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Activation of focal adhesion kinase via M1 muscarinic acetylcholine receptor is required in restitution of intestinal barrier function after epithelial injury

Md Rafiqul Islam Khan [a,i], Takashi Yazawa [a], Abu Syed Md Anisuzzaman [i], Shingo Semba [a], Yanju Ma [k], Junsuke Uwada [c], Hisayoshi Hayashi [f], Yuichi Suzuki [f,g], Hiroki Ikeuchi [j], Motoi Uchino [j], Atsuo Maemoto [b,h], Ikunobu Muramatsu [c,d,e], Takanobu Taniguchi [a,*]

[a] Division of Cellular Signal Transduction, Department of Biochemistry, Asahikawa Medical University, Asahikawa, Japan
[b] Department of Gastrointestinal Immunology and Regenerative Medicine, Asahikawa Medical University, Asahikawa, Japan
[c] Division of Pharmacology, Department of Biochemistry and Bioinformative Sciences, University of Fukui, Fukui, Japan
[d] Organization for Life Science Advancement Programs, University of Fukui, Fukui, Japan
[e] Research Center for Child Mental Development, University of Fukui, Fukui, Japan
[f] Laboratory of Physiology, School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Japan
[g] Division of Health and Nutrition, Sendai Shirayuri Women's College, Sendai, Japan
[h] Inflammatory Bowel Disease Center, Sapporo Higashi Tokushukai Hospital, Sapporo, Japan
[i] Department of Pharmacy, University of Rajshahi, Rajshahi, Bangladesh
[j] Inflammatory Bowel Disease Center, Hyogo College of Medicine, Nishinomiya, Japan
[k] Department of Medical Oncology, The First Hospital of China Medical University, Shenyang, China

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ABSTRACT

Impairment of epithelial barrier is observed in various intestinal disorders including inflammatory bowel diseases (IBD). Numerous factors may cause temporary damage of the intestinal epithelium. A complex network of highly divergent factors regulates healing of the epithelium to prevent inflammatory response. However, the exact repair mechanisms involved in maintaining homeostatic intestinal barrier integrity remain to be clarified. In this study, we demonstrate that activation of M1 muscarinic acetylcholine receptor (mAChR) augments the restitution of epithelial barrier function in T84 cell monolayers after ethanol-induced epithelial injury, via ERK-dependent phosphorylation of focal adhesion kinase (FAK). We have shown that ethanol injury decreased the transepithelial electrical resistance (TER) along with the reduction of ERK and FAK phosphorylation. Carbachol (CCh) increased ERK and FAK phosphorylation with enhanced TER recovery, which was completely blocked by either MT-7 (M1 antagonist) or atropine. The CCh-induced enhancement of TER recovery was also blocked by either U0126 (ERK pathway inhibitor) or PF-228 (FAK inhibitor). Treatment of T84 cell monolayers with interferon-γ (IFN-γ) impaired the barrier function with the reduction of FAK phosphorylation. The CCh-induced ERK and FAK phosphorylation were also attenuated by the IFN-γ treatment. Immunological and binding experiments exhibited a significant reduction of M1 mAChR after IFN-γ treatment. The reduction of M1 mAChR in inflammatory area was also observed in surgical specimens from IBD patients, using immunohistochemical analysis. These findings provide important clues regarding mechanisms by which M1 mAChR participates in the maintenance of intestinal barrier function under not only physiological but also pathological conditions.

1. Introduction

Epithelial integrity of the gut is essential for preventing the invasion of microorganisms and the development of inflammation in intestinal submucosa. The intestinal epithelium is a highly selective barrier that permits the absorption of nutrients from the gut lumen into the circulation and at the same time restricts the passage of harmful and potentially toxic compounds [1,2]. Disruption of intestinal barrier integrity (leaky gut) may lead to the penetration of luminal bacterial products into the submucosa to initiate local inflammation [1]. Mild form of intestinal epithelial injury commonly occurs in many diseases, which is rapidly repaired to reform the integrity of epithelial monolayers to prevent invasion of noxious compounds.

Findings on various tissues or cells reveal that focal adhesion kinase (FAK) is one of the key regulators for the maintenance and repair of barrier functions [3–7]. FAK is a non-receptor tyrosine kinase that modulates various cell functions, including survival, proliferation, and

* Corresponding author at: Division of Cellular Signal Transduction, Department of Biochemistry, Asahikawa Medical University, Asahikawa 078-8510, Japan. Tel.: +81 166 68 2340; fax: +81 166 68 2349.
E-mail address: takotago@asahikawa-med.ac.jp (T. Taniguchi).

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migration [8,9]. On the other hand, the MAP kinase family makes up a group of important intracellular mediators of signal transduction to various stimuli. The classical MAP kinase, ERK1/2 (ERK) has been associated with the regulation of cellular proliferation and differentiation [10,11]. The role of MAP kinase pathway in the regulation of the paracellular permeability across epithelial cells has not been explored extensively. However, several studies have reported the positive regulation of epithelial barrier function as a result of the phosphorylation of ERK [12,13].

Carbachol (CCh), an agonist of muscarinic acetylcholine receptors (mAChRs), increased the phosphorylation of ERK and FAK in T84 cells [14]. Recent evidence implies the participation of mAChRs in the tightness of epithelial integrity in the proximal colon [15]. These findings suggest the crucial role of mAChRs in the intestinal epithelium to maintain and/or to repair barrier functions. However, the mechanisms and signaling molecules downstream from mAChRs in the regulation of barrier functions are still unknown.

The family of mAChRs belongs to a G-protein–coupled-receptor superfamily. Five subtypes of mAChRs (M1–M5), with difference in signal transduction, have been cloned [16,17]. Numerous studies revealed that intestinal infection and inflammation impaired the muscarinic cholinergic response to the gut epithelium [18]. The exact signaling mechanism of cholinergic hyporesponsiveness to the gut epithelium under inflammatory condition remains to be clarified. We reported previously that ERK and FAK are localized in the intestinal epithelial cells [19,20] and may be involved in the modulation of barrier functions under normal as well as inflammatory conditions. Impaired epithelial barrier function is a common feature of the inflammatory bowel diseases (IBD) and is caused, at least in part, by the elevated level of various cytokines. Treatment of T84 epithelial cell monolayers with interferon-γ (IFN-γ), one of those cytokines, has been shown to compromise their barrier integrity with a decrease in transepithelial electrical resistance (TER) and an increase in epithelial permeability [21,22]. Recently, it has been demonstrated that AMP-activated protein kinase and phosphatidylinositol 3′-kinase may be involved in IFN-γ-induced epithelial barrier dysfunction [23–25]. However, the precise mechanism of barrier dysfunction under the inflammatory condition is yet to be elucidated.

In this study, we aimed to uncover the role of mAChRs and the downstream signaling pathway in the maintenance and restitution of the barrier function in human intestinal epithelial cells. We also sought to investigate the cause of barrier dysfunction under inflammatory condition in T84 cell monolayers. Our data suggest that the IFN-γ-induced barrier dysfunction is associated at least in part, with the reduction of FAK phosphorylation presumably via the downregulation of M1 mAChR signaling. We also tested surgical specimens of colons from IBD patients in immunohistochemical analysis.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12, trypsin-EDTA solution and CCh (Sigma Aldrich, St. Louis, USA), fetal bovine serum (FBS) and Lipofectamine 2000 (Invitrogen, Life Technologies Corp., CA, USA), atropine sulfate, U0126 and human IFN-γ (Wako Pure Chemical Industries Ltd., Osaka, Japan), muscarinic toxin 3 (MT-3) and 7 (MT-7) (Peptide Institute, Inc., Osaka, Japan), PF-573228 (PF-228, Tocris Cookson Ltd., Bristol, UK), [^{3}H]-*N*-methyl scopolamine chloride (PerkinElmer, Boston, USA) were obtained from the sources noted. Antibodies against MAP kinases (ERK, p38 and JNK), phosphorylated MAP kinases, MAP2K1, FAK, phosphorylated FAK at tyrosine 397 (Y397), and β actin were from Cell Signaling Technology, Inc. (Massachusetts, USA) and those against mAChR M1 (H-120) and mAChR M3 (H-210) were from Santa Cruz Biotechnology, Inc. (CA, USA).

2.2. Cell culture

A human colon cancer cell line, T84, from the Health Protection Agency Culture Collection (Salisbury, UK) was grown in a humidified atmosphere with 5% CO_2 at 37 °C in DMEM and Ham's F12 medium supplemented with 2 mM glutamine, 15 mM HEPES (pH 7.2), 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were separated by trypsinization, and 1×10^6 cells were seeded onto 12-mm diameter semipermeable filter supports (Millicell-PCF, 0.4 μm pore size, Millipore, Ireland) for TER measurement and immunoblot analysis. Cells were cultured for at least 14–21 days prior to use. Human small intestine epithelial cells (PD015-F, DV Biologics, CA, USA) were grown in pro-conditioned medium (D-PRO-015, DV Biologics) in collagen coated culture plates or collagen coated transwell as above.

2.3. Receptor binding assay

After development of 100% confluent monolayers with complete differentiation, cells were scraped with rubber policeman and washed with modified Krebs–Henseleit solution (KHS, comprising NaCl, 120.7 mM; KCl, 5.9 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2.0 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 25.5 mM; and (+)-glucose, 11.5 mM, pH 7.4), which had been bubbled with a mixture of 95% O_2 and 5% CO_2. The whole-cell suspension (1×10^6 cells/ml in KHS) was then incubated for 2 h with [^{3}H]-*N*-methyl scopolamine chloride (NMS) and appropriate chemicals in a final volume of 1 ml at 4 °C. The assays were performed in duplicate and a nonspecific binding was defined in the presence of 1 μM atropine. [^{3}H]-NMS concentrations ranging from 30 to 2500 pM were used in saturation binding experiments, while 600 pM was used in the competition binding experiment with the addition of increasing concentrations of the unlabeled drugs. The reactions were terminated by centrifuging the incubation solution at 700 ×*g* for 2 min. The cell pellets were then washed once with 1 ml KHS and were dissolved in 0.3 M NaOH. The radioactivity was measured by a liquid scintillation counter (Hitachi Aloka Medical Ltd., Mitaka, Japan). The mock incubation, in which cells were omitted, gave less than 40 counts per minute per tube and was similar to the background reading. Protein concentration was measured using a protein assay kit (Bio-Rad Inc., CA, USA).

2.4. Immunoblotting

Cells were cultured onto 12-mm Millicell-PCF for 14–21 days. The monolayers on filters were washed with KHS for three times and allowed to equilibrate in KHS for 30 min at 37 °C with or without inhibitors. Cells were then stimulated with 100 μM carbachol (CCh) for 5 min. For receptor activation or inhibition by muscarinic ligands, all drugs were added basolaterally as described previously [26]. Reaction was terminated by aspirating the medium and rinsed with ice-cold PBS twice. Cells were then lysed by adding pre-heated 1× SDS sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 1% β-mercapto ethanol, 0.1% bromophenol blue) to the monolayers and the lysates were collected into Ependorf tubes and heated for 3 min at 100 °C. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were probed with appropriate concentrations of primary antibody. The immunoreactive proteins were detected by horseradish-peroxidase-labeled secondary antibody with Amersham ECL advance Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK). The ratio of intensities of signals was quantified by densitometry.

2.5. Knockdown of M1 and MAP2K1 by siRNA

We used predesigned siRNAs (Life Technologies) as follows: CHRM1 (5′–3′)-AGGUCAACACGGAGCUCAAtt and MAP2K1 (5′–3′) GGCCUGAC AUAUCUGAGGGtt. Transient transfections of siRNA into T84 cells were