Cyclooxygenase 2 mediates post-inflammatory colonic secretory and barrier dysfunction

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Background and aims: The colonic epithelium plays a key role in host defence. During colitis, epithelial function is impaired, leading to elevated bacterial translocation and exacerbation of inflammation. We previously documented perturbation of epithelial function, in terms of secretion and as a barrier to bacterial translocation, that persisted long after resolution of a bout of colitis in the rat. The mechanisms underlying the epithelial dysfunction are not completely understood.

Methods: Given the ability of prostaglandin (PG) D₂ to suppress colonic epithelial secretion, we investigated the potential roles of this eicosanoid and of cyclooxygenase 2 (COX-2) in mediating post-colitis epithelial secretory and barrier dysfunction.

Results: Six weeks after induction of colitis with trinitrobenzene sulphonic acid, there was marked elevated chloride secretion, including nitric oxide (NO), histamine, serotonin, and leukotrienes. Previous studies using experimental colitis and radiation enteritis models have pointed to an important contribution of NO derived from the inducible form of NO synthase in prolonged epithelial secretory dysfunction. For example, expression of iNOS was found to be upregulated in the colon of rats six weeks after administration of trinitrobenzene sulphonic acid (TNBS), and the epithelial hyporesponsiveness to electrical field stimulation (EFS) and to isobutylmethylxanthine (IBMX) could be partially reversed by pre-exposure to a selective inducible nitric oxide synthase (iNOS) inhibitor.

Prostaglandins (PG) also appear to play an important role in modulating colonic epithelial secretion. These mediators are closely associated with acute inflammatory conditions, contributing to oedema formation through their vasodilator effects, and to the generation of hyperalgesia. In recent years, the anti-inflammatory properties of PG have become better appreciated. For example, in a model of pleuritis in the rat, cyclooxygenase 2 (COX-2) expression was increased to the greatest extent during resolution of the inflammatory reaction. Moreover, generation of PGD₂ from COX-2 was shown to be crucial in reducing leucocyte infiltration to the site of inflammation during the resolution phase. These effects may have been in part due to the actions of a metabolite of PGD₂, namely 15-deoxy-Δ12-14-PGJ₂ (subsequently abbreviated as APGJ₂). We previously reported that colonic PGD₂ generation via COX-2 downregulates granulo-

Abbreviations: COX, cyclooxygenase; MPO, myeloperoxidase; NO, nitric oxide; TNBS, trinitrobenzene sulphonic acid; EFS, electrical field stimulation; IBMX, isobutylmethylxanthine; iNOS, inducible nitric oxide synthase; PG, prostaglandins; APGJ₂, 15-deoxy-Δ12-14-PGJ₂; PD, potential difference; Iₑ, short circuit current; LPS, lipopolysaccharide; CFU, colony forming units
Role of COX-2 and PGD₂ in epithelial hyporesponsiveness

To examine the role of COX-2 and PGD₂ in the hyporesponsiveness of colonic tissue to stimulation by IBMX and EFS, we tested the effects of selective COX-2 (celecoxib 10 μM) and COX-1 (SC-560 300 nM) inhibitors. After mounting in the Ussing chambers, the tissues were exposed to one of these inhibitors or vehicle for 10 minutes prior to stimulation with IBMX or EFS and the short circuit current responses were measured, as above. Celecoxib and SC-560 were initially dissolved in dimethylsulphoxide. The final concentration of dimethylsulphoxide in the Ussing chamber was 0.1%.

Additional studies were performed using colonic tissue from control rats in which the tissue was preincubated with PGD₂ (1 μM), the metabolite APGJ₂ (1 μM), or vehicle. These prostanooids were initially dissolved in ethanol. The final concentration of ethanol in the Ussing chamber was 0.1%. Isc responses to IBMX and EFS were then measured.

Prostaglandin synthesis and COX-2 expression

Groups of 5–6 rats each were killed on day 1, and one, two, and six weeks after intracolonic TNBS or saline (control) administration. Colonic tissue was collected and processed for measurements of PGE₂ and PGD₂, as described previously. Levels of prostaglandins generated by the tissue samples were measured using specific commercially available ELISA kits.

Colonic tissue was also taken from rats killed six weeks after TNBS or saline administration for examination of COX-2 expression by western blotting. For all western blot experiments, tissue samples were homogenised in lysis buffer (0.1% Triton X-100, 50 μM pepstatin-A, 0.2 mM leupeptin, 1 μg/ml aprotinin, 10 mg/ml phenylmethyl sulphonyl fluoride, 50 mM Tris, and 10 mM EDTA). Samples were then centrifuged and the protein concentration of the supernatant was determined by colorimetric assay (BioRad, Hercules, California, USA). Protein (50 μg) was separated on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was incubated for one hour with working buffer (20 mM Tris, 100 mM NaCl, 0.5% Tween 20, and 5% non-fat dried milk) and then probed overnight with a polyclonal rabbit antibody against COX-2 (1:500; Cayman Chemical, Ann Arbor, Michigan, USA). The membrane was then incubated with a donkey antirabbit IgG secondary antibody conjugated to horseradish peroxidase (Amersham, Little Chalfont, UK) for one hour at room temperature. A chemiluminescence reagent (Amersham) was added to visualise the labelling according to the manufacturer’s instructions. Densitometry was done using a GS-710 Calibrated Imaging Densitometer (BioRad) and analysed with Quantity One software (BioRad).

Bacterial translocation and colonic bacterial colonisation

Bacterial translocation and colonic bacterial colonisation in rats six weeks after TNBS administration and in age matched controls were determined as described previously. For bacterial translocation, we examined colonisation of the spleen. As in our previous study, any sample having more than 20 colony forming units (CFU) per gram of tissue was classified as positive for translocation of bacteria. For bacterial colonisation, we measured total numbers of aerobes, as in our previous study we had observed changes in aerobic colonisation and translocation, but not in anaerobic colonisation or translocation. A segment of colon including...
luminal contents was placed in 5 ml of sterile phosphate buffered saline (pH 7.4). The samples were weighed, homogenised, and diluted prior to plating of 0.1 ml of each dilution on blood agar plates. The number of CFU/g tissue was recorded for both control and post-colitis rats.

To examine the contribution of COX-2 to any observed changes in bacterial translocation and/or bacterial colonisation, groups of 10–11 rats each were treated twice daily with rofecoxib (3 mg/kg orally) during the sixth week after TNBS administration. A group of control rats (no TNBS; n = 10) was treated with vehicle at the same times. The choice of rofecoxib as a selective COX-2 inhibitor for these in vivo studies, as opposed to celecoxib in the in vitro studies, was entirely based on our having an adequate supply of the former for this multiple dosing study. The dose of rofecoxib was selected based on our previous demonstration that it produced significant inhibition of COX-2 in the rat without affecting COX-1 activity.21

Materials
TNBS was obtained from Fluka Chimika (Buchs, Switzerland). IBMX, carbachol, and LPS were purchased from Sigma Chemical Company (St Louis, Missouri, USA). PGD2 and ΔPGJ2 were obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA). Rofecoxib and celecoxib were generous gifts from NicOx (Sophia Antipolis, France), while SC-560 was provided by Boehringer-Ingelheim (Ingelheim, Germany).

Statistical analysis
All data are expressed as mean (SEM). Comparisons among groups of data were made using a one way analysis of variance followed by the Student-Newman-Keuls test. An associated probability (p value) of less than 5% was considered significant.

RESULTS
As in our previous studies,5,9 colonic tissue taken from rats six weeks after administration of TNBS did not exhibit any macroscopic or histological signs of damage or inflammation. Colonic damage scores in the post-colitis group (1.2 (0.4); n = 14) were not significantly different from those in controls (0.9 (0.2); n = 12). Colonic MPO activity in post-colitis rats (3.9 (0.6) U/mg; n = 14) was not significantly different from that in controls (3.2 (0.4) U/mg; n = 12).

Prostaglandin synthesis and COX-2 expression
Administration of TNBS resulted in a marked increase in colonic synthesis of both PGF2 and PGD2 (fig 1). Synthesis of PGF2 was significantly elevated above basal levels at one day and one week post-TNBS, but had returned to basal levels by two weeks post-TNBS. Changes in PGD2 after TNBS administration followed a very different pattern. There was a significant increase in PGD2 synthesis one day after TNBS, a return towards basal levels at one week, and a second increase in PGD2 synthesis at two weeks that remained evident at six weeks post-TNBS. Thus the greatest increase in PGD2 synthesis was observed during the period when healing of the colonic injury occurred.

To determine the contribution of COX-2 to the elevated synthesis of PGD2 in post-colitis rats, we examined the effects of twice daily treatment with rofecoxib (3 mg/kg orally) during the sixth week post-TNBS on colonic PGD2 synthesis. As shown by the square symbols in fig 1, rofecoxib suppressed colonic PGD2 synthesis to basal levels. These observations therefore suggest that colonic synthesis of PGD2 at six weeks post-TNBS occurred primarily via COX-2.

To further explore the possibility that the elevated colonic PGD2 synthesis was occurring via COX-2, expression of COX-2 in the colon was examined by western blotting. In post-colitis rats, COX-2 was elevated by only 56% above that in controls (p<0.05) (fig 2). However, it was possible that
COX-2 was “primed” for induction in the post-colitis state. To test this possibility, we administered LPS to control and post-colitis rats, and then measured COX-2 expression. When control rats were given LPS, it caused a small (44%) but significant increase in COX-2 expression in colonic tissue. In contrast, similar stimulation with LPS in the post-colitis group produced a 700% increase in COX-2 expression (fig 2).

Role of COX-1 and COX-2 in post-colitis epithelial secretory hyporesponsiveness

Consistent with previous observations of no difference between control and post-colitis colonic tissue in terms of permeability to small molecules, there were no differences between the two groups of rats in terms of conductance, as measured in the Ussing chamber experiments (control: 11.5 (1.2) mS/cm²; post-colitis: 13.6 (1.3) mS/cm²; n = 19 per group). However, the ability of the colonic epithelium of post-colitis rats to secrete was markedly altered compared with controls. As shown in table 1, the basal short circuit current was significantly reduced in post-colitis rats compared with controls. Moreover, as observed in previous studies, the distal colon from rats six weeks after TNBS administration exhibited hyporesponsiveness, in terms of chloride secretion, to both EFS and IBMX (fig 3). Preincubation of the tissue with SC-560, a selective COX-1 inhibitor, had no effect on the \( I_{sc} \) response to either EFS or IBMX in control or post-colitis tissue. In contrast, preincubation of distal colon with celecoxib, a selective COX-2 inhibitor, reversed the hyporesponsiveness seen with both EFS and IBMX (fig 4). There was no significant difference between post-colitis tissue treated with celecoxib and control tissue treated with either

**Figure 3** Effects of selective inhibition of cyclooxygenase 1 on changes in colonic short circuit current (\( I_{sc} \)) in response to electrical field stimulation (EFS; A) or isobutylmethylxanthine (IBMX) (B). Tissues harvested from rats six weeks after induction of colitis (post-colitis) and from age matched controls were studied. The serosal side of the tissues was exposed to SC-560 (300 nM) for 10 minutes prior to stimulation with EFS or IBMX. Values are mean (SEM), with 6–10 rats per group. *\( p < 0.05 \) versus the corresponding control group.

**Figure 4** (A) Representative traces of short circuit current recording from colonic tissue mounted in Ussing chambers. The effects of vehicle and of celecoxib (10 \( \mu \)M) are shown in both control and six week post-colitis rats. In each case the tissue was stimulated with isobutylmethylxanthine (IBMX 300 \( \mu \)M), eliciting an increase in the short circuit current. (B, C) Effects of selective inhibition of cyclooxygenase 2 on changes in colonic short circuit current (\( I_{sc} \)) in response to electrical field stimulation (EFS; B) or IBMX (C). Tissues harvested from rats six weeks after induction of colitis (post-colitis) and from age matched controls were studied. The serosal side of the tissues was exposed to celecoxib (Celecoxib 10 \( \mu \)M) for 10 minutes prior to stimulation with EFS or IBMX. Values are mean (SEM), with 6–10 rats per group. *\( p < 0.05 \) versus the corresponding control group; †\( p < 0.05 \) versus the corresponding vehicle treated group.
vehicle or celecoxib. Neither celecoxib nor SC-560 significantly affected basal I_{sc} (table 1) or basal conductance (with both drugs basal conductance stayed within the range 10–14 mS/cm², in controls and post-colitis rats).

**Effects of PGD₂ and ΔPGJ₂ on colonic chloride secretion**

Pre-exposure of distal colon from control rats to PGD₂ (1 μM) resulted in ~60% decrease in basal short circuit current (table 1) and a similar decrease in the chloride secretory responses to EFS or IBMX (fig 5). Exposure to PGD₂ did not significantly affect conductance (vehicle: 10.1 (1.2) mS/cm²; PGD₂: 8.8 (0.8) mS/cm²; n = 5 per group). In contrast, pre-exposure of the distal colon to ΔPGJ₂ (1 μM), one of the major metabolites of PGD₂, did not significantly affect basal I_{sc} or conductance, or the I_{sc} responses to EFS or IBMX (fig 5). Thus acute exposure of normal colonic tissue to PGD₂ could mimic the hyporesponsiveness to EFS and IBMX that was observed in colonic tissue from post-colitis rats.

**Effects of COX-2 inhibition on bacterial colonisation and translocation**

Consistent with previous observations,⁶ aerobic bacterial colonisation of the distal colon was significantly increased in post-colitis rats compared with age matched controls. In this study, we found that the bacterial counts in the post-colitis group were 12-fold greater than in controls (table 2). Furthermore, there was significantly greater (threefold) translocation of bacteria in the post-colitis group than in controls (table 2). Twice daily treatment with a selective inhibitor of COX-2 (rofecoxib) did not significantly change the numbers of aerobic bacteria in the distal colon of post-colitis rats. However, the incidence of bacterial translocation was reduced from 73% to 18% (p<0.05), not significantly different from that observed in control rats (table 1).

**DISCUSSION**

The ability of the gastrointestinal epithelium to act as a barrier is critically important in terms of preventing infection. There are many components to epithelial defence, including the ability of the epithelium to secrete electrolytes, water, and mucus, and the ability (by virtue of tight junctions) to physically restrict the movement of microbes and microbial products into the lamina propria. Impairment of these defensive functions could therefore predispose an organism to bouts of infection and inflammation. Intestinal inflammation has been shown to produce prolonged alterations in smooth muscle function, which could contribute to symptom generation in disorders such as irritable bowel syndrome.²⁴ In a series of studies over the past few years, we have sought to determine if a bout of inflammation of the intestine would similarly result in prolonged epithelial dysfunction.⁷⁸ Indeed, colitis induced by a hapten (TNBS) and enteritis induced by *Nippostrongylus brasiliensis* were both found to result in prolonged colonic epithelial secretory dysfunction, and in increase in bacterial translocation.²⁴ In the present study, we provide evidence for a key role of COX-2, possibly via PGD₂, in mediating the epithelial dysfunction that persists after resolution of TNBS induced colitis. Despite the absence of macroscopic and histological evidence of damage or inflammation, the colon of rats examined six weeks after TNBS administration did not function normally. Basal short circuit current and, as demonstrated previously,⁷ the colonic secretory responses to two stimuli were significantly reduced compared with those in healthy rats. Moreover, we confirmed our previous observation⁷ that there was a marked increase in bacterial translocation in the post-colitis rats. Treatment for one week with a selective COX-2 inhibitor reduced bacterial translocation to normal levels. Moreover, the epithelial hyposecretion was mimicked in vitro by PGD₂ but not by its metabolite, ΔPGJ₂, and was reversed by a selective COX-2 inhibitor, but not a selective COX-1 inhibitor.

PGD₂ has been suggested to be important in the resolution of acute inflammation, and it has further been suggested that this prostanoid is primarily derived from COX-2 in this setting.¹⁵ We previously showed that COX-2 derived PGD₂ played an important role in downregulating neutrophil infiltration into the mucosa in acute colitis,¹⁶ while Gilroy and colleagues¹⁵ showed an important role of PGD₂ and ΔPGJ₂, derived from COX-2, in downregulating leucocyte

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**Table 2. Role of cyclooxygenase 2 in elevated colonic bacterial colonisation and translocation following resolution of colitis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total aerobes (log_{10} CFU/g)</th>
<th>Incidence of translocation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control+vehicle</td>
<td>5.40 (0.34)</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>Post-colitis+vehicle</td>
<td>6.47 (0.31)*</td>
<td>8/11 (73)*</td>
</tr>
<tr>
<td>Post-colitis+rofecoxib</td>
<td>6.05 (0.29)</td>
<td>2/11 (18)*</td>
</tr>
</tbody>
</table>

Colonic bacterial colonisation and translocation to the spleen were measured six weeks after trinitrobenzene sulphanic acid administration (controls were age matched). Treatment with rofecoxib (3 mg/kg) or vehicle was performed twice daily for the week prior to the rats being killed.

* p<0.05 versus the control group; †p<0.05 versus the post-colitis+vehicle group.

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Figure 5  (A) Representative traces of short circuit current recording from colonic tissue from normal rats mounted in Ussing chambers. The effects of vehicle (control) or of prostaglandin D₂ (PGD₂ 1 μM) are shown on the response to subsequent serosal application of isobutylmethylxanthine (IBMX 300 μM). (B) Effect of serosal pre-exposure to PGD₂ or 15-deoxy-Δ^{12-14}PGJ₂ (ΔPGJ₂ 1 μM for 10 minutes) on the change in colonic short circuit current (I_{sc}) in response to electrical field stimulation (EFS) or IBMX. Values are mean (SEM), with 6–10 rats per group. *p<0.05, **p<0.01 compared with vehicle group response to the same secretagogue.

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influx in pleuritis. In the present study, we observed marked upregulation of PGD₂ synthesis by the colon during the period of resolution of colitis and healing of colonic ulcers. The increase in PGD₂ synthesis was completely abolished by treatment with rofecoxib, indicating that it was derived primarily from COX-2. A role for COX-2 in the resolution of colitis is also consistent with our previous observation that selective inhibitors of COX-2 exacerbated TNBS induced colitis in the rat.13 Surprisingly, the increase in COX-2 expression in post-colitis tissue was only of the order of ~60% above that in colonic tissue from healthy rats. However, expression of COX-2 appeared to be “primed” in the post-colitis setting as administration of LPS resulted in a much more robust (700%) increase in expression in post-colitis rats compared with that in healthy controls stimulated with LPS.

PGD₂ has well characterised inhibitory effects on epithelial secretion in the dog and rat.17 18 Our observation that PGD₂ reduced IBMX and EFS stimulated chloride secretion is consistent with previous observations from rat studies.31 There is some controversy as to which of the two identified PGD₂ receptors mediate the inhibitory effects of PGD₂ on epithelial secretion,26 and from the results of the present study we cannot shed light on this issue. However, we did observe that ΔPGJ₂ did not suppress epithelial secretion when tested at the same concentration as PGD₂. This indicates that the inhibitory effects of PGD₂ did not occur as a consequence of metabolism to ΔPGJ₂.

The observed changes in colonic epithelial secretion may have been produced by changes in the epithelium itself, and/or through changes in several mucosal elements that can influence epithelial function. For example, the enteric nervous system and mucosal stromal and immune cells (for example, myofibroblasts, mast cells) can influence epithelial responses to secretagogues and to EFS. In a previous study,7 we noted that secretory hyporesponsiveness to EFS, IBMX, and carbacol in tissues from post-colitis rats was not influenced by pre-exposure of the tissues to tetrodotoxin. While this suggests that enteric neurones are not responsible for the post-colitis hyporesponsiveness, one cannot rule out a contribution of tetrodotoxin insensitive nerves, or of other extra-epithelial cells in modulating epithelial function in the post-colitis state. Similarly, the mechanism underlying the prevention of bacterial translocation in post-colitis rats treated with rofecoxib has not been identified. It is possible that normalisation of colonic secretion, as the in vitro studies demonstrated, resulted in the decrease in bacterial translocation. However, it is also possible that rofecoxib reduced bacterial translocation through other actions on the epithelium or on non-epithelial cells.

In an earlier study, we observed that selective suppression of iNOS could also significantly reduce post-colitis bacterial translocation, and partially reverse secretory hyporesponsiveness.7,9 There may be a link between the role of NO production in post-colitis tissue and the observed importance of COX-2. NO derived from iNOS has been reported to stimulate COX-2 activity in a mouse macrophase cell line and in vivo in the rat.30 31 Thus increased iNOS can lead to increased COX-2 activity while suppression of iNOS activity results in lower COX-2 activity.39 Interestingly, iNOS and COX-2 have recently been suggested to mediate alterations in barrier and transport properties of the human intestinal epithelium caused by enteroinvasive bacteria, and these alterations could be partially reversed by inhibitors of either iNOS or COX-2.32 Moreover, COX-2 has been implicated as a mediator of the smooth muscle dysfunction that persists after a bout of inflammation.33

While not the focus of the present study, it is important to note that prolonged elevation of COX-2 has been implicated in proliferative disorders of the large intestine. Also, metabolites of PGD₂ (such as ΔPGJ₂) acting at the PPARγ receptor, have similarly been implicated as contributing to such disorders.34 COX-2 appears to play an important role in the pathogenesis of colorectal cancer,35 the incidence of which is markedly elevated in colitis patients.36 We observed that COX-2 was “primed” in the post-colitis scenario, such that expression was increased profoundly following stimulation with LPS (relative to healthy controls). Thus we have observed two key alterations that could contribute to proliferative changes in the colon of rats following resolution of colitis, namely, increased bacterial translocation (thus exposure to bacterial endotoxin) and an increased sensitivity to stimulation by bacterial endotoxin in the context of COX-2 expression. The underlying mechanisms for the priming of COX-2 have not yet been identified, and certainly warrant further study.

In summary, the results of the present study further demonstrate the important role the COX-2 derived products in regulating epithelial function. The marked elevation of bacterial translocation in post-colitis rats was reversed by selective inhibition of COX-2, as was epithelial secretory hyporesponsiveness. Whether or not there is a causative link between secretory hyporesponsiveness and impaired barrier function is not yet clear. Colonic secretion of water, which is driven by electrolyte secretion, has been suggested to be very important in mucosal defence, by diluting toxins, flushing away luminal microbes, and delivering secretory IgA.37 In addition to the potential role of COX-2 in mediating epithelial proliferative changes following colitis, this enzyme and one of its products, PGD₂, appear to contribute significantly to the prolonged alterations in epithelial function that follow a bout of colitis. COX-2 and possibly PGD₂ may therefore represent rational therapeutic targets for preventing these prolonged alterations in epithelial function, which may contribute to the relapses of colonic inflammation that are common in ulcerative colitis and Crohn’s disease.

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