Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E₁ and E₂ production by intestinal myofibroblasts

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Background: The mucus layer protects the gastrointestinal mucosa from mechanical, chemical, and microbial challenge. Mucin 2 (MUC-2) is the most prominent mucin secreted by intestinal epithelial cells. There is accumulating evidence that subepithelial myofibroblasts regulate intestinal epithelial cell function and are an important source of prostaglandins (PG). PG enhance mucin secretion and are key players in mucoprotection. The role of bacterial fermentation products in these processes deserves further attention.

Aims: We therefore determined whether the effect of short chain fatty acids (SCFA) on MUC-2 expression involves intermediate PG production.

Methods: Both mono- and cocultures of epithelial cells and myofibroblasts were used to study the effects of SCFA on MUC-2 expression and PG synthesis. Cell culture supernatants were used to determine the role of myofibroblast derived prostaglandins in increasing MUC-2 expression in epithelial cells.

Results: Prostaglandin E₁ (PGE₁) was found to be far more potent than PGE₂ in stimulating MUC-2 expression. SCFA supported a mucoprotective PG profile, reflected by an increased PGE₁/PGE₂ ratio in myofibroblast supernatants and increased MUC-2 expression in mono- and cocultures. Incubation with indomethacin revealed the latter to be mediated by PG.

Conclusions: SCFA can differentially regulate PG production, thus stimulating MUC-2 expression in intestinal epithelial cells. This mechanism involving functional interaction between myofibroblasts and epithelial cells may play an important role in the mucoprotective effect of bacterial fermentation products.

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Chronic relapsing mucosal inflammation is a hallmark of inflammatory bowel disease (IBD). Concentrations of proinflammatory cytokines are dramatically increased in the intestinal mucosa of IBD patients. Local production of proinflammatory cytokines may compromise intestinal barrier integrity (for example, increase epithelial permeability and change mucus production and quality). The mucus layer forms a physical-chemical barrier on the epithelial layer separating the gut lumen from the lamina propria, thus having an important function in scavenging dietary and microbial antigens. Mucus secretion by epithelial cells can be influenced by a variety of physiological and immune mediators, such as prostaglandins (PG). It is now well established that epithelial cell functions (for example, proliferation, differentiation, secretion, and motility) are regulated by myofibroblasts which form a thin layer of cells underlying the epithelium. These myofibroblasts are an important source of PG and therefore may play a crucial role in mucoprotection. Myofibroblasts constitutively express cyclooxygenase (COX)-1 whereas during inflammation COX-2 is induced, and these enzymes are responsible for prostaglandin E₁ (PGE₁) and PGE₂ production. COX inhibition with non-steroidal anti-inflammatory drugs (NSAIDs) can result in detrimental side effects such as induction of gastrointestinal lesions. Administration of PGE₂ analogues in this respect is believed to support mucoprotection. Certain bacteria of the intestinal flora are beneficial for gut health. Apart from immunomodulating capacities, these bacteria can also improve mucosal barrier integrity. The mucoprotective effects of metabolic products from the intestinal flora deserve further study. Short chain fatty acids (SCFA) are the end products of microbial fermentation of non-digestible carbohydrates and have been reported to increase mucus secretion. SCFA are absorbed by the distal ileum and colon, and butyrate in particular is an important source of nutrition for epithelial cells. Butyrate has gained much attention as it promotes mucosal restitution, induces differentiation, and inhibits inflammation and tumour growth. Hence in this study we determined the effect of SCFA on PGE₁ and PGE₂ production and assessed the implications for epithelial mucin 2 (MUC-2) expression.

To do this, we used a coculture model representing the spatial interaction between epithelial and mesenchymal cells.

MATERIAL AND METHODS

Cell culture

Monolayers (MC) of intestinal epithelial T84 (passages 57–64) and LS174T (passages 110–120) (ATCC, Manassas, USA) cells or intestinal myofibroblasts CCD-18Co (passages 10–14) were cultured in 24 or 96 well tissue culture plates (Corning BV, Acton, USA). Parallel with MC, cocultures (CC) were set up by culturing epithelial cells directly on a confluent layer of CCD-18Co cells, as previously described. In brief, CCD-18Co cells were seeded in a twofold dilution in the culture plates and grew confluent within one week. T84 and LS174T cells were added in a fivefold dilution on top of the CCD-18Co layer (CC) or in a separate plate (MC). Cells were cultured in DMEM/F12 glutamax I (Invitrogen Life Technologies, Carlsbad, USA) with penicillin (100 IU/ml), streptomycin (100 μg/ml) (Invitrogen Life Technologies), and 5% heat inactivated fetal bovine serum (Invitrogen Life Technologies).

Abbreviations: IBD, inflammatory bowel disease; COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; CC, coculture; MC, monoculture; PG, prostaglandins; SCFA, short chain fatty acids; MUC-2, mucin 2; BLU, light units.
Medium was refreshed every two days. CCD-18Co monolayers were used when grown confluent while CC and MC were used when the epithelial cell layer had grown subconfluent.

**Stimulation of CC and MC with PG or SCFA**

CCD-18Co monolayers or MC/CC T84 or LS174T were incubated for 24 hours with a concentration range (0.025-4.0 mM) of acetic acid, propionic acid, or butyric acid (VWR International, West Chester, USA). To determine the effects of PG on MUC-2 expression, MC/CC T84 were incubated for 24 hours with 0.01–100 ng/ml PGE1 or PGE2 (dissolved in 100% ethanol, diluted to 1 mg/ml stocks in phosphate buffered saline, and stored at −80°C, Sigma-Aldrich BV, St Louis, Missouri, USA). In addition, supernatants of CCD-18Co which had been stimulated for 24 hours with butyrate in the absence or presence of 10−10 M indomethacin (to block prostaglandin production; Sigma-Aldrich BV) were transferred to MC T84. Supernatants and/or cells were collected and PG concentration or MUC-2 expression was determined.

**Dot blotting MUC-2**

We used a dot blot technique to determine MUC-2 expression in cell cultures as mucins are extremely large glycoproteins (over 500 kDa) which makes them difficult to handle in western blotting techniques.28, 29 We used the anti-HCM (human colon mucin) antibody raised against purified epitopes of MUC-2 in colonic goblet cells.28 Our method was validated using preimmune serum (T84 stained negative), negative control cells (CCD-18Co), and bovine serum albumin. Cell samples were collected in laemmli (protein isolation buffer) and protein determination was performed using the DC protein assay (Biorad, Hercules, California, USA) according to the manufacturer’s protocol with minor modifications. Samples (0.3–0.7–1.0 µg/2 µl) were dotted onto nitrocellulose membranes (Schleicher and Schuell, Riviera Beach, USA). Membranes were blocked in TBST/5% Protivar (Nutricia Roetermeer, the Netherlands) followed by one hour of incubation with anti-MUC-2 antibody (kindly donated by Dr Einerhand, Erasmus University, Rotterdam, the Netherlands). After washing, blots were incubated with goat antirabbit-horseradish peroxidase (Santa Cruz Biotechnology Santa Cruz, USA) and for substrate detection ECL (Roche Diagnostics Indianapolis, Indiana, USA) was used. Densitometry was performed using the Lumi-Imager (Roche Diagnostics) and the signal was expressed in light units (BLU). BLUs were also expressed relative to control incubations (%BLU). SCFA incubations were performed in parallel with MC and CC, and MUC-2 expression was analysed within the same dot blot. To compare the stimulatory effect of SCFA on MUC-2 expression in MC and CC, we deducted basal MUC-2 expression levels.

**Measurement of PGE1 and PGE2**

Supernatants from CCD-18Co were analysed for PG production using ELISAs for PGE1 (R&D Minneapolis, USA; 21% cross reactivity with PGE2) and PGE2 (Biotrak Amersham Biosciences corp., Piscataway, USA; 4% cross reactivity with PGE1).20 Concentrations measured during SCFA incubations were expressed relative to basal secretion. The PGE1/PGE2 ratio was calculated to determine a shift towards a more mucoprotective PG profile.

**Viability measurements**

As SCFA have been reported to be cytotoxic at high doses, we measured the viability of CCD-18Co, MC, and CC using a WST-1 assay (Roche Diagnostics). After 24 hours of incubation, the medium was refreshed and cells were incubated with WST-1. After one hour for MC and CC, and three hours for CCD-18Co, 100 µl sample of the supernatant was measured at A450-A635 in a spectrophotometer (Biorad).

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**Figure 1** Prostaglandin stimulation of mucin 2 (MUC-2) expression in monocultures (MC)/cocultures (CC) of T84. (A) Dot blot analyses showed MC T84 cells to be positive for MUC-2 whereas CCD-18Co were negative. (B) Representative dot blot showing the effects of prostaglandins PGE1 and PGE2 on MUC-2 expression in CC T84. (C) Densitometric analysis revealed PGE1 to enhance MUC-2 expression in both MC and CC T84 (1–100 ng/ml; **p<0.002**) whereas PGE2 only marginally affected MUC-2 expression in CC T84 (10–100 ng/ml; *p<0.01*). MUC-2 expression is presented relative to controls (%BLU).
Short chain fatty acids (SCFA) increased the prostaglandin PGE1/PGE2 ratio produced by CCD-18Co. (A) Butyrate increased PGE1 and decreased PGE2 concentrations (p<0.01, *p<0.002); acetate and propionate followed the same tendency. Prostaglandin production is presented relative to controls (% control). (B) SCFA increased the PGE1/PGE2 ratio (n = 5; *p<0.01, **p<0.002), resulting in a preferred mucoprotective profile.

### Data analysis
All data are presented as mean (SEM). Data were analysed with the univariate ANOVA using SPSS software version 10.

### RESULTS
#### MUC-2 expression of MC/CC T84 after incubation with PG
Protein titration of T84 or CCD-18Co homogenates revealed T84 to be positive for MUC-2, and as expected CCD-18Co were negative in the dot blot analyses (fig 1A). The differential effects of PGE1 and PGE2 on MUC-2 expression by CC T84 are shown in a representative dot blot (fig 1B). Densitometry revealed that MUC-2 expression in both MC and CC T84 was stimulated by PGE1 rather than PGE2 (fig 1C). MUC-2 expression in both MC and CC T84 was dose dependently increased after 24 hours of incubation with PGE1 (1–100 ng/ml; p<0.002) whereas PGE2 only marginally increased MUC-2 expression in CC T84 cultures at the highest doses (10–100 ng/ml; p<0.01).

#### Effect of SCFA on PGE1 versus PGE2
Supernatants of unstimulated CCD-18Co contained more PGE2 than PGE1 (633 (371) v 891 (437) pg/ml; p<0.05, n = 6). Butyrate in particular enhanced PGE1 and reduced PGE2 concentrations in all dosage groups (fig 2A: p<0.01, p<0.002). Acetate and propionate incubations followed the same trend. As a result, the PGE1/PGE2 ratio increased after SCFA incubation (fig 2B: p<0.01, p<0.002). Culture medium levels for PGE1 and PGE2 were low or undetectable.

**Figure 2** Increased mucin 2 (MUC-2) expression in cocultures (CC) of T84 compared with monocultures (MC) of T84. Basal MUC-2 expression increased when T84 cells were cocultured with CCD-18Co, the latter however was not apparent using LS174T cells (n = 4, *p<0.05). BLU, light units.
MUC-2 expression of MC and CC T84 or LS174T after SCFA incubation

T84 and LS174T cells were cultured directly on CCD-18Co monolayers and MUC-2 expression was determined. Basal MUC-2 expression was significantly higher in CC T84 compared with MC T84 while MUC-2 expression in CC LS174T was not increased compared with MC (fig 3; p<0.05).

SCFA enhanced MUC-2 expression more in CC compared with MC in both cell lines, except for propionate in MC/CC T84 (p<0.05). Propionate and acetate effectively induced MUC-2 expression in MC and CC of both cell lines (fig 4A, B; p<0.01, p<0.002). Butyrate stimulated MUC-2 expression in MC/CC T84 and CC LS174T but not in MC LS174T (p<0.01, p<0.002). As it is likely that intestinal epithelial cells are exposed to higher levels of SCFA than subepithelial myofibroblasts, we used a broader concentration range in our epithelial mucin expression studies. WST data revealed no toxicity of these SCFA concentrations in both MC and CC (data not shown).

MUC-2 expression of MC T84 after incubation with CCD-18Co supernatant

SCFA enhanced MUC-2 expression more in CC compared with MC. Therefore, we tested whether mucin expression in cocultures was regulated by CCD-18Co derived PG by blocking PG production with indomethacin. MUC-2 expression in MC T84 incubated with supernatants of CCD-18Co stimulated with butyrate in the presence or absence of indomethacin are shown in fig 5A. Densitometric analysis showed butyrate to enhance MUC-2 expression in MC T84 (fig 5B; 1 mM, p<0.002). T84 incubation with CCD-18Co supernatants increased MUC-2 expression at lower butyrate concentration.
Figure 5  Mucin 2 (MUC-2) stimulation by butyrate is mediated by prostaglandins. (A) Representative dot blot of MUC-2 expression in monolayers of T84 incubated with supernatants of CCD-18Co that had been stimulated with butyrate in the presence or absence of indomethacin. (B) Densitometric analysis of four different experiments. Stimulation of MUC-2 expression by butyrate was found to be mediated by CCD-18Co and T84 derived prostaglandins as indomethacin (indo) blocked this effect (0.5–1 mM; **p < 0.002). CCD-18Co supernatants (sup) increased MUC-2 expression at lower butyrate concentrations (0.5–1 mM; *p < 0.002) compared with MC T84 incubated with butyrate (1 mM; **p < 0.002).

**DISCUSSION**

Bacterial fermentation products may play an important role in mucoprotection, being an energy source for intestinal epithelial cells and stimulating mucin secretion. Butyrate enemas have been found to reduce clinical symptoms in patients with ulcerative colitis, and high butyrate concentrations in the colon are protective against colon cancer. Butyrate most effectively stimulated PGE$_1$ production by CCD-18Co cells and when transferring these CCD-18Co supernatants to MC T84, MUC-2 expression was further increased compared with incubation of epithelial cells with butyrate alone. The butyrate effects mediated through CCD-18Co supernatant or directly by T84 (data not shown) were abrogated by indomethacin. This implies that enhanced mucin synthesis by SCFA is mediated by PG derived from both subepithelial myofibroblasts and intestinal epithelial cells. The latter is supported by the fact that mucosal epithelial cells constitutively express COX-1 and indeed epithelial cells have been reported to produce PG. SCFA infusion in these studies was one hour or less. We observed that, independent of cholinergic activation, prolonged incubation with SCFA induced MUC-2 expression in epithelial cells, thus skewing the balance towards mucoprotection. Our finding is also supported by a study in which butyrate was found to increase mucin synthesis in colonic biopsies.

In general, SCFA enhanced MUC-2 expression more potently in CC compared with MC. Monolayer LS174T cells were even unresponsive to butyrate incubations while cocultures were effectively stimulated. These data strongly support a role for subepithelial myofibroblasts in the regulation of MUC-2 production by epithelial cells.

Butyrate most effectively stimulated PGE$_1$ production by CCD-18Co cells and when transferring these CCD-18Co supernatants to MC T84, MUC-2 expression was further increased compared with incubation of epithelial cells with butyrate alone. The butyrate effects mediated through CCD-18Co supernatant or directly by T84 (data not shown) were abrogated by indomethacin. This implies that enhanced mucin synthesis by SCFA is mediated by PG derived from both subepithelial myofibroblasts and intestinal epithelial cells. The latter is supported by the fact that mucosal epithelial cells constitutively express COX-1 and indeed epithelial cells have been reported to produce PG.

In conclusion, SCFA increased the PGE$_1$/PGE$_2$ ratio produced by subepithelial myofibroblasts and PGE$_1$ was found to be superior to PGE$_2$ in enhancing MUC-2 expression in epithelial cells. SCFA stimulated epithelial MUC-2 expression was mediated by PG derived from subepithelial myofibroblasts and epithelial cells, as proved by our indomethacin inhibition experiments. The present study therefore suggests that bacterial fermentation products may have beneficial effects on gut health by supporting mucosal barrier integrity.
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