Colorectal leukotriene B\textsubscript{4} synthesis in vitro in inflammatory bowel disease: inhibition by the selective 5-lipoxygenase inhibitor BWA4C

A B Hawthorne, N K Boughton-Smith, B J R Whittle, C J Hawkey

Abstract
The in vitro synthesis of leukotriene B\textsubscript{4} (LTB\textsubscript{4}) was evaluated in colorectal biopsy specimens and resection tissue from patients with inflammatory bowel disease. The in vitro formation of LTB\textsubscript{4} from biopsy tissues stimulated with calcium ionophore A23187 correlated with the degree of mucosal inflammation assessed at sigmoidoscopy, and with neutrophil infiltration measured as myeloperoxidase activity. Biopsy specimens from patients taking prednisolone formed less LTB\textsubscript{4} than those from patients not on prednisolone, with comparable levels of inflammation seen at sigmoidoscopy. The formation of LTB\textsubscript{4} was reduced dose-dependently by the acetoxyhydroxamic acid 5-lipoxygenase inhibitor BWA4C, with no significant inhibition of prostaglandin E\textsubscript{2} or thromboxane B\textsubscript{2} synthesis. In inflamed colonic resection tissue from colitic patients, the IC\textsubscript{50} for inhibition of LTB\textsubscript{4} formation by BWA4C was 0.03 \textmu mol/l, compared with an IC\textsubscript{50} of 0.8 \textmu mol/l for NDGA. Thus, BWA4C is a potent and selective inhibitor of LTB\textsubscript{4} synthesis in colonic tissue from patients with ulcerative colitis. Acetoxyhydroxamic acid 5-lipoxygenase inhibitors, exemplified by BWA4C, may be useful to evaluate the clinical importance of LTB\textsubscript{4} in ulcerative colitis, and offer a novel therapy for the disease.

Amplification of the inflammatory response in the colonic mucosa in ulcerative colitis is likely to be controlled by a number of soluble mediators including prostaglandins and leukotrienes, histamine, serotonin, and complement pathway products. The formation of the arachidonic acid 5-lipoxygenase metabolite leukotriene B\textsubscript{4} (LTB\textsubscript{4}) a potent neutrophil chemotactant and activator, is increased in colonic mucosal homogenates\textsuperscript{1,2} and in rectal dialysates\textsuperscript{3} in ulcerative colitis. In colonic mucosa from patients with ulcerative colitis, most of the neutrophil chemo-tactic activity is lipid extractable, and coelutes with LTB\textsubscript{4} on high performance liquid chromatography,\textsuperscript{4} suggesting that LTB\textsubscript{4} is quantitatively important in the amplification of inflammatory responses in ulcerative colitis.

Oral prednisolone reduces the synthesis of lipooxygenase products in ulcerative colitis,\textsuperscript{5} as does sulphasalazine\textsuperscript{6,7} and 5-aminosalicylic acid (5-ASA),\textsuperscript{8} but these drugs have a number of other actions contributing to their anti-inflammatory effects. Selective 5-lipoxygenase inhibitors accelerate healing in animal models of colitis.\textsuperscript{9,10} Furthermore, in animal models of colitis, topical LTB\textsubscript{4} enhances colonic inflammation.\textsuperscript{11} We have investigated the ex vivo synthesis of eicosanoids from human colonic and ileal tissue in inflammatory bowel disease and studied the in vitro effects of the acetoxyhydroxamic acid BWA4C, a potent and selective inhibitor of 5-lipoxygenase activity.\textsuperscript{12}

Methods
The study was approved by the South Nottingham Hospital ethical committee and informed consent was obtained from patients. Two methods were used to investigate mucosal LTB\textsubscript{4} synthesis from patients with inflammatory bowel disease. In vitro synthesis of LTB\textsubscript{4} was measured using colorectal biopsy specimens to investigate parameters affecting synthesis and to show the effect of 5-lipoxygenase inhibitors. Because the specimens were small, dose-response studies were also performed using larger amounts of inflamed colonic or ileal tissue obtained from ulcerative colitis or Crohn's disease patients undergoing operative resection.

LTB\textsubscript{4} SYNTHESIS IN COLORECTAL BIOPSY SPECIMENS

Biopsy specimens were taken from 47 patients with ulcerative colitis undergoing sigmoidoscopy or colonoscopy for clinical evaluation. Eleven of the patients had total colitis, 15 left sided colitis, 15 sigmoid colitis, and six proctitis. Twenty three patients were taking sulphasalazine, and 16 mesalazine; 25 were taking prednisolone. A visual assessment of mucosal inflammation and vascularity was made, and a score given as shown in Table I.\textsuperscript{13} Tissue specimens were then taken with St Marks rectal
biopsy forceps (Key Med, Southend on Sea, UK), 10 cm above the dentate line, or with colonoscopic biopsy forceps.

Calcium ionophore (A23187) is widely used as a potent stimulus to LT\textsubscript{B}4 synthesis from neutrophils. Ionophore stimulation of colorectal biopsy specimens is a simple and reliable method of increasing LT\textsubscript{B}4 synthesis, and a suitable way of evaluating 5-lipoxygenase inhibitors. Basal LT\textsubscript{B}4 synthesis, followed by ionophore-stimulated LT\textsubscript{B}4 synthesis was measured. Biopsy tissues were placed in 0.5 ml fresh Tyrode’s solution in Eppendorf vials on ice. Within 10 minutes they were transferred to a sealed incubation chamber and were pre-incubated (20 minutes at 37°C in 95% O\textsubscript{2}, 5% CO\textsubscript{2}). Tissue specimens were then centrifuged (10 seconds at 10000 g) and the supernatant was saved. Biopsy specimens were then washed twice in Tyrode’s solution, ionophore (dissolved in DMSO) was added to the vials (incubation volume 0.5 ml, 10 μmol/l final concentration), and the supernatant was saved after a further 20 minute incubation. For some biopsy specimens, a further 20 minute stimulation with freshly added ionophore followed (that is 20–40 minutes). In time course experiments, samples of supernatant (0.25 ml), were taken at four minute intervals during the 20 minute period, each time replaced with an equal volume of fresh oxygenated Tyrode’s solution containing ionophore.

Supernatants were stored at −40°C before radioimmunoassay. After stimulation, biopsy specimens were weighed and the protein and myeloperoxidase contents were assayed.

\textit{Lipoxygenase inhibitors.} In drug studies, the lipoxygenase inhibitors (dissolved in DMSO, 0.1% final concentration), were added to the Tyrode’s solution for basal and stimulated incubations, as well as the wash solution. Parallel control biopsy specimens were stimulated in the presence of DMSO alone.

DOSE RESPONSE STUDIES IN RESECTION TISSUE

Fresh resection specimens were obtained from three patients undergoing colectomy for ulcerative colitis (two taking prednisolone), and six patients undergoing resection for Crohn’s disease (five on prednisolone): five from the terminal ileum and one from the ileoceleal area. The bowel was immediately washed with saline, dabbed dry, and mucosa (at 4°C) cut from underlying tissue with scissors, and chopped finely with a scalpel. Aliquots (wet weight 100 mg or 200 mg), were placed in Tyrode’s solution (1 or 2 ml). Drug or vehicle (DMSO), were added and tubes were shaken and incubated at 37°C for five minutes. Ionophore (10 μmol/l final concentration) or vehicle (DMSO) was then added. Tubes were vortex mixed and incubated (37°C for 30 minutes), centrifuged (1150 g for 10 minutes at 4°C), and the supernatants saved.

\textit{Extraction.} Supernatants (1 ml) were acidified with 10% acetic acid (50 μl), and loaded onto 1 ml Bond-Elut C8 cartridges with steel frits (Analytichem, Harbor City CA, USA) which had been prewashed with 1 ml methanol and 1 ml water pH 3.5. Cartridges were washed with 1 ml of water, 10% ethanol, hexane, and then eluted with 1 ml methanol. Samples were stored at −40°C before radioimmunoassay.

\textbf{Radioimmunoassay}

LT\textsubscript{B}4 was assayed by kit (Amersham, Bucks, UK). The detection limit was 1 pg per tube. The intra-assay variation was 8.3% and interassay variation was 15.6%. Thromboxane B\textsubscript{2} assay (TXB\textsubscript{2}) used antiserum supplied by Professor L Levine (Brandeis University, MA, USA). The prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) radioimmunoassay used antiserum from the Sigma Chemical Co (St Louis, MI, USA). Leukotriene C\textsubscript{4} (LTC\textsubscript{4}) antiserum cross reacted by up to 40% with leukotriene D\textsubscript{4} (LTD\textsubscript{4}). All samples were assayed in duplicate. Addition and parallelism experiments confirmed that there was no interference in the assays from any of the drugs, ionophore, or the vehicle used in the study.

\textbf{Myeloperoxidase assay}

Biopsy specimens were homogenised in 1 ml hexadecyltrimethylammonium bromide 0.5% w/v in potassium phosphate buffer, in a Dual ground glass homogeniser (10 strokes), freeze thawed three times, and assayed for myeloperoxidase by degradation of hydrogen peroxide in a spectrophotometric assay using α-dianisidine.

\textbf{Protein assay}

Protein content of biopsy specimen homogenates was measured by Peterson’s modification of the modified Lowry method (Sigma P5656, St Louis, MI, USA).

\textbf{statistics}

Data are given to two significant figures, and are expressed as median and interquartile range, except where data are sufficient to show a normal distribution, when they are shown as mean (SEM). Non-parametric data were compared by the Mann-Whitney U test, Kruskal-Wallis, or

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Grade of inflammation} & \textbf{Basal} & \textbf{0–20 mins} & \textbf{20–40 mins} \\
\hline
0 & \textbf{3.0} & 10 & \textbf{6.0} \\
(n=4) & (9-9-8) & (10-11) & \\
\hline
1 & \textbf{11} & 24 & 26** \\
(n=18) & (5-5-19) & (7-6-43) & (13-44) \\
2 & \textbf{68*} & 160** & 88** \\
(n=10) & (10-120) & (87-280) & (24-200) \\
3 & \textbf{43*} & 154** & 122* \\
(n=6) & (26-53) & (38-210) & (10-170) \\
\hline
\end{tabular}
\caption{Ex vivo LT\textsubscript{B}4 synthesis (pg/mg wet weight) from rectal or colonic biopsy specimens, according to sigmoidoscopic grade of inflammation.}
\end{table}

Basal: level present in supernatant after 20 minute incubation without stimulation. Ionophore stimulation: release into supernatant when 10 μmol/l ionophore present in buffer (see text for details). Data are expressed as median (interquartile range). All patients were taking sulphasalazine or mesalazine except two with grade 1, one with grade 2, and one with grade 3 biopsies. The effect of ionophore stimulation, first and second 20 minute periods compared with basal was significant (p<0.05) at all grades of inflammation (except grade 0, basal < 20–40 minutes). Differences compared with grade 0 biopsies for each column:

*p<0.05; **p<0.01 (Mann-Whitney test). †n=5 only.
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Friedman’s non-parametric analysis of variance for several groups where appropriate. Parametric data were compared by paired t-test and Pearson correlation coefficient. The level of statistical significance was taken as p<0.05.

Results

LTB₄ SYNTHESIS IN COLORECTAL BIOPSY SPECIMENS

The mean (SD) wet weight of biopsy tissues used was 4.9 mg (2.2 mg) (n=61). There was a close correlation between protein content and wet weight (r=0.83, p<0.001, n=61), which did not vary with the sigmoidoscopic grade of inflammation.

Basal incubation of biopsy specimens for 20 minutes without ionophore stimulation resulted in LTB₄ synthesis of 68 (10-120) pg/mg wet weight for grade 2 biopsy specimens (median and IQR) (n=10), which was increased to 160 (87-280) pg/mg wet weight by subsequent stimulation with ionophore for 20 minutes (Table II).

There were similar increases for specimens graded 1 and 3. In time course experiments ionophore stimulated synthesis of LTB₄ was linear over 20 minutes (Fig 1). In experiments in which a further 20 minute stimulation period was also used (that is, 20-40 minutes), LTB₄ synthesis was reduced to 54% compared with the first 20 minute stimulation for grade 2 tissue specimens (p<0.01), and reduced to 79% compared with the first 20 minute stimulation for grade 3 specimens (p<0.05). Differences for grade 0 and grade 1 specimens were not significant, Table II.

Biopsy specimens of non-inflamed tissue (from patients with irritable bowel syndrome or those undergoing sigmoidoscopy for other reasons) which were histologically normal, synthesised 8.9 (3.7-15) pg/mg wet weight LTB₄ (n=9) on stimulation with ionophore over 20 minutes. This was similar to synthesis by un-inflamed ulcerative colitis tissue (10 pg/mg, IQR 1-0-10 pg/mg (n=4)) and those specimens with a sigmoidoscopic score of 1. Inflamed biopsy specimens from patients with a sigmoidoscopic score of 3 produced 15-fold higher levels (Table II). Ionophore stimulated LTB₄ synthesis correlated with the myeloperoxidase activity of the biopsy (R=0.8, p=0.01, n=21) (Fig 2).

BWA4C (10 μmol/l) markedly reduced basal LTB₄ synthesis by median (IQR) of 79% (73-94%) of control (n=6, p<0.01), while ionophore stimulated LTB₄ synthesis was inhibited by 96% (80-98%, n=6, p<0.01). Inhibition was selective for this 5-lipoxygenase product in that no significant suppression of the cyclo-oxygenase products thromboxane B₂ (TXB₂) or PGE₂ occurred (n=6). NDGA (10 μmol/l) inhibited basal LTB₄ synthesis by median (IQR) 69% (57-79%) (n=4, p=0.05), and inhibited ionophore stimulated synthesis by 87% (65-94%) (n=9, p<0.01). At this concentration of NDGA there was a 27% (14-40%) inhibition of TXB₂ synthesis (n=7, p<0.05) and 34% (19-54%) inhibition of PGE₂ synthesis (n=7, p=0.05).

When BWA4C (10 μmol/l) and indomethacin (10 μmol/l) were coincubated with the colonic tissue, there was a median 91% (87-96%) inhibition of LTB₄ formation (n=4, p=0.05), and a 76% (63-89%) inhibition of TXB₂ formation (n=4, p=0.06). In the presence of indomethacin (10 μmol/l) alone, there was a median 109% (14-350%) increase in LTB₄ (n=4, p=NS) and an 80% (37-92%) inhibition of TXB₂ (n=8, p=0.02).

Concentration response studies used a series of three biopsy specimens incubated with different concentrations of inhibitor. BWA4C (0.01-1 μmol/l) inhibited LTB₄ with an IC₅₀ of 0.06 μmol (n=5), BW755C (0.1-10 μmol/l) inhibited LTB₄ with an IC₉₀ of 10 μmol/l (n=2), and 5-ASA (0.01-1 mmol/l) inhibited with an IC₉₀ of 0.5 mmol/l (n=1). There was no significant inhibition of PGE₂ or TXB₂ synthesis by BWA4C at 0.01-1 μmol/l (n=5). In some supernatants, the formation of LTC₄ was also measured. The median (IQR) control levels of LTC₄, 680 (380-750) pg/mg wet weight were inhibited 52% (n=7, p<0.05) by BWA4C.

Most patients were regularly taking sulpha-
TABLE III  Ex vivo LTB4 synthesis (pg/mg wet weight), during 20 minutes of basal incubation and 20 minutes of ionophore stimulation, for colonic biopsies according to degree of inflammation assessed sigmoidoscopically, and whether patients were receiving prednisolone therapy.

<table>
<thead>
<tr>
<th>Patient treatment</th>
<th>Nil</th>
<th>Prednisolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1 Basal</td>
<td>13 (3-8-14)</td>
<td>9 (5-8-21)*</td>
</tr>
<tr>
<td>Stimulated</td>
<td>50 (10-61)</td>
<td>24 (7-6-25)**</td>
</tr>
<tr>
<td>n=8</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td>Grades 2-3 Basal</td>
<td>60 (7-7-100)</td>
<td>40 (14-51)**</td>
</tr>
<tr>
<td>Stimulated</td>
<td>190 (29-300)</td>
<td>160 (64-170)**</td>
</tr>
<tr>
<td>n=10</td>
<td>n=13</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 compared with biopsies from patients not on prednisolone (median (IQR)) dose 20 mg (10-30 mg), (Mann-Whitney U test).

salazin or mesalazin, and therefore their effect on ex vivo LTB4 synthesis could not be assessed. However, biopsy specimens from patients taking prednisolone for active disease (median (IQR) dose 20 mg (10-30 mg)), synthesised less LTB4 both in basal incubations and after ionophore stimulation compared with those with comparable inflammation seen at sigmoidoscopy who were not taking prednisolone (Table III).

DOSE RESPONSE STUDIES IN RESECTION TISSUE
Basal synthesis of LTB4 by resected tissue was mean (SEM) 23 (9.2) pg/mg wet weight over 20 minutes (n=9). Stimulation with ionophore (10 μmol/l) gave significantly higher levels (76 (26) pg/mg, p<0.02). In concentration response studies (shown in Fig 3), the IC50 for inhibition of LTB4 synthesis for BWA4C was 0.03 μmol/l, the IC50 for BW755C was >10 μmol/l and the IC50 for NDGA was 0.8 μmol/l. The basal synthesis of PGE2 and TXB2 (212 (49) pg/mg and 107 (31) pg/mg respectively over 20 minutes were not significantly affected by stimulation with ionophore (250 (68) and 130 (39) pg/mg respectively). BWA4C (0.01-1 μmol/l), BW755C (0.1-10 μmol/l), and NDGA (0.1-10 μmol/l) did not significantly reduce the levels of PGE2 and TXB2 in the supernatants (n=6).

Discussion
Ionophore stimulated LTB4 synthesis in colorectal biopsy specimens was significantly correlated with the myeloperoxidase values in the tissue, suggesting that LTB4 is largely produced by neutrophils, quantitatively the major source of myeloperoxidase in the colonic mucosa. Similar correlations between myeloperoxidase values and LTB4 synthesis have been shown for homogenates of colonic mucosa. Both basal and ionophore stimulated LTB4 synthesis were also correlated with the sigmoidoscopic grade of inflammation, reflecting vascularity of mucosa. Grade 3 biopsy specimens (showing erythematous inflamed mucosa with spontaneous bleeding at sigmoidoscopy) did not, however, produce significantly more LTB4 than grade 2 specimens (contact bleeding only), and this may be because specimens from grade 3 mucosa contained more necrotic tissue and debris than grade 2, resulting in less viable tissue able to synthesise lipoygenase products.

Although the rate of LTB4 synthesis was linear over the first 20 minutes of ionophore stimulation, the amount of LTB4 produced in a second 20 minute period of ionophore stimulation was less than that of the first. Prolonged exposure to ionophore is unphysiological, and reasons for the decrease could include exhaustion of substrate, alteration in the physiological milieu of the tissue fragments (for example, hypoxia or acidosis within the biopsies), or even build up of products leading to feedback inhibition of the lipoygenase pathway (for example, lipoxins or 15-HETE and 15-HPETE).

The LTB4 values in ionophore stimulated sigmoidoscopic grade 2 or 3 colorectal biopsy specimens by our method produced levels of LTB4 rather higher than those of Peskar et al, who reported levels of 3 pg/mg/20 minutes for active ulcerative colitis. These values are much closer to those produced by uninfamed or grade 1 specimens in our studies. The discrepancy may be attributable to the degree of inflammation of the biopsy specimens studied and also methodological differences. Thus, in our study, we were able to incubate samples within 10 minutes of being obtained at sigmoidoscopy. Synthesis of the most inflamed tissue (sigmoidoscopic grade 3) is similar to the levels reported to be formed by mucosal homogenates of tissue from active colitis (254 pg/mg).

The acetylhydroxamic acid BWA4C was a potent inhibitor of LTB4 synthesis in human colonic tissue in inflammatory bowel disease in our study. The methods using individual colorectal biopsy series, and the chopped resection tissue incubations gave comparable IC50 values for the inhibition of LTB4 synthesis, although the resection tissue method, using larger amounts of tissue, is likely to be more reliable. The IC50 values of BWA4C (0.03-0.06 μmol/l) are of the same order as values for human leukocyte homogenates (0.1 μmol/l). The complete inhibition of immunoreactive LTB4 by BWA4C implies de novo in vitro synthesis, and that the LTB4 is authentic. De novo synthesis is also supported by the linear synthesis over 20 minutes of ionophore stimulation.

BWA4C has been shown to be a powerful inhibitor of LTB4 synthesis in human leukocyte homogenates, whole blood ex vivo and in vitro.
and rat gastric tissue ex vivo, and also to reduce leukotriene dependent anaphylactic bronchoconstriction in anaesthetised guinea pigs. The observed inhibition of colonic LTC4 formation as well as LTB4 in the present study implies that the drug acts at or before the LTA4 synthetase step in the lipoxigenase pathway, as previously shown in rat gastric mucosa.

BWA4C was selective for the lipoxigenase pathway, and had no effect on cyclooxygenase products. The specific action of BWA4C was shown in the dual inhibitor experiments in which BWA4C alone inhibited LTB4 but had no effect on TXB2 synthesis, while a combination of BWA4C and indomethacin also significantly inhibited TXB2.

Biopsy specimens from patients taking prednisolone produced significantly less LTB4, when biopsies of the same degree of inflammation sigmoidoscopically were compared. This suggests that inhibition of LTB4 synthesis by prednisolone may be a primary action rather than secondary to a reduction in inflammation, and is in keeping with rectal dialysis studies showing a reduction in LTB4 levels within 72 hours of prednisolone administration. Despite most of the patients taking sulphasalazine or mesalazine at the time of biopsy, there were still high levels of LTB4 synthesis ex vivo. It is of note that preliminary clinical findings with the 5-lipoxygenase inhibitor A64077 (zileuton) showed a more pronounced effect in patients not taking sulphasalazine or mesalazine.

BWA4C was considerably more potent in vitro than BW755C or NDGA. If leukotrienes are important proinflammatory mediators in ulcerative colitis, then acetohydroxamic acids such as BWA4C could be therapeutically active anti-inflammatory agents. There are some animal data to support this proposition. In the TNB model of colitis in rats, it has been shown that the specific 5-lipoxygenase inhibitor L651,392 when administered intraperitoneally to rats before TNB, and then daily for four days, reduced colonic LTC4 synthesis, the colon damage score, and the colon wet weight. It should be noted that this drug also has antioxidant properties, which may also contribute to its overall biological effect. A similar effect was shown with MK886, an orally active 5-lipoxygenase inhibitor without antioxidant actions, and likewise, anti-inflammatory effects have been noted in rat TNB colitis, with pretreatment with enemas of 5-ASA, NDGA, or another lipoxygenase inhibitor, Revlon 5901.

However, treatment of established inflammation with the combined lipoxigenase/cyclooxygenase inhibitor BW755C in the same model does not reduce colonic LTC4 synthesis, despite inhibition of LTB4 synthesis. It may be that the balance of eicosanoids is relevant, and selective inhibition preferable. Preliminary clinical findings with the oral 5-lipoxygenase inhibitor A64077 has shown reduction in LTC4 synthesis measured by rectal dialysis, and some clinical benefit for patients with active colitis.

Further investigation of the potency and effects of 5-lipoxygenase inhibitors are therefore required in inflammatory bowel disease.
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