Intestinal barrier function: regulation of epithelial permeability and mucin expression
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Short chain fatty acids stimulate epithelial mucin-2 expression through differential effects on prostaglandin $E_1$ and $E_2$ production by intestinal myofibroblasts

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Abstract
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 for bacterial fermentation products in these processes deserves further attention. We therefore determined whether the effect of short chain fatty acids (SCFA) on MUC-2 expression involves intermediate PG production. 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. 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. Incubations with indomethacin revealed the latter to be mediated by prostaglandins. 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.
Introduction

Chronic relapsing mucosal inflammation is a hallmark of inflammatory bowel disease (IBD). Concentrations of pro-inflammatory cytokines are dramatically increased in the intestinal mucosa of IBD patients. Local production of pro-inflammatory 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 physiologic 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 PGE₁ 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 the 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 the 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 MUC-2 expression. To do this, we used a coculture model representing the spatial interaction between epithelial and mesenchymal cells.

Materials and methods

Cell culture

Monolayers (MC) of intestinal epithelial T84 (passage 57-64) and LS174T (passage 110-120) (ATCC, Manassas, USA) cells or intestinal myofibroblasts CCD-18Co (passages 10-14) were cultured in 24 or 96 wells 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). 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 prostaglandins or short chain fatty acids**

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 1mg/ml stocks in PBS, and stored at -80°C, Sigma-Aldrich BV, St. Louis, USA). In addition, supernatants of CCD-18Co which had been stimulated for 24 hours with butyrate in the absence or presence of 10^-6 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 the 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 mucins from mucosal scrapings which recognises peptide epitopes of MUC-2 in colonic goblet cells\(^{28}\). Our method was validated using pre-immune serum (T84 stained negative), negative control cells (CCD-18Co) and bovine serum albumin. Cell samples were collected in laemmlii (protein isolation buffer) and protein determination was performed using the DC-protein assay (Biorad, Hercules, 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 & Schuell, Riviera Beach, USA). Membranes were blocked in TBST/5% Protivar (Nutricia, Zoetermeer, 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 anti-rabbit-HRP (Santacruz Biotechnology, Santa Cruz, USA) and for substrate detection ECL (Roche Diagnostics, Indianapolis, 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 in MC and CC and MUC-2 expression was analysed within the same dotblot. To
compare the stimulatory effect of SCFA on MUC-2 expression in MC and CC, we deducted basal MUC-2 expression levels.

**Measurement of PGE\textsubscript{1} and PGE\textsubscript{2}**

Supernatants from CCD-18Co were analysed for PG production using ELISAs for PGE\textsubscript{1} (R&D, Minneapolis, USA; 21% cross reactivity with PGE\textsubscript{2}) and PGE\textsubscript{2} (Biotrak, Amersham Biosciences Inc, Piscataway, USA; 4% cross reactivity with PGE\textsubscript{1})\textsuperscript{30}. Concentrations measured during SCFA incubations were expressed relative to basal secretion. The PGE\textsubscript{1}/PGE\textsubscript{2} ratio was calculated to determine a shift towards a more mucoprotective prostaglandin profile.

![Prostaglandin stimulation of mucin-2 (MUC-2) expression in monocultures (MC) or 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 effects of PGE\textsubscript{1} and PGE\textsubscript{2} on MUC-2 expression in CC T84. C) Densitometric analysis revealed PGE\textsubscript{1} to enhance MUC-2 expression in both MC and CC T84 (1-100 ng/ml, \(**p<0.002\)), whereas PGE\textsubscript{2} 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).](image)

**Viability measurements**

Since 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-A655 in a spectrophotometer (Biorad).

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

A) 

Figure 2 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 increase the PGE₁/PGE₂ ratio (n=5; *p<0.01, **p<0.002), resulting in a preferred mucoprotective profile.

Results

MUC-2 expression of MC/CC T84 after incubation with prostaglandins
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 PGE$_1$ rather than PGE$_2$ (fig 1C). MUC-2 expression in both MC and CC T84 was dose-dependently increased after 24 hours of incubation with PGE$_1$ (1-100 ng/ml, **p<0.002), whereas PGE$_2$ 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 PGE$_1$ versus PGE$_2$**

Supernatants of unstimulated CCD-18Co contained more PGE$_2$ than PGE$_1$ (633 (371) vs 891 (437) pg/ml; p<0.05, n=6). Butyrate in particular enhanced PGE$_1$ and reduced PGE$_2$ 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 PGE$_1$/PGE$_2$ ratio increased after SCFA incubation (fig 2B; *p<0.01, **p<0.002). Culture medium levels for PGE$_1$ and PGE$_2$ were low or undetectable. PG levels as measured in butyrate containing culture medium spiked with PG did not differ from the spiked medium controls. Moreover, none of the incubations compromised cell viability, as determined by WST analysis (data not shown).

**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 as compared to MC T84 while MUC-2 expression in CC LS174T was not increased as compared to MC (fig 3, #p<0.05). SCFA enhanced MUC-2 expression more in CC as compared to 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 4 A/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).
Figure 4 Short chain fatty acids (SCFA) increased mucin-2 (MUC-2) expression in monocultures (MC) and cocultures (CC) of T84 and LS174T. A,B) SCFA stimulation of MUC-2 expression in the presence of CCD-18Co was found to be more pronounced compared with epithelial monolayers alone (p<0.05). Acetate and propionate dose dependently increased MUC-2 expression in MC/CC T84 (n=4) and MC/CC LS174T (n=3) (*p<0.01; **p<0.002). Butyrate was effective in MC/CC T84 and CC LS174T (*p<0.01; **p<0.002) but did not enhance MUC-2 expression in MC LS174T cells. MUC-2 expression was presented after deduction of basal expression levels of either MC or CC. BLU, light units.
MUC-2 expression of MC T84 after incubation with CCD-18Co supernatant

SCFA stimulated MUC-2 expression to a higher extent in CC as compared to MC. Therefore, we tested whether the 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 presence or absence of indomethacin are shown in figure 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 supernatans increased MUC-2 expression already at lower butyrate dose, representing the additional effect of CCD-18Co derived soluble mediators (0.5-1 mM, **p<0.002). The butyrate effect was found to be mediated by PG derived from both CCD-18Co and T84 since indomethacin completely blocked stimulation of MUC-2 expression (0.5-1 mM, #p<0.01). In additional analyses, we confirmed that indomethacin indeed reduced the PG concentrations in butyrate stimulated CCD-18Co culture supernatants (PGE1 p<0.02, PGE2 p<0.02; n=5). Enhanced MUC-2 expression in MC T84 incubated with butyrate, acetate or propionate was also abrogated with indomethacin (data not shown).

**Figure 5** Mucin 2 (MUC-2) stimulation by butyrate is mediated by prostaglandins. A) Representative dot blot of MUC-2 expression in monocultures of T84 incubated with supernatans 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 prostaglandin as indomethacin (indo) blocked this effect (0.5-1 mM; #p<0.01). CCD-18Co supernatants (sup) increased MUC-2 expression already at lower butyrate concentration (0.5-1 mM; **p<0.002) compared with MC T84 incubated with butyrate (1 mM; **p<0.002).
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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. Also PG are implicated in sustaining mucosal integrity as revealed by the observation that PGE₁ analogues were found to prevent formation of gastroduodenal lesions induced by NSAIDs.

Our data revealed differential effects of PGE₁ and PGE₂ on MUC-2 expression in epithelial cells. PGE₁ and PGE₂ are known stimulators of mucin secretion by T84 and LS174T cells and the rat colon. In our experiments PGE₁ was more effective than PGE₂ in enhancing MUC-2 expression. Mucin expression within the cells is the net effect of synthesis and secretion. This implies that PGE₁ but not PGE₂ incubation resulted in net MUC-2 synthesis. To our knowledge, the effect of PGE₁ on mucin synthesis has not been studied before; however a PGE₁ analogue has been reported to increase the intracellular mucus content of stomach epithelium. Our PGE₂ data are in agreement with a study in which dmPGE₂ enhanced mucin secretion by HT29-18N2 cells but reduced the amount of newly synthesised mucins, resulting even in decreased intracellular mucus stores at 24 hours. The observed difference between PGE₁ and PGE₂ can not be contributed to differential bioactivity as in our lab they were found to be equally active in inhibiting cytokine secretion.

There is consistent evidence that butyrate induces cell differentiation and downregulates inflammatory responses. Our data showed that SCFA enhanced the PGE₁/PGE₂ ratio secreted by subepithelial myofibroblasts, a profile that may support mucoprotection by enhancing epithelial mucin expression. Our concept of exposure of subepithelial myofibroblasts to bacterial fermentation products is biologically relevant as following epithelial absorption SCFA are found in the bloodstream.

To study MUC-2 expression we used colonic carcinoma cell lines T84 (columnar crypt epithelium) and LS174T (goblet cell type). When cocultured with subepithelial myofibroblasts, T84 expressed more MUC-2 compared to MC T84. Cell-to-cell contact, extracellular matrix and soluble factors like TGF-β are known to induce epithelial differentiation which might cause T84 cells to increase basal MUC-2 expression. Basal MUC-2 expression of CC LS174T was not enhanced as compared to MC LS174T, possibly because LS174T cells are not capable of further differentiation.

SCFA dose-dependently induced MUC-2 expression in MC/CC T84 and LS174T. SCFA induce mucin exocytosis in the rat colon through activation of cholinergic nerves. SCFA

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infusion in these studies was one hour or less. We observed that, independent from 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 as compared to MC. Monolayers 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 PGE₁ 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 implicates 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 epithelial cells indeed have been reported to produce prostaglandins.

In conclusion, SCFA increased the PGE₁/PGE₂ ratio produced by subepithelial myofibroblasts and PGE₁ was found to be superior to PGE₂ 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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**References**

Chapter 3


Bacterial fermentation products and mucoprotection


