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Bradykinin stimulates protein kinase D–mediated colonic myofibroblast migration via cyclooxygenase-2 and heat shock protein 27

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ABSTRACT

Background: Inflammatory bowel disease is characterized by episodic intestinal injury and repair. Myofibroblasts are gastrointestinal tract stromal cells that regulate the reparative process and are known targets of inflammatory mediators including bradykinin (BK). However, the mechanisms through which inflammation regulates myofibroblast-induced wound healing remain incompletely understood. Here, we demonstrate, for the first time, that BK stimulates myofibroblast migration through protein kinase D (PKD)–mediated activation of the cyclooxygenase-2 (COX-2) and heat shock protein 27 (Hsp27) pathways.

Materials and methods: CCD-18Co is a human colonic myofibroblast cell line used from passages 8 to 14. An *in vitro* scratch assay assessed the effect of BK (100 nM) on myofibroblast migration over 24 h in the presence or absence of several inhibitors (CID755673 [10 μM] and NS398 [10 μM]). Hsp27 small interfering RNA evaluated the effect of Hsp27 on colonic myofibroblast migration. Antibodies to pPKD, pHsp27, and COX-2 evaluated expression levels by Western blot.

Results: BK stimulated myofibroblast migration over 24 h. BK also led to rapid and sustained phosphorylation of PKD at Ser-916, rapid phosphorylation of Hsp27 at Ser-82, and increased COX-2 expression over 4 h. BK-mediated COX-2 expression and Hsp27 phosphorylation were both inhibited by the PKD inhibitor CID755673. Similarly, BK-induced myofibroblast migration was significantly inhibited by CID755673 ($P < 0.05$), by the direct COX-2 inhibitor NS398 ($P < 0.05$), and by Hsp27 small interfering RNA ($P < 0.05$).

Conclusions: BK stimulates myofibroblast migration through PKD-mediated activation of COX-2 and Hsp27. PKD, COX-2, and Hsp27 all appear to regulate myofibroblast cell migration, a stromal population that may play an important role in mucosal healing in the setting of inflammation.

Introduction

Inflammatory bowel disease (IBD) describes two clinical entities, Crohn's disease and ulcerative colitis. Though distinct in many ways, they share overlapping characteristics that include repetitive inflammatory injury that is immune mediated and chronic. Intestinal repair is a complex process that involves stromal–epithelial cell communication to restore the

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integrity of the bowel wall. Myofibroblasts are stromal cells of the gastrointestinal tract that are a known target of inflammatory mediator signaling[1-4] and appear to play an important role in restoring intestinal homeostasis.[5-7] Myofibroblasts migrate to areas of injury and interact with the overlying epithelium to regulate cell proliferation as well as deposition and remodeling of the underlying extracellular matrix (ECM).[6,8-11]

We have recently demonstrated that myofibroblast migration can be stimulated by tumor necrosis factor (TNF)-α,[12] a potent 17-kDa proinflammatory cytokine that is known to regulate myofibroblast function[1-3] and has been strongly implicated in the pathogenesis of IBD.[3,13] TNF-α was found to stimulate myofibroblast migration through signaling pathways involving cyclooxygenase-2 (COX-2) and heat shock protein 27 (Hsp27).[12] COX-2, the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs) and thromboxanes, is upregulated in the setting of colitis and plays an important role in mucosal repair.[1,8,12,14] Hsp27 is a molecular chaperone and a member of the small HSP group encoded by the *HspB1* gene that regulates the stress response, wound healing, and cell migration.[12,15-17]

Interestingly, both COX-2 and Hsp27 are known downstream targets of protein kinase D (PKD), a ubiquitous serine–threonine kinase that is involved in biological responses to inflammation and oxidative stress, but is not independently activated by TNF-α.[1,2,18] Structurally, PKD is composed of a cysteine rich domain and a pleckstrin homology domain, which keep PKD in a basal, inactive state.[2] PKD can be activated by multiple G protein–coupled receptor (GPCR) agonists, including bradykinin (BK), in the setting of inflammation,[1,2] but the role of PKD in the regulation of myofibroblast migration is unknown. Consequently, the purpose of this study was to determine whether GPCR-mediated PKD activation can regulate colonic myofibroblast migration and to determine the underlying cell signaling mechanisms that are involved in this process.

Here, we demonstrate that BK-mediated PKD activation stimulates the migration of human colonic myofibroblasts (18Co) through signaling pathways that involve COX-2 and Hsp27. Our results support the notion that PKD signaling may play an important role in myofibroblast migration and intestinal repair. Furthermore, COX-2 and Hsp27 appear to be conserved signaling targets that regulate myofibroblast migration through activation by multiple upstream agonists.

Materials and methods

Cell culture

The human myofibroblast cell line CCD-18Co was purchased from American Type Culture Collection (Manassas, VA). These cells share structural and functional similarities with primary colonic subepithelial myofibroblasts and have been validated by several previous studies.[8,19,20] CCD-18Co cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, penicillin, streptomycin, fungizone, and glutamine at 37°C in 5% CO_2 humidified air. Cells were passaged when confluent and experiments were performed with cells from passages 8 through 14.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

Cell lysis was performed using Triton buffer (50 mM Tris, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1% Triton X-100, 50 mM NaF plus 1% Calbiochem protease inhibitor cocktail) and lysates were assayed for protein using the Bradford protein assay, then diluted with 5× Laemmli loading buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Equal amounts of protein were loaded in 4%-20% Tris–glycine gels and electrophoresed for 120 min at 130 V constant voltage. The gel was blotted onto a polyvinylidene difluoride membrane by electrophoretic transfer at 25 V constant voltage overnight. The membrane was washed, blocked with 5% milk, and probed with primary antibodies. Appropriate secondary antibodies conjugated to horseradish peroxidase (Pierce, Rockford, IL) and a chemiluminescent substrate (SuperSignal; Pierce) were used to visualize immunoreactive bands. The primary antibodies against pHsp27, pPKD, and COX-2 were purchased from Cell Signaling (Danvers, MA), antibodies against Hsp27 and GAPDH were purchased from Santa Cruz (Santa Cruz, CA). Donkey anti-mouse and donkey anti-rabbit secondary antibodies were purchased from Pierce. Protein expression was analyzed with software ImageJ and was normalized relative to total protein for each lane ("relative expression").

Scratch assay

For migration assays, cells were plated in 35-mm dishes until confluent. Under varying conditions, a scratch gap was then created by scratching the cell monolayer with a sterile pipette tip, and the cells were incubated in serum-free medium. Three pictures were randomly taken along the scratch gap (time 0 h), and these positions were marked for subsequent re-evaluation (time 24 h). The scratch area was measured with software ImageJ and normalized to time 0 h control. Cells were treated with CID755673 (10 μM), NS398 (10 μM), or HSP27 small interfering RNA (siRNA; 100 nM) before exposure to BK during the migration assay.

SiRNA transfection

HSP27 siRNA (Cat # 4392420; Ambion, Carlsbad, CA) or negative control siRNA (Cat # 4611; Ambion) was used to transfect 80%-90% confluent CCD-18Co cells with Lipofectamine 2000 (Invitrogen, CA) at 100 nM according to the manufacturer's instructions. Western blotting confirmed the silencing effect of siRNA on the expression levels of Hsp27.[12] Transfected cells were then used for migration experiments.

Materials and reagents

Dulbecco's Modified Eagle's Medium, fetal bovine serum, penicillin G potassium, streptomycin, fungizone, and