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Effects of pro-inflammatory cytokines, lipopolysaccharide and COX-2 mediators on human colonic neuromuscular function and epithelial permeability

B.K. Safdari [a], T.C. Sia [b], D.A. Wattchow [b], S.D. Smid [a,*]

[a] Discipline of Pharmacology, School of Medicine, Faculty of Health Sciences, The University of Adelaide, South Australia, Australia
[b] Department of Surgery, Flinders Medical Centre, Bedford Park, South Australia, Australia

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ABSTRACT

Chronic colitis is associated with decreased colonic muscle contraction and loss of mucosal barrier function. Pro-inflammatory cytokines and bacterial lipopolysaccharide (LPS) are important in the generation and maintenance of inflammation. While colitis is associated with upregulated COX-2 -derived prostanoids and nitric oxide (NO), the direct activity of pro-inflammatory cytokines on human colonic neuromuscular function is less clear. This study investigated the effects of IBD-associated pro-inflammatory cytokines IL-17, TNF-α, IL-1β and LPS on human colonic muscle strip contractility, alone and following inhibition of COX-2 or nitric oxide production. In addition, human colonic epithelial Caco-2 cell monolayers were treated with LPS or COX-2 mediators including prostaglandins (PGE_2, $PGF_{2\alpha}$) or their corresponding ethanolamides (PGE_2-EA or $PGF_{2\alpha}$-EA) over 48 h and trans-epithelial electrical resistance used to record permeability changes. Longitudinal muscle strips were obtained from healthy colonic resection margins and mounted in organ baths following IL-17, TNF-α, IL-1β and bacterial LPS incubations in an explant setting over 20 h. Contraction in response to acetylcholine (ACh) was then measured, before and after either COX-2 inhibition (nimesulide; 10^{-5} M) or nitric oxide synthase (NOS) inhibition (L-NNA; 10^{-4} M). None of the cytokine or LPS explant incubations affected the potency or maximum cholinergic contraction *in vitro*, and subsequent COX-2 blockade with nimesulide revealed a significant but similar decrease in potency of ACh-evoked contraction in control, LPS and cytokine-incubated muscle strips. Pre-treatment with L-NNA provided no functional differences in the potency or maximum contractile responses to ACh in cytokine or LPS-incubated colonic longitudinal smooth muscle. Only PGE_2 transiently increased Caco-2 monolayer permeability at 24 h, while LPS (10 μg/ml) increased permeability over 24–48 h.

These findings indicate that cholinergic contractility in the human colon can be decreased by the blockade of COX-2 generated excitatory prostanoids, but major pro-inflammatory cytokines or LPS do not alter the sensitivity or amplitude of this contraction *ex vivo*. While PGE_2 transiently increase epithelial permeability, LPS generates a significant and sustained increase in permeability indicative of an important role on barrier function at the mucosal interface.

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1. Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing inflammation of the gut with symptoms such as diarrhoea, bloating, cramping and abdominal pain. Clinical and animal studies suggest that a dysregulated immune response to endogenous bacterial flora or normal constituents of the gut can both initiate and maintain inflammation [1,2]. Colitis is accompanied by the production of inflammatory mediators such as cytokines, prostaglandins and nitric oxide (NO), factors which can disrupt both colonic muscle contraction and mucosal barrier function [3].

The source of cytokines are not only the resident immune cells in the gastrointestinal mucosa and submucosa, but also distinct population of immune cells such as macrophages localized in the muscularis externa [4,5]. The immunological events that occur in inflammation can directly expose the muscle layer to increased concentrations of cytokines, particularly in CD where transmural

* Corresponding author at: Discipline of Pharmacology, School of Medical Sciences, Faculty of Health Sciences, The University of Adelaide, Adelaide, SA 5005, Australia.
E-mail address: scott.smid@adelaide.edu.au (S.D. Smid).

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inflammation can directly affect smooth muscle. In addition, cytokines such as IL-1β are produced in the muscle layer following inflammation and myocytes have been shown to directly produce TNF-α under ischemic and inflammatory conditions [6,7]. Bacterial lipopolysaccharide (LPS) has also been shown to directly impair isolated human colonic smooth muscle contraction [8]. However, whether cytokines and potential inflammatory initiators such as LPS have a direct effect on human colonic muscle contractility remains unclear.

In the present study we incubated pro-inflammatory cytokines associated with IBD such as IL-17, TNF-α, IL-1β and LPS in healthy human colonic muscularis tissue in an explant setting to determine effects on myogenic contractility. We then investigated whether any functional alterations were attributable to upregulated inflammatory mediators such as NO or COX-2 derived prostanoids via pharmacological blockade of endogenous production. Additionally, we tested the effects of selected prostanoid metabolites of COX-2 on epithelial permeability using a colonic epithelial (Caco-2) cell line, including prostaglandins E_2, $F_{2\alpha}$ and their respective ethanolamide derivatives, as we have previously shown these so-called 'prostamide' COX-2 based mediators of the endocannabinoid anandamide may influence gastrointestinal mucosal integrity [9]. Given our previous evidence of cytokine-evoked increases in epithelial permeability [10], and considering that TLR-4 receptors are expressed on human colonic Caco-2 epithelial cells [11], this study collectively would provide further insight into the role for such inflammatory mediators on both human colonic neuromuscular and epithelial barrier function.

2. Materials and methods

2.1. Human colon tissue collection and preparation

Specimens of human colonic tissue were obtained from 12 consenting patients undergoing resective surgery for colonic malignancy at Flinders Medical Centre, Adelaide. Patients provided informed consent and the use of specimens was approved by the Human Research Ethics Committee of Flinders Medical Centre (050.11). Full thickness tissue was obtained at the surgical margins distant from the tumour site as confirmed macroscopically and verified histologically. The tissue was transferred to the laboratory within 1 h of resection in ice-cold carbogenated Kreb's solution. The muscularis was dissected free from the mucosal and submucosal layer and then dissected into longitudinally-oriented muscle strips approximating 3 mm by 10 mm.

Isolated muscle strips were then placed into 5 ml of cell culture media (RPMI-1640) supplemented with 1% penicillin/streptomycin and 0.01% BSA as previously described for human colonic mucosal explants [12]. Each muscle strip was incubated in capped borosilicate glass vials for 20 h at 37 °C in the explant media, with continuous supply of carbogen introduced through perforations in the cap in order to minimise media loss through evaporation and aspiration. In this *ex vivo* (explant) model, muscle strips were incubated either alone or with a combination of IBD-associated pro-inflammatory cytokines TNF-α and IL-1β (TNF-α + IL-1β), IL-17 (all 10 ng/ml each) or LPS (10 μg/ml) prior to functional assessment of contractility. These cytokines have been previously established in our laboratory as producing alterations in mucosal integrity and epithelial permeability [10,12]. Following explant incubation, some additional tissue sections were placed in 10% neutral buffered formalin followed by haematoxylin and eosin (H&E) staining-based histological assessment, with some specimens also stained for nuclear labelling using DAPI and for neuronal degeneration using a Fluoro Jade C labelling kit (Biosensis; Thebarton, South Australia) according to manufacturer's instructions.

2.2. Organ bath colonic muscle strip contractility

Following the explant incubation period, muscle strips were mounted in Kreb's-filled organ baths at 37 °C and gassed with carbogen as previously described [13]. Briefly, one end of the sutured muscle strip was fastened to a tissue support and the other end was attached to an isometric force transducer (FT03, Grass Instruments, Quincy, MA). Platinum wire electrodes were attached to the tissue support for electric field stimulation (EFS) of muscle strips, whereby neurogenic contraction was initially used to functionally confirm neuronal viability. The muscle was then placed at an initial tension of 1 g and equilibrated for 45 min with replacement of Kreb's solution every 15 min. Isometric muscle responses were amplified and digitised via an analogue-digital interface before acquisition and management onto a computer hard drive using Chart software (ADInstruments, Sydney, Australia). At the end of the experiment, muscle strips were snap frozen and stored at −70 °C for further biochemical analysis.

To evaluate the effects of cytokine and LPS explant incubations on colonic smooth muscle contractility, exogenous acetylcholine (ACh: 10^{-9}–10^{-3} M) was added to the organ bath using a cumulative protocol and muscle contractions recorded. Subsequently, to investigate the role of COX-2-derived prostanoids on muscle contraction in explant muscle strips previously incubated with cytokines or LPS, the COX-2 selective inhibitor nimesulide (10^{-5} M) was used [14]. Additionally, to evaluate the effects of nitric oxide on smooth muscle contractility, the non-selective NOS inhibitor L-NNA was used in separate experiments at 10^{-4} M [13]. Nimesulide and L-NNA were allowed to equilibrate in the organ baths for 15 min prior to recording ACh-mediated contractile responses.

2.3. Caco-2 cell culture and treatment

Caco-2 cells derived from human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in 75 cm^2 tissue culture flasks (Corning Life Sciences, Lowell, MA, USA) at 37 °C with 5% CO_2 in DMEM supplemented with 10% foetal calf serum and 1% penicillin/streptomycin solution (complete DMEM). Cells were passaged every 3–4 days when at approximately 80% confluence. Experiments were carried out using Corning Costar Transwell Inserts (6.5 mm diameter, 0.4 μm pore size, tissue culture treated polyester membrane) (Corning Life Sciences, Lowell, MA, USA). Cells were seeded at a density of 16,500 cells per insert in 0.1 ml of media in the apical compartment**.** An insert on the plate was left blank and contained media only. The Transwell plate was then incubated at 37 °C and 5% CO_2. Media in the apical and basolateral well was replaced every 2–3 days for up to 25 days as the cells reached confluence and differentiated. During the period of cell growth the trans-epithelial electrical resistance (TEER) was measured using the EVOM2 epithelial voltohmeter with chopstick electrode (World Precision Instruments, Sarasota, FL, USA) to determine the integrity of the monolayer. The formula:

$$TEER\ Monolayer\,(\mathrm{cm}^2) = [TEER\ total\,(\Omega) - TEER\ blank\,(\Omega)] \times A\,(\mathrm{cm}^2)$$

was used to calculate TEER. Only inserts with TEER values over 500 Ω per cm^2 indicative of optimal monolayer confluence were used in experiments. Prostaglandins PGE_2 and $PGF_{2\alpha}$, and the COX-2 endocannabinoid prostaglandin ethanolamide metabolites PGE_2-EA and $PGF_{2\alpha}$-EA (all 10^{-6} M) in 0.1% ethanol vehicle were added on the basolateral compartment of the well and incubated for 48 h, with TEER measurements taken at 2, 4, 6, 24 and 48 h following prostanoid and LPS administration. LPS was applied to the apical surface of Caco-2 cells at 10 μg/ml.

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