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PAR-2 activation increases human intestinal mucin secretion through EGFR transactivation

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Abstract

PAR-2 (protease-activated receptors-2) are G protein-coupled receptors whose action on mucin secretion by intestinal epithelial cells is still unknown. The aim of this study was to examine the effect of PAR-2 activation on mucin secretion in the human colonic goblet cell line HT29-Cl.16E and the intracellular pathways involved. We found that PAR-2 mRNA was constitutively expressed by HT29-Cl.16E cells as well as by isolated human normal colonocytes. The PAR-2-activating peptide SLIGKV-NH_2 elicited rapid mucin secretion in HT29-Cl.16E, which was partially inhibited by calcium chelator BAPTA. Inhibitors of MAPK activation (PD98059) and EGFR tyrosine kinase activity (AG1478) abrogated PAR-2-induced ERK1/2 and EGFR tyrosine phosphorylation, respectively, and subsequent mucin secretion. Finally, PAR-2-induced EGFR transactivation was involved upstream of ERK1/2 activation. Our results show that the activation of PAR-2 expressed by human intestinal epithelial cells enhances mucin secretion, a component of the intestinal innate defence, via a pathway involving EGFR transactivation.

Keywords: Protease-activated receptor-2; Trypsin; Human colonic epithelial cells; Mucus secretion; EGFR; MAP kinases; Calcium

The gastrointestinal (GI) tract is exposed to a large array of proteases, under both physiological and pathophysiological conditions. The discovery of G protein-coupled receptors activated by proteases, the protease-activated receptors (PARs) [1–5] have highlighted new signalling functions for proteases in the GI tract [6,7].

Protease-activated receptors (PARs) belong to a growing family of G protein-coupled receptors (GPCRs), activated by extracellular proteases which currently consist in four members (PARs 1–4). Trypsin, mast cell tryptase and other tissue specific serine proteases activate PAR-2 by proteolytic unmasking of the N-terminal extracellular cryptic receptor activating sequence [5]. A short synthetic peptide (activating peptide-2 or AP2) corresponding to the new amino-terminus exposed after trypsin cleavage is able to activate selectively the PAR-2 receptor and mimics cellular effects of trypsin [2,8]. PAR-2 activation initiates signal transduction resulting in stimulation of phosphoinositide breakdown and cytosolic calcium mobilization [1,4].

The presence of PAR-2 has been revealed in a variety of tissues [1,9]. In particular, high expression has been observed in the GI tract [1,9–11], where it is expressed on many cell types including epithelial and endothelial cells, enteric neurons, myocytes, and immune cells [2,9,11,12]. In the intestine, activation of PAR-2 has been involved in ion [13] and prostaglandin E2 (PGE2) secretion [11]. Other effects of PAR-2 in the GI tract have been described, for example on gastric mucus secretion [14] and on inflammation [15], which are however mediated, at least in part, by the release of neuromediators [14,16].

Mucin secretion is important in gastrointestinal mucosal cytoprotection and is considered as part of the gut innate

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defences. Whether PAR-2 activation is able to regulate mucin secretion from intestinal goblet cells, by direct or indirect effects, is so far unknown. To examine this issue, we used the HT29-Cl.16E cell line, a human colonic epithelial cell line that differentiates into polarized goblet cells [17]. This allows assessing the effects of PAR-2 agonists, without the interference of neuromediators or immune-derived inflammatory cytokines. In this study, we investigated (1) PAR-2 expression in HT29-Cl.16E cells and human normal colonocytes, (2) the functional coupling of PAR-2 to mucin secretion in HT29-Cl.16E cells, and (3) the intracellular pathways involved in PAR-2-induced mucin secretion in HT29-Cl.16E cells.

Materials and methods

Cell culture and treatments. The HT29-Cl.16E epithelial cell line [17] was grown on porous filters (12-well Transwell Clear, 0.45 μm porosity, Costar, France) and formed monolayers of polarized cells at post-confluence, which differentiated into goblet cells expressing the secretory mucin genes MUC2 and MUC5AC [18]. Cells were seeded at high density in DMEM (4.5 g/L glucose)/10% heat-inactivated FCS (Invitrogen, Cergy-Pontoise, France) and cultured until post-confluence and full differentiation. Experiments were conducted at day 18 after seeding. HT29-Cl.16E cells formed polarized monolayers, secreting mucins into the apical compartment. After a 48 h serum-deprivation, the PAR-2 agonists SLIGKV-NH_2 (100 μM, Neosystem, Strasbourg, France) or trypsin (16,000 U mg^{-1}, Sigma, St. Louis, MO) were added to the basolateral compartment, at the indicated time points. For some experiments, monolayers were pre-treated for 1 h with the EGFR tyrosine kinase inhibitor AG1478 (3 μM, Calbiochem, San Diego, CA), or with the MEK inhibitor PD98059 (25 μM, Calbiochem), or with the intracellular Ca^{2+} chelator BAPTA-AM (20 μM, Sigma), before treatment with the PAR-2 synthetic peptide SLIGKV-NH_2.

Isolation of epithelial cells from human normal colonic mucosa was performed as previously described [19].

Reverse-transcription polymerase chain reaction (RT-PCR). RNA extraction, reverse transcription, and PCR amplifications with human PAR-2 or GAPDH primers were conducted as previously described [12]. PCR products were identified by electrophoresis in 2% agarose gel followed by ethidium bromide staining. MUC2 and MUC5AC mRNAs were quantified by real-time PCR, using commercially available kits (TaqMan Gene expression assays Hs00159374 for MUC2, Hs00873638 for MUC5AC, and Hs99999903 for β-actin; Applied Biosystems, Courtaboeuf, France) and a 7000 thermocycler (Applied Biosystems).

Fura-2/AM loading and intracellular calcium measurement. Intracellular calcium concentrations were measured using Fura-2/AM in HT29-Cl.16E cells, as previously described [12]. Briefly, cells were grown onto glass coverslips and cultured to 70–90% confluency. These coverslips were then loaded with 5 μM Fura-2/AM in Na–Hepes-buffered saline containing 0.01% pluronic acid for 45–60 min at 37 °C. They were then washed in Na–Hepes buffer and placed at 37 °C in a fluorimeter. The fluorescence was measured with a dual wavelength excitation fluorimeter at 340 and 380 nm for excitation and 510 nm for emission.

Immunoradiometric assay (IRMA) of mucin secretion. HT29-Cl.16E monolayers were stimulated with SLIGKV-NH_2 or with the vehicle and the apical medium was flushed several times on the monolayers in order to recover the secreted mucins. As the mucin exocytotic response of HT29-Cl.16E cells consists both in MUC2 and MUC5AC, we chose to measure MUC5AC, due to the lack of assays specific for MUC2. Mucins were measured in the collected media using a solid-phase double-antibody-sandwich IRMA for MUC5AC gene [20,21].

MAP-kinase Western blots. For assessment of ERK1/2 phosphorylation, post-confluent filter-grown HT29-Cl.16E cells were serum-deprived for 48 h prior to their treatment with 100 μM SLIGKV-NH_2 for 5 min. In some experiments, cells were pre-treated with PD98059 (25 μM) or AG1478 (3 μM), for 1 h. Cells were then lyzed with RIPA buffer, proteins separated by SDS–PAGE, and immunoblots using phospho-specific antibodies to ERK1/2 (New England biolabs, MA) were performed as previously described [22]. The same membranes were reprobed with a polyclonal anti-ERK1/2 antibody (Santa Cruz, CA), and served as a loading control.

EGFR immunoprecipitation assay and Western blot. Filter-grown HT29-Cl.16E cells, serum-starved for 48 h, were treated with SLIGKV-NH_2 (100 μM) for 5 min at 37 °C before being washed with cold PBS and lyzed. EGFRs were precipitated and detected as previously described [22]. In some experiments, cells were pre-treated with AG1478 (3 μM) for 1 h, prior to the stimulation with SLIGKV-NH_2 for 5 min.

Statistical analysis. Results were expressed as means ± SEM. Statistical analyses were performed using the Mann–Whitney *U* test. A p value of 0.05 was considered as statistically significant.

Results

Expression of functional PAR-2 receptors by the HT29-Cl.16E cell line

We first investigated the expression of PAR-2 transcripts by RT-PCR analysis, both in epithelial cells isolated from human normal colon and in HT29-Cl.16E cells. As shown in Fig. 1A, a single PCR product of the expected size (505 bp) was observed in normal colonocytes as well as in HT29-Cl.16E cells.

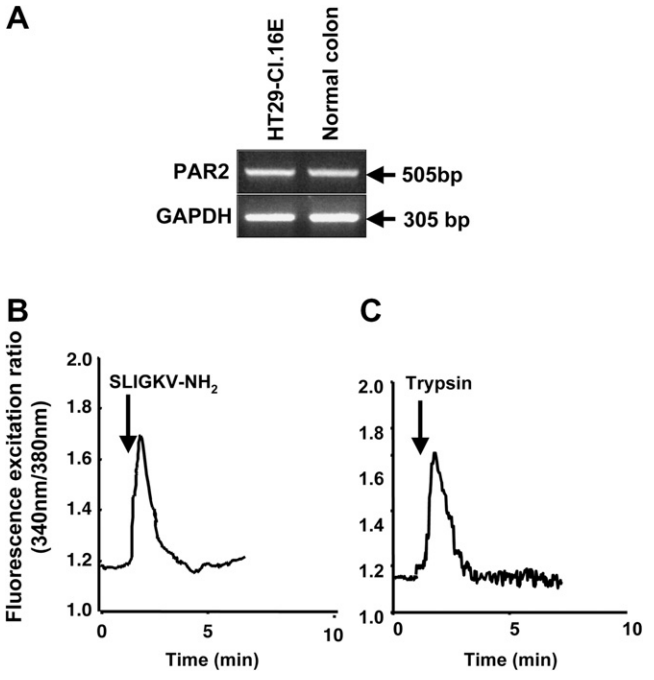

Fig. 1. Expression of PAR-2 mRNA in HT29-Cl.16E cells and normal human colonocytes, and PAR-2-mediated Ca^{2+} mobilization in HT29-Cl.16E cells. (A) Total RNA was extracted from HT29-Cl.16E cells or normal human colonocytes, reverse transcribed and PCR amplified with PAR-2 or GAPDH primers. PCR products were visualized on a 2% agarose gel electrophoresis. (B, C) Intracellular Ca^{2+} mobilization was induced by treatment with 100 μM SLIGKV-NH_2 (B) or 10 nM trypsin (C). Ca^{2+} concentration was determined by monitoring fluorescence changes in cells loaded with 5 μM Fura-2 AM. The compounds are given at the arrow.