immunoblotting using a site-specific antibody that detects the activated form of ERK1/2 phosphorylated on Thr202 and Tyr204 and another antibody to detect COX-2 protein. As shown in Fig. 8, exposure of 18Co cells to the combination of TNF-α and BK produced synergistic stimulation of ERK activation and, as expected from the previous results shown in this study, a striking increase in COX-2 protein expression.

The salient feature of the results shown in Fig. 8 is that exposure to U-0126, at a concentration that completely prevented ERK activation, markedly reduced COX-2 expression in response to stimulation with the combination of TNF-α and BK. We verified that cell treatment with U-0126 did not inhibit BK-induced PKD activation (scored by Ser916 phosphorylation), demonstrating that ERK signaling is downstream of PKD (results not shown). Collectively, these results suggest that the ERK pathway contributes to PKD signaling leading to COX-2 expression.

**DISCUSSION**

The association between chronic inflammation and the development of cancer is a well-established but incompletely understood phenomenon (20). Numerous epidemiologic, cell culture, and animal models have supported this association, with one of the best-known examples seen in patients with ulcerative colitis. Although the fundamental mechanism is not known, the pathophysiology likely involves complex interactions between neighboring cells of the inflammatory microenvironment (34). These interactions are regulated by an intricate network of signaling pathways that, in a particular context and combination of interacting cells, may predispose to tumor development. In this context, there has been a growing awareness of the importance of the underlying intestinal stroma on the development of colon cancer (1, 2, 45, 62). However, the signal transduction pathways involved remained incompletely understood.

In the present study, we show that treatment of 18Co cells, a model of human colonic myofibroblasts, with the potent proinflammatory mediators TNF-α and BK induces a synergistic increase in the expression of COX-2 protein. Stimulation of myofibroblasts with TNF-α and BK also induced a synergistic increase in the levels of mRNA encoding COX-2 and mPGES-1 and in the production of PGE2. Intestinal myofibroblasts are recognized as a major source of stromal-derived COX-2, and increased numbers of these cells have been seen underlying adenomas and carcinomas in parallel with elevated levels of COX-2 (1, 2, 45, 62). A large body of evidence supports a causal relationship between COX-2 expression, PGE2, and colon carcinogenesis (7, 13, 36, 60), and recent results indicate that TNF-α is a major contributor to colitis-associated cancer (36) through as yet unidentified mechanisms.

The BK signaling system has also been associated with colitis in experimental models (3, 19) and in human tissues (48). Interestingly, our results showed that exposure of myofibroblasts to TNF-α alone induced a relatively small increase in COX-2 expression, but exposure to this cytokine dramatically amplified the ability of BK to induce COX-2 expression and PGE2. Consequently, it is conceivable that interactions between the signaling pathways initiated by TNF-α with other inflammatory mediators, including BK, may provide a mechanism for exaggerated cellular responsiveness (e.g., COX-2 expression) in response to stimulation.
PKD MEDIATES EXPRESSION OF COX-2 IN COLONIC MYOFIBROBLASTS

In an effort to elucidate signaling pathways involved in colonic myofibroblasts, we found that the increase in COX-2 expression induced by BK and TNF-α in these cells was prevented by the B₂ receptor antagonist HOE-140 and the PKC inhibitors Ro31-8220, GF-109203X, and Gö-6976, indicating that PKCs have a major role in mediating COX-2 induction in myofibroblasts. These results are consistent with the notion that PKC isoforms mediate numerous cellular responses elicited by proinflammatory peptides, including the synthesis and release of chemokines and eicosanoids that amplify the inflammatory response (10, 23, 31, 63, 64). However, the mechanism(s) by which PKC-mediated signals are propagated to critical downstream targets remains incompletely understood in most cell types, and this has not been explored in intestinal myofibroblasts.

Many studies have demonstrated that PKD is rapidly activated by GPCR agonists through a Gα/PLC/PKC-dependent pathway. At baseline, PKD is maintained in a catalytically inactive state through the suppressive actions of its cysteine-rich domain and pleckstrin homology domain (16, 54, 58). PKD is activated by phosphorylation at sites (Ser744 and Ser748) located in the activation loop of the catalytic domain (17, 21, 39, 41, 55). Our results showed that BK-induced PKD phosphorylation on Ser744 and Ser916, a major autophosphorylation site, was prevented by PKC inhibitors (Ro31-8220, GF-109203X). Furthermore, treatment of the cells with Gö-6976, an inhibitor of classic PKCs and PKD, greatly diminished autophosphorylation on Ser916 but did not prevent phosphorylation on Ser744. These results confirmed that PKD is directly inhibited by Gö-6976 and suggest that PKD phosphorylation on the activation loop is mediated by novel (Gö-6976 insensitive) PKCs. Interestingly, exposure of myofibroblasts to TNF-α alone did not produce any detectable increase in PKD phosphorylation either on Ser744 or on Ser916, but this cytokine greatly increased the intensity and duration of BK-induced PKD phosphorylation.

Fig. 8. PKD-mediated synergistic COX-2 expression is inhibited by U-0126. I8Co cells were treated with TNF-α (8.3 ng/ml), BK (100 nM), or both for 6 h with or without preincubation with U-0126 (5 μM), a selective MEK inhibitor, for 60 min. Combined treatment with TNF-α and BK led to synergistic COX-2 expression, as previously described, and also markedly augmented ERK phosphorylation. Preincubation with U-0126 inhibited ERK phosphorylation and significantly reduced COX-2 expression in response to TNF-α and BK. Result shown is a representative autoluminogram; band intensity was analyzed by densitometric scanning of 3 independent experiments, presented as mean ± SE, and expressed as % of maximum level of p42/44 and COX-2. Equal protein loading was verified with an antibody that detects ERK2. *Statistical significance (P < 0.05).

Accumulating evidence demonstrates that PKD plays an important role in several cellular processes and activities, including signal transduction, chromatin organization, Golgi function, gene expression, immune regulation and cell survival, adhesion, motility, differentiation, and DNA synthesis and proliferation (reviewed in Refs. 40, 41). However, none of the previous studies demonstrated a link between PKD and COX-2 expression. Our results, from multiple pharmacological interventions, suggested that PKD could be a point of integration of inputs from TNF-α and BK leading to dramatic COX-2 expression in colonic myofibroblasts. To test this possibility further, we used siRNA targeting PKD in myofibroblasts and then challenged these cells with BK and TNF-α. Under conditions that resulted in siRNA-mediated depletion in PKD protein, the synergistic effect of these proinflammatory mediators on COX-2 expression was completely blocked. These results, together with the potent inhibitory effects on COX-2 expression and PKD activation produced by preferential PKC inhibitors (Ro31-8220, GF-109203X) and a direct PKD inhibitor (Gö-6976), imply that PKD is a critical downstream target that links TNF-α and BK to COX-2 expression.

In a variety of cell types, COX-2 gene expression is upregulated by several transcription factors, including cAMP-response element-binding protein (CREB), CCAAT/enhancer-binding protein (C/EBP), activator protein 1 (AP-1), and NF-κB (see Ref. 11) and repressed by histone deacetylases (HDACs), including HDAC4 (61). A mechanism by which PKD mediates Gα-coupled receptor signaling is by increasing the duration of the MEK/ERK/p90RSK activation (47), one of the major pathways leading to COX-2 transcriptional activation via AP-1 (28, 46, 59). Here we show that suppression of ERK activation markedly reduced COX-2 expression in response to stimulation with the combination of TNF-α and BK, suggesting that the ERK pathway contributes to PKD signaling leading to COX-2 expression. Recently, PKD has been implicated in the regulation of NF-κB and CREB activity and in promoting nuclear extrusion of class II HDACs (41). Thus PKD could act as a switch of COX-2 expression in colonic myofibroblasts through different transcriptional pathways, a proposition that warrants further experimental work.

In conclusion, our results demonstrate potent synergistic effects between TNF-α and BK leading to COX-2 expression through PKD in colonic myofibroblasts. Given that TNF-α, BK,
COX-2, and myofibroblasts are increasingly implicated in gastrointestinal inflammation and cancer, the cross talk between TNF-α and BK, identified here, leading to synergistic enhancement of COX-2 expression through PKD provides an attractive mechanism potentially leading to colitis-associated cancer. A better understanding of the contribution of stromal cells to inflammation and tumorigenesis could lead to new therapeutic approaches and novel treatment strategies. In this context, PKD emerges as a potential novel target for the prevention and therapy of inflammatory bowel diseases and colorectal cancer.

REFERENCES

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DISCLOSURES

No conflicts of interest are declared by the author(s).


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