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Activation of muscarinic cholinoceptor ameliorates tumor necrosis factor-α-induced barrier dysfunction in intestinal epithelial cells

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ABSTRACT

Impaired intestinal barrier function is one of the critical issues in inflammatory bowel diseases. The aim of this study is to investigate muscarinic cholinoceptor (mAChR)-mediated signaling for the amelioration of cytokine-induced barrier dysfunction in intestinal epithelium. Rat colon challenged with TNF-α and interferon γ reduced transepithelial electrical resistance (TER). This barrier injury was attenuated by muscarinic stimulation. In HT-29/B6 intestinal epithelial cells, muscarinic stimulation suppressed TNF-α-induced activation of NF-κB signaling and barrier disruption. Finally, muscarinic stimulation promoted the shedding of TNFR1, which would be a mechanism for the attenuation of TNF-α/NF-κB signaling and barrier disruption via mAChR.

1. Introduction

Intestinal epithelial cells (IECs) functions, as a barrier to restrict the passage of pathogenic antigens into the interstitium. Disruption of intestinal barrier integrity may lead to the penetration of luminal bacterial products into the submucosa to trigger local inflammation [1]. Barrier dysfunction is one of the main indicators of the inflammatory bowel diseases (IBD). Therefore, modulation of the epithelial barrier function is currently viewed as a potentially positive pharmacological outcome.

The intestinal epithelium not only provides a physical barrier against infectious agents but also functions as a sensor of infection for the immune system. During gut inflammation, IECs receive their activating signals from basically two sources: [1] the humoral factors, such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) and [2] bacteria and bacterial products [2,3]. TNF-α is a potent activator of IECs to produce the proinflammatory cytokine interleukin-8 (IL-8), which is an important mediator of inflammation of the CXC chemokine family [4,5]. An enhanced synthesis of IL-8 has been shown in the intestinal mucosa from patients with

Abbreviations: mAChR, muscarinic cholinoceptor; IEC, intestinal epithelial cell; TER, transepithelial electrical resistance; IBD, inflammatory bowel diseases; FD-4, fluorescein isothiocyanate-labeled 4kDa dextran; FBS, fetal bovine serum; IFN γ, interferon γ; MT-3, muscarinic toxin 3; MT-7, muscarinic toxin 7; KHS, Krebs–Henseleit solution; TAPI-0, TNF-alpha protease inhibitor-0; NMS, *N*-methylscopolamine chloride; ATR, atropine; CCh, carbachol

Author contributions: Author contributions are as the follows: M.I.K. and T.T. designed the study and wrote the paper. M.I.K. and J.U. performed TER measurement and ELISA assays. J.U., S.K., Y.S. and A.K. prepared rat colonic membrane, performed permeability study including Ussing chamber experiments and dye leakage, and analyzed the data. S.M.K. and M.F. provided HT-29/B6 cells and optimized culture conditions. T.Y. and M.T.I. designed and constructed the plasmids, and performed PCR and luciferase assays. M.I.K., H.Y., K.S., A.M.A. and I. M. performed binding and immunoblotting experiments, and analyzed the data.

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IBD [6–9]. The expression of this proinflammatory cytokine is upregulated by the transcription factor nuclear factor κB (NF-κB) [10]. In its inactive state, NF-κB predominantly exists as a heterodimer of p65 (RelA) and p50 subunits and resides in the cytoplasm associated with an inhibitory protein, IκB. Upon exposure to various stimuli, IκB undergoes rapid degradation and free NF-κB translocates into nucleus where it binds to the promoter region of target genes to induce transcription [11,12].

In IECs, acetylcholine is considered as one of the most important regulators, which controls numerous physiological functions including ion transport and cell proliferation, presumably via nicotinic and/or muscarinic cholinoceptor (mAChR) [13,14]. Several previous reports including those from our laboratory demonstrated that IECs express mAChRs [15–18], in which five subtypes of mAChRs (M1–M5) which differ in signal transduction have been cloned [19,20]. Emerging evidence indicates positive effects of cholinergic activation for the intestinal barrier function [14,16,21,22]. However it remains unclear whether muscarinic stimulation contributes to protection against inflammatory cytokines like TNF-α.

In this study, we attempted to uncover the potential benefits of the mAChR response on barrier function in IECs using native tissues of rat colon and HT-29/B6 intestinal epithelial cells. Our results indicate that activation of mAChR ameliorates TNF-α-induced barrier dysfunction by the regulation of NF-κB signaling in IECs.

2. Materials and methods

2.1. Reagents

Carbachol (CCh), lucifer yellow, fluorescein isothiocyanate-labeled 4kDa dextran (FD-4) and piperacillin from Sigma Aldrich (MO, USA), fetal bovine serum (FBS) from Invitrogen (CA, USA), recombinant human TNF-α from PEPROTECH (NJ, USA), atropine sulfate, recombinant rat TNF-α, recombinant rat interferon γ (IFNγ) and imipenem *n*-Hydrate from Wako (Osaka, Japan), TNF-alpha protease inhibitor-0 (TAPI-0), muscarinic toxin 3 (MT-3) and 7 (MT-7) from Peptide Institute (Osaka, Japan), [^{3}H]-*N*-methylscopolamine chloride (NMS) from PerkinElmer (MA, USA), BAY 11-7082 from Focus Biomolecules (PA, USA) were obtained from the sources noted.

2.2. Measurements of TER in vitro

The handling and killing of animals were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals* of the University of Shizuoka, and this study was approved by the University of Shizuoka Animal Usage Ethics Committee. Mucosal-submucosal sheets of distal colon were prepared from adult male SD rats (SLC, Hamamatsu, Japan) weighing between 300 and 340 g, as described previously [23]. Separated samples were mounted in Ussing chambers that provided an exposed area of 0.64 cm^2. The mucosal and serosal surfaces of the tissue were bathed with 10 ml of bathing solution and maintained at 37 °C in a water-jacketed reservoir. The bathing solution was composed of 119 mM NaCl, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, 21 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D(+)-glucose, 0.5 mM β-OH-butyrate, 2.5 mM glutamine and 10 mM D(+)-mannose, supplemented with heat inactivated FBS (final concentration, 10%) and antibiotics (50 mg/l piperacillin and 50 mg/l imipenem). The solution was gassed with 95% O_2 and 5% CO_2. The tissue was continuously short-circuited with compensation for the fluid resistance between the two potential sensing electrodes by using an automatic voltage-clamping device (CEZ9100; Nihon Kohden, Tokyo, Japan). TER was measured by recording the current resulting from short duration, square bipolar voltage pulses (5 s and ± 10 mV) for each 10 min cycle. Atropine (10 μM) and CCh (100 μM) were applied to serosal side 60 or 30 min prior to pro-inflammatory cytokine stimulation, respectively. TER attained plateau within 6–8 h after the start of incubation and remained there until at least 21 h under control conditions. After the addition of rat cytokines (100 ng/ml TNF-α and 100 ng/ml IFN γ) to serosal side, measurements were continued for 21 h.

2.3. Cell culture

HT-29/B6 cells are a subclone of the human colon cell line HT-29, which grow as highly differentiated polarized epithelial monolayers [24]. Cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C in RPMI 1640 medium supplemented with 2 mM glutamine, 15 mM HEPES (pH 7.2), 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.4. 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. Binding experiments were performed as described in a previous report [16].

2.5. Immunoblotting

Cells were cultured onto 12-mm Millicell-PCF (Millicell-PCF, 0.4 μm pore size, Millipore, Cork, Ireland) for 5–7 days. Cells were then stimulated with 100 ng/ml TNF-α for 5 min with or without the presence of mAChR ligands. Atropine (ATR, 10 μM) or TAPI-0 (10 μM) was added 20 min prior and agonist, CCh was added 5 min prior to the stimulation by TNF-α. All agonists and antagonists were applied to serosal side. Reaction was terminated by aspirating the medium and rinsed with ice-cold PBS twice. Cells were then lysed by adding a pre-heated SDS sample buffer and analyzed by immunoblotting with antibodies against IκB, phospho-IκB, NF-κB, phospho-NF-κB, and GAPDH (Cell Signaling Technology., MA, USA).

2.6. Measurement of TER and paracellular permeability

TER across the cell monolayers was measured using a Millicell ERS-2 epithelial volt-ohm meter (Millipore, Darmstadt, Germany). The values ($\Omega \times cm^2$) were obtained by subtracting the resistance of blank filters without cells from the resistance of filters with cells multiplied by the effective membrane area of the filter insert. Inserts having TER of at least 600 $\Omega \times cm^2$ was used for the experiments. For the measurement of paracellular permeability, medium was replaced with KHS with Ca^{2+} and Mg^{2+}. Lucifer yellow or FD-4 was added to the apical compartment at final concentration of 1 or 0.4 mM, respectively. Then cells were incubated for 2 h. Samples were collected from basolateral compartment at every 30 min. Fluorescence leakage was determined for Lucifer yellow by 430 nm excitation and 535 nm emission or for FD-4 by 494 nm excitation and 521 nm emission using a fluorescence spectrophotometer (Hitachi F-4500; Hitachi, Tokyo, Japan).