Inter-relationships between inflammatory mediators released from colonic mucosa in ulcerative colitis and their effects on colonic secretion

T D Wardle, L Hall, L A Turnberg

Abstract
Metabolites of arachidonic acid have been implicated in the pathophysiology of ulcerative colitis—they can stimulate intestinal secretion, increase mucosal blood flow, and influence smooth muscle activity. The influence on the mucosal transport function of culture medium in which colonic mucosal biopsy specimens had been incubated was investigated using rat stripped distal colonic mucosa in vitro as the assay system. Colonic tissue from patients with colitis and from control subjects was cultured. Medium from inflamed tissue contained more prostaglandin E2 (PGE2) and leukotriene D4 (LTD4) and evoked a greater electrical (secretory) response in rat colonic mucosa than control tissue medium. In inflamed tissue, cyclo-oxygenase inhibition (indomethacin) attenuated PGE2 but increased LTD4 production; conversely lipoxygenase inhibition (ICI 207968) inhibited LTD4 production but enhanced PGE2 output. Each inhibitor alone enhanced the electrical response in the rat colon. Inhibition of both enzymes (indomethacin plus ICI 207968) caused a fall in both PGE2 (82%) and LTD4 (89%) production and in the electrical response (57%). Inflamed tissue treated with a phospholipase A2 inhibitor (mepacrine) produced less PGE2, LTD4, and electrical responses when compared with inflamed tissue, either untreated (91%, 92%, and 79% respectively) or treated with cyclooxygenase and lipoxygenase inhibition. Incubation with bradykinin stimulated eicosanoid release and electrical response, while a bradykinin antagonist caused a modest inhibition. Analysis of these observations suggests that a combination of arachidonic acid derivatives accounts for about half the secretory response. Other products of phospholipase A2 activity are probably responsible for much of the remainder, leaving up to 20% the result of types of mediator not determined in this study.

High concentrations of prostaglandins and leukotrienes have been found in stool water and colonic mucosal biopsy specimens from patients with ulcerative colitis and these have been implicated in the pathophysiology of inflammatory bowel disease. They may not only be involved in the mediation and amplification of the immune response but several have also been shown to stimulate mucosal secretion, increase mucosal blood flow, and influence smooth muscle activity, each of which may be relevant to the diarrhoea that these patients suffer.

Because such a wide variety of inflammatory mediators is liberated in colitis it is difficult to ascertain which, either alone or in combination, might be responsible for the associated changes in intestinal function.

We describe studies of the influence on intestinal secretion of inflammatory mediators released into the medium in which biopsy specimens of inflamed colonic mucosa were cultured. We used rat colonic mucosa in vitro as our 'assay' system for determining secretory responses. Studies of the effect of a variety of inhibitors on these responses, and on the release of a number of mediators, have allowed us to show that a combination of prostaglandins and leukotrienes is probably responsible for over half of the secretory response, and that other products of phospholipase A2 activity are probably responsible for much of the remainder.

Methods

PATIENT DETAILS
Thirty patients underwent colonoscopy after bowel preparation. Preparation consisted of a three day low residue diet, and one day before the examination a combination of X prep (purified senna extract; 1 ml/kg body weight) and 10% mannitol (500 ml) modified according to the patient’s symptoms.

Ten of the patients (four men and six women, median age 36 years) had a clinical diagnosis of irritable bowel syndrome. They all had endoscopically and histologically normal mucosa.

Twenty patients (13 men and seven women, median age 39-4 years) with active distal proctosigmoiditis had biopsy specimens taken from inflamed mucosa. Six patients were taking mesalazine (400 mg three times daily), four prednisolone (5 to 15 mg once daily), and seven topical steroids at the time of colonoscopy. All biopsy specimens were taken with non-spiked forceps to minimise tissue trauma.

Ethical approval for these studies was given by the Salford Health Authority Ethics Committee.

EXPLANT CULTURE
A series of cultures was performed for each patient. Mucosal biopsy specimens were immediately placed in transport medium (L15, with added penicillin G and streptomycin sulphate), transferred to the laboratory, washed gently three times in the L15 medium, carefully
botted, weighed (range 3–8 mg), and placed in a 5 cm culture dish containing 1 ml of culture medium (CMRL) 1066, plus glucose 5 µg/ml, methionine 1 µM/ml, Tris buffer 20 mM, glutamine 3 µM/ml, β-retinyl acetate 1 µg/ml, penicillin G 100 units/ml, streptomycin sulphate 100 µg/ml, gentamicin 50 µg/ml, and amphotericin β 0.25 µg/ml. Individual mucosal biopsy specimens were cultured with either no additives or in the presence of one of the following: indomethacin (cyclo-oxygenase inhibitor); ICI 207968 (lipooxygenase inhibitor); a combination of indomethacin and ICI 207968; mepacrine (phospholipase A2 inhibitor) (all at 10^{-3}M); and bradykinin, or des arg leu bradykinin (bradykinin antagonist) (both at 10^{-4}M).

The culture dishes were placed in a humidified chamber maintained at 37°C, supplied with a mixture of 95% oxygen/5% carbon dioxide, and rotated at 10 cycles/minute. After 4 hours of culture the medium was removed and divided into three aliquots for measurement of PGE₂, LTD₄, and electrical responses in rat colonic mucosa.

**Eicosanoid Measurements**

PGE₂ and LTD₄ were measured using commercially available radioimmunoassay kits (PGE₂, du Pont UK; stevenage, Herts, UK; LTD₄, Amersham, Aylesbury, Bucks, UK). Eicosanoids were extracted from the culture medium using solid phase sorbant extraction (mini columns, Amersham). The resultant sample competes with a fixed amount of radioactively labelled eicosanoid analogue (iodinated PGE₂ or tritiated LTD₄) for a limited number of binding sites. The sample PGE₂ antibody complex is separated from the free antigen by polyelectrolyte glycol precipitation and centrifugation, and then counted on a gamma counter. Separation of the leukotriene bound antibody complex was facilitated using dextran coated charcoal. After centrifugation the quantity of antibody bound radioactive ligand was measured on a beta counter.

**Assay performance characteristics**

Assay performance characteristics were as follows. PGE₂ intra and interassay variation values were 11 pg/ml and 60 pg/ml respectively; recovery was 96% and sensitivity 0.8 pg/ml. Cross reactivity (non-E prostaglandins) was <0.4%. LTD₄ intra and interassay variation values were 14 pg/ml and 39 pg/ml respectively; recovery was 91% and sensitivity 5 pg/ml. Cross reactivity (non-sulphidopeptide leukotrienes) was <0.001%.

Eicosanoid concentrations were calculated by interpolation from a standard curve. All results were expressed in pmol/mg wet tissue/hour.

**RAT DISTAL COLON PREPARATION**

An in vitro technique modified from that of Ussing and Zerahn was used. Unfasted male Sprague-Dawley rats were killed and the distal colon was removed immediately and bathed in oxygenated buffer. Muscle layers were stripped and the two most distal pieces of mucosa were mounted as sheets, between Perspex flux chambers, with a surface area of 0.64 cm² (VT Plastics Ltd, Warrington, UK).

The spontaneous, basal transmucosal potential difference (PD) was measured, on a high impedance digital voltmeter via fine tipped electrode bridges (3M KCl in 3% agar) connected to matched calomel half cells. The short circuit current (Isc) was delivered by silver/silver chloride electrodes via 1M NaCl in 1% agar bridges. The electrodes were connected to a voltage clamp for automatic short circuiting. The clamp was corrected for fluid resistance between the PD sensing bridges. Tissue conductance and resistance were calculated from the PD and Isc according to Ohm’s law.

Each mucosal sheet was bathed on both sides with 5 ml of isotonic buffer containing: Na: 146 mM; K: 4.2 mM; Cl: 125.8 mM; HCO₃: 26.6 mM; H₃PO₄: 0.2 mM; HPO₄: 1.2 mM; Ca 1.2 mM; Mg 1.2 mM; and glucose 10 mM, at pH 7.4. The bathing media were stirred and oxygenated via a bubble lift system using 95% O₂/5% CO₂ and were maintained at a constant temperature of 37°C.

Culture medium (100 µl) was added to the bathing fluid on the serosal aspect of rat colonic mucosa after electrical stability had been reached, usually after 30 minutes.

**SECRETORY AGONISTS**

**Eicosanoids**

PGE₂ or LTD₄ in final concentrations ranging from 10^{-8} to 10^{-4}M, were added to the serosal aspect of stripped rat distal colon. Changes in PD, Isc, and resistance were recorded.

**Culture medium**

Culture medium (100 µl) was added to the serosal aspect of stripped rat colon and electrical measurements, as described above, were recorded. The process was repeated using either medium incubated with the inhibitors to act as controls or medium from biopsy specimens cultured with the inhibitors as detailed above. The resultant rise in Isc was compared with the PGE₂ and LTD₄ dose response curves.

**CHEMICALS**

The 5 lipooxygenase inhibitor, ICI 207968, was kindly supplied by Dr R Dowell, Imperial Chemical Company, Alderley Edge, UK. Prostaglandin E₂, bradykinin, des arg leu bradykinin, mepacrine, and indomethacin were obtained from Sigma Chemical Co, Poole, Dorset, UK. LTD₄ was purchased from Cascade Biochem Ltd, University of Reading, Berks, UK.

**CALCULATIONS**

All values are expressed as the mean (SEM). Statistical comparisons were performed using paired and unpaired t tests.
Results

EICOSANOID MEASUREMENTS

Inflamed tissue produced significantly more PGE$_2$ and LTD$_4$ than control tissue (2-79 (0-11) vs 0-54 (0-04); 1-73 (0-11) vs 0-44 (0-04) respectively; p<0-01) (all values are given in pmol/mg wet tissue weight/hour). The production rate of eicosanoids is also expressed graphically in both pmol and pg/mg wet tissue weight/hour for example in inflamed tissue PGE$_2$ production is 2-79 pmol or 1012 pg/mg wet tissue weight/hour and LTD$_4$ is 1-73 pmol or 837 pg/mg wet weight/hour. (Figs 1 and 2). Cyclo-oxygenase inhibition (indomethacin, 10$^{-5}$M) significantly reduced PGE$_2$ production when values were compared with those in the untreated groups (inflamed 0-511 (0-06) vs 2-79 (0-11); control 0-17 (0-02) vs 0-54 (0-04); p<0-01), whereas the yield of LTD$_4$ was increased (2-4 (0-17) v 1-73 (0-11); control 0-74 (0-07) v 0-44 (0-04); p<0-01).

A significant increase in PGE$_2$ production, by all groups, followed lipoxygenase inhibition (ICI 207968, 10$^{-5}$M) (inflamed 3-9 (0-22) v 2-79 (0-11); control 0-91 (0-13) v 0-54 (0-04); p<0-01), while LTD$_4$ generation was reduced (inflamed 0-16 (0-07) v 1-73 (0-11); control 0-1 (0-01) v 0-44 (0-04); p<0-01).

Combined inhibition of cyclo-oxygenase and lipoxygenase produced almost identical PGE$_2$ results to those found after indomethacin alone (Fig 1), and LTD$_4$ results to those found after ICI 207968 alone (Fig 2).

In inflamed tissue, phospholipase A$_2$ inhibition (mepacrine) appreciably reduced the production of both PGE$_2$ and LTD$_4$ when compared with values in untreated mucosa (PGE$_2$ 0-21 (0-03) v 2-79 (0-11); LTD$_4$ 0-14 (0-04) v 1-73 (0-11) respectively; p<0-001). The reduction after mepacrine was also greater than that after combined cyclo-oxygenase and lipoxygenase inhibition (PGE$_2$ 0-21 (0-03) v 0-5 (0-05); LTD$_4$ 0-14 (0-04) v 0-19 (0-03) respectively; p<0-04).

Mepacrine also significantly attenuated eicosanoid output by control tissue (PGE$_2$ 0-11 (0-04) v 0-54 (0-04); LTD$_4$ 0-1 (0-08) v 0-44 (0-04); p<0-01).

In comparison with the untreated group, bradykinin stimulated a significant increase in both PGE$_2$ production (control 1-1 (0-18) v 0-54 (0-04); inflamed 4-1 (0-24) v 2-79 (0-11); p<0-001) and LTD$_4$ production (control 1-1 (0-12) v 0-44 (0-4); inflamed 4-1 (0-17) v 1-7 (0-11); p<0-001).

Inhibition of bradykinin resulted in a fall in PGE$_2$ and LTD$_4$ generation by all groups but only the reduction found in the inflamed group reached statistical significance (inflamed PGE$_2$ 2-3 (0-14) v 2-79 (0-11); LTD$_4$ 1-1 (0-1) v 1-73 (0-11) respectively; p<0-01, control PGE$_2$ 0-34 (0-05) v 0-54 (0-04); LTD$_4$ 0-29 (0-05) v 0-44 (0-04); NS).

EFFECT OF CULTURE MEDIUM ON STRIPPED RAT DISTAL COLON

Fresh culture medium applied to the serosal half chamber did not produce any change in baseline electrical activity (Fig 3). Culture medium incubated with the inhibitors did not influence
the basal Isc. Culture fluid from inflamed tissue evoked a significantly larger Isc increase than fluid from control mucosa (31 (2-6) vs 6-3 (1-3) μA.cm⁻²; p<0-001).

Cyclo-oxygenase inhibition
Medium from control biopsy specimens cultured with or without indomethacin produced similar rises in electrical measurements. However, a significantly greater increase in Isc occurred with fluid derived from inflamed mucosal biopsy specimens treated with indomethacin, compared with the untreated group (40 (2-5) vs 31 (2-6) μA.cm⁻²; p<0-01).

Lipoxygenase inhibition
Lipoxygenase inhibition with ICI 207968 did not influence the modest rise in electrical activity seen with the control biopsy medium. However, after lipoxygenase inhibition medium from inflamed tissue produced a significantly greater Isc response than that from untreated control tissues (43-5 (5) vs 31 (2-6) μA.cm⁻²; p<0-01).

Combined lipoxygenase and cyclo-oxygenase inhibition
There was no significant difference between the short circuit response evoked by culture medium from untreated control tissue and control biopsy specimens exposed to combined cyclo-oxygenase and lipoxygenase inhibition. Culture medium from inflamed tissue treated in the same way, however, produced a significantly lower Isc response than medium from untreated tissues (13-4 (2-1) vs 31 (2-6) μA.cm⁻²; p<0-005).

Phospholipase A₂ inhibition
Control biopsy specimens incubated with mepacrine produced media which provoked a significantly smaller Isc response than untreated mucosal media (2-5 (1) vs 6-3 (1-3) μA.cm⁻²; p<0-01). Medium from inflamed tissue exposed to mepacrine evoked an Isc response that was significantly lower than that from either medium from untreated tissue or from tissues exposed to the combined effects of cyclo-oxygenase and lipoxygenase inhibition. (6-4 (1-1) vs 31 (2-6) and 13-4 (2-1) μA.cm⁻²; respectively, p<0-005).

Bradykinin agonist
The addition of bradykinin to the culture medium bathing both types of tissue produced a highly significant increase in Isc compared with values in the untreated groups (control 17-5 (2-8) vs 6-3 (1-3); inflamed 66-1 (4-9) vs 31 (2-6) μA.cm⁻²; p<0-01).

Bradykinin antagonism
Bradykinin receptor blockade produced a fall in the Isc for both tissue types, but only in the inflamed group did the change reach statistical significance (31 (2-6) vs 19-3 (3-1) μA.cm⁻²; p<0-01).

DOSE RESPONSE CURVES

PGE₂
PGE₂ added to the serosal, but not the mucosal, side of stripped rat distal colon caused a rapid increase in Isc, which peaked after 2½ to 3 minutes. The dose response curve for PGE₂ gave an EC₅₀ of 5×10⁻⁶M (Fig 4). PGE₂ generated a parallel but smaller increase in PD and a modest rise in tissue conductance.

LTD₄
LTD₄ added to the serosal aspect of stripped rat distal colon, evoked a rapid rise in Isc, which peaked after 2½ minutes (Fig 5). The EC₅₀ for this response was 8×10⁻⁷M. The transmucosal PD also increased while conductance rose to a modest extent.

Combined PGE₂ and LTD₄
At the peak Isc response to PGE₂ at 10⁻⁷M and 10⁻⁸M, LTD₄ 10⁻⁷M and 10⁻⁸M respectively were added to the serosal chamber. The combined Isc for 10⁻⁷M was 43.4 μA.cm⁻² and for 10⁻⁸M it was 20.8 μA.cm⁻². These values were not significantly different from those expected from the dose response curves (41.5 μA.cm⁻²).

Figure 4: Prostaglandin E₂ dose response curve in stripped rat distal colon. Values are mean (SEM). Number of rat mucosal preparations under each point.

Figure 5: Leukotriene D₄ dose response curve in stripped rat distal colon. Values mean (SEM). Number of rat mucosal preparations under each point.
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18.5 μA cm² respectively – that is, there was no evidence of potentiation at either maximal or half maximal concentration.

Discussion
In these studies we have shown that inflamed tissue releases more eicosanoids into the culture medium than control mucosa, and that medium from any of the tissues will evoke an electrical response in stripped rat distal colon. A rise in electrical potential difference and Isc in this mucosal preparation is associated with secretion of chloride ions and we have used a rise in electrical measurements as a proxy for anion secretion.

Inflamed tissue produced significantly more PGE₂ and LTD₄ than control mucosa, but the difference was not as great as that shown by rectal dialysis. Although the degree of indomethacin induced cyclo-oxygenase inhibition is variable, all groups showed significant attenuation of PGE₂ production and a reciprocal, significant increase in LTD₄ generation. Conversely, inhibition of lipoygenase activity resulted in a suppression of LTD₄ generation and stimulation of PGE₂ synthesis. Presumably, the increased availability of arachidonic for one enzyme system when the alternative route was blocked is responsible for these reciprocal effects on PGE₂ and LTD₄ production.

Because PGE₂ and LTD₄ have each been shown to stimulate intestinal secretion it is not surprising to find that inhibiting the production of one of these did not reduce the secretory (electrical) response to medium, in which a reciprocal rise in the other had occurred. More difficult to explain is the greater Isc response to medium in which one stimulant was appreciably depressed while the other was only moderately increased. It is possible that the inhibition of the lipoygenase or cyclo-oxygenase pathways caused the synthesis of more potent secretagogues than simply the two measured in this study. Other potential contenders for this role include prostacyclin, a more potent secretagogue than PGE₂ in the case of lipoygenase inhibition, and other leukotrienes (B₄, C₄) in the case of cyclo-oxygenase inhibition.

This type of analysis leads to the conclusion that there is a finely balanced production of the major metabolites of arachidonic acid and that subtle alterations in the relative activities of the enzymes concerned will cause variable responses. Blockade of a major pathway, as in this study, can thus produce unpredictable changes in functional responses. The failure of early attempts to treat ulcerative colitis with cyclo-oxygenase inhibitors may be explicable on this basis.

Simultaneous blockade of cyclo-oxygenase and lipoygenase pathways reduced both PGE₂ and LTD₄ production, as might be expected, and also attenuated the electrical response of rat colonic mucosa to this culture medium. However, the 82% reduction in PGE₂ production and the 89% reduction in LTD₄ production was associated with only a 57% reduction in transmucosal Isc suggesting that other factors are probably involved in provoking the electrical response. One possibility is that potentiation between the effects of low concentrations of these metabolites might occur. Such a potentiation would have to be greater at low than at high concentrations to account for this observation and we did not find evidence of potentiation between PGE₂ and LTD₄ in our study. Thus, this seems a less likely explanation than the simultaneous generation of other types of secretory agonists. In favour of this are the results of phospholipase A₂ inhibition with mepacrine. Here blockade of the enzyme responsible for arachidonic acid liberation caused a much greater fall in the electrical response of rat colonic mucosa (from 57% after combined lipoygenase and cyclo-oxygenase inhibition to 79% after phospholipase A₂ inhibition). PGE₂ and LTD₄ production were also further reduced (82% to 92% and 89% to 91% respectively), but these falls were much less impressive than the fall in ISc.

It is interesting to compare the electrical responses to culture media (with their measured eicosanoid concentrations) with the dose response curves for PGE₂ and LTD₄. It is clear that the concentrations of both eicosanoids measured in the culture medium are much lower than those that might be expected to provoke the electrical responses which were observed if their effects were simply additive. The mean eicosanoid concentrations derived from culture medium from inflamed tissue were in the order of 4.45×10⁻¹¹M for PGE₂ and 2.77×10⁻¹¹M for LTD₄. As indicated by the dose response curves, no change in ISc would be expected with these amounts, even if these concentrations were summated. The mean rise in ISc evoked by medium from inflamed cultures was 30 μA cm⁻² which would be expected at concentrations of some 10⁻⁹M. PGE₂ or 10⁻⁸M LTD₄, if these were the only mediators present. Clearly other mediators must also be involved in the electrical response, but the degree of inhibition by mepacrine (79%) suggests that most are likely to be products of phospholipase A₂ activation.

It may be concluded from these observations that cyclo-oxygenase and lipoygenase products account for most (57%) of the electrical response induced by medium from cultured colonic mucosa. Only PGE₂ and LTD₄ were measured in these studies and since they could only be held responsible for part of the response, other cyclo-oxygenase and lipoygenase products are likely to have contributed. Moreover, other phospholipase A₂ metabolites are probably responsible for a further 22% of the electrical response. It seems most likely that this is due to a non-arachidonic acid derivative, platelet activating factor being a reasonable contender for this role.

The remaining 21% of the electrical response produced by medium from cultured inflamed biopsy specimens could be caused by a variety of other mediators such as histamine, 5-hydroxytryptamine and, possibly, transmitters liberated from neural tissues.

Because much of the electrical, secretory response to culture medium could be ascribed to the release of products of phospholipase A₂ activity it was of interest to investigate the
influence of one potentially important stimulus to phospholipase A2 activity. Inflammatory cells, in particular macrophages have receptors for bradykinin, a potent secretagogue acting almost entirely by stimulating arachidonic acid release via phospholipase A2 activation. Its effect on intestinal mucosa, at least in the rat ileum, is indirect, its site of action being on subepithelial cells.28,29 In our study bradykinin caused a noticeable rise in eicosanoid release from control and inflamed biopsy specimens and culture medium from these caused a greater rise in Isc in the rat colonic mucosa model. Bradykinin receptor blockade with des arg leu bradykinin caused a fall in eicosanoid output and the associated Isc response, suggesting that bradykinin may be a stimulus to endogenous phospholipase A2 activity in biopsy specimens of normal and inflamed colonic mucosa. The fall in PGE2 and LTD4 production after bradykinin receptor blockade was, however, less than that found after direct phospholipase A2 inhibition with mepracine or after combined lipoxigenase and cyclo-oxygenase inhibition. Reasons for this smaller effect include the possibility that receptor blockade with des arg leu bradykinin was incomplete in these specimens. It is also likely that the other stimuli to phospholipase A2 activity such as interleukin 1β, interleukin 8, and other monocyte derived growth factors,10 are liberated in these tissues.

Although we have focussed on the secretory effects of these inflammatory mediators, it is clear that they are likely to have a number of other effects that will contribute to the pathophysiology of the disease. The effects, on secretion, described here, however, provide one indicator of the tissue response in inflammatory disease.

In conclusion, we have provided evidence in favour of the view that eicosanoids are the major inflammatory mediators causing secretory responses in colonic mucosa and that their production can be significantly reduced by combined cyclo-oxygenase and lipoxigenase inhibition or by mepracine or by a bradykinin antagonist. The maximal reduction in eicosanoid release and secretory response was achieved with mepracine and this may warrant further clinical evaluation of its therapeutic role in inflammatory bowel disease. Care should be taken in the assessment of drugs which influence inflammatory mediator metabolic pathways since disturbance of these complex interactions and balances may produce unpredictable results.

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