Biosynthesis of lipoxygenase and cyclo-oxygenase products from $^{14}$C-arachidonic acid by human colonic mucosa

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SUMMARY The human colon synthetises several prostanoids which may have a role in inflammatory bowel diseases. As lipoxygenase products of arachidonate metabolism have been implicated in inflammatory processes, we have now investigated the formation of both lipoxygenase and cyclo-oxygenase metabolites from $^{14}$C-arachidonic acid ($^{14}$C-AA) by human colonic tissue. Homogenates of human colonic mucosa were incubated with $^{14}$C-AA and after extraction into diethyl ether, separated by thin layer chromatography using two solvent systems that allowed resolution of cyclo-oxygenase and lipoxygenase products. The predominant cyclo-oxygenase products, as identified by their chromatographic mobility, were PGE$_2$$>$PGF$_{2\alpha}$$>$PGD$_2$$>$TXB$_2$$>$6-keto-PGF$_{1\alpha}$. The formation of these products was inhibited both by indomethacin (1–10 µM) and the dual pathway inhibitor, BW755C (1–30 µM). The predominant lipoxygenase products formed, which had the chromatographic mobility of 11-, 12-, 15-HETE (which ran together) were inhibited by BW755C (19 µM) but not by indomethacin (3 µM). Further resolution of this TLC band, performed using normal phase HPLC, indicated that both 12-HETE and 15-HETE were major lipoxygenase products formed by human colonic homogenate. The present findings indicate that human colonic tissue can convert $^{14}$C-AA into lipoxygenase products as well as cyclo-oxygenase products and support the suggestion that lipoxygenase products may have a role in inflammatory bowel disease.

Previous studies of arachidonic acid metabolism in human colon mucosa have concentrated on products of the cyclo-oxygenase (prostaglandin synthetase) pathway. The synthesis of prostanoids such as prostaglandin E$_2$ (PGE$_2$), PGF$_{2\alpha}$, prostacyclin, its breakdown product 6-keto PGF$_{1\alpha}$, and thromboxane B$_2$ (TXB$_2$) is increased during relapse in inflammatory bowel disease.¹⁻⁵ These compounds may play a role in the inflammation – for example, by enhancing vasodilatation and oedema formation.⁶⁻⁷ They have little or no effect, however, in activating human leucocytes: indeed, prostacyclin (PGI$_2$) in low concentrations has been shown to prevent polymorphonuclear leucocyte chemotaxis.⁸ Prostaglandin synthesis alone is therefore unlikely to lead to the accumulation of leucocytes during relapse in inflammatory bowel diseases. Indeed, specific potent inhibitors of prostaglandin synthesis such as indomethacin or flurbiprofen have not been shown to be of benefit in ulcerative colitis.⁹⁻¹¹

The metabolism of arachidonic acid via lipoxygenase enzymes has been recently described in leucocytes and platelets.⁷¹²⁻¹⁴ The products formed include the leukotrienes and 5-hydroxyeicosatetraenoic acid (5-HETE) from the 5-lipoxygenase pathway, and 12- and 15-HETE formed by the 12- and 15-lipoxygenase enzymes. In addition non-prostaglandin products of the cyclo-oxidase pathway such as 11-HETE and 12-hydroxy-heptadecatrienoic acid (HHT) can be formed. In contrast with prostaglandins, some of these lipoxygenase products can stimulate locomotion, lysosomal enzyme release, and superoxide production by human leucocytes.⁷¹²¹⁵⁻¹⁷ As the synthesis of lipoxygenase
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products by solid tissues such as skin, pericardium, pleura, and peritoneum have also been shown\textsuperscript{18,19} and because of the potential importance of such compounds in inflammatory bowel diseases, we have investigated whether human colonic mucosa also possesses the ability to metabolise radiolabelled arachidonic acid by lipoxygenase pathways.

**Methods**

**Patients**

Human colon was obtained at operation from five patients undergoing resection of carcinomas in the rectum, sigmoid, or descending colon and from two patients undergoing colectomy for ulcerative colitis. The colonic mucosa of the patients with ulcerative colitis had the histological appearances of severe inflammation. The mucosa from the other patients (control tissue) was taken >5 cm away from the tumour, and was histologically normal. Both patients with ulcerative colitis were untreated at the time of operation, while none of the patients was receiving any drug known to affect arachidonic acid metabolism.

Mucosal tissue (which had been stripped from the resected human colon and stored at \(-70^\circ \text{C}\)) was manually homogenised in 50 mM Tris (pH 8-0) using a 'Dual' ground glass homogeniser, to give a 50–85 mg/ml w/v final suspension. Aliquots (2 ml) were incubated for 20 minutes at 37\(^{\circ}\)C with 840 ng (160 nCi) of \([^{14}\text{C}]\)-arachidonic acid (\([^{14}\text{C}]\)-AA).

**Inhibition of \([^{14}\text{C}]\)-AA Metabolism**

Fresh stock solutions of indomethacin (200 \(\mu\)g/ml; 5.7×10\(^{-3}\)M dissolved in 50 mM Tris; pH 8-0) and BW755C (1 mg/ml; 3.8×10\(^{-3}\)M dissolved in distilled water) were diluted in 50 mM Tris (pH 8-0) and incubated with tissue suspension for 20 minutes (at \(0^\circ\)C) before incubation with \([^{14}\text{C}]\)-AA.

**Extraction and Thin Layer Chromatography**

After acidification to pH 3-5 with citric acid (2-3M), the products were extracted twice into two volumes of diethyl ether (total volume 8 ml). Extracted samples were dried under nitrogen, redissolved in 75 \(\mu\)l of chloroform:methanol (2:1) and applied to multi-lane thin layer chromatography plates (Whatman LK 5D). In each experiment duplicate thin layer chromatography plates were prepared and were developed using two separate solvent systems (Fig. 1).

**Solvent System ‘A’**

This consisted of ether:hexane:acetic acid (60:40:1, v/v/v). The system separates TXB\(_2\) and prostaglandins (which remain together on the origin) from the lipoxygenase products. The Raa values (chromatographic mobility of products with respect to arachidonic acid) were for leukotriene \(\text{B}_4\) (0-15), 5-HETE (0-44), HHT (0-63), and 11-, 12-, 15-HETE (which run together, 0-75).

**Solvent System ‘B’**

This was the organic phase of ethyl acetate: trimethyl pentane:water:acetic acid (110:50:100:20, v/v/v/v). The system facilitated the separation of the individual prostaglandin and TXB\(_2\). The Raa values were: 6-keto PGF\(_{1\alpha}\) (0-29), PGF\(_{2\alpha}\) (0-39), TXB\(_2\) (0-50), PGE\(_2\) (0-59), and PGD\(_2\) (0-73).

Radioactive products were labelled using autoradiography (three days' contact with Kodak NS2T
photographic film). The silica gel zone corresponding to each radioactive band was scraped off, and the radioactivity was determined by liquid scintillation counting (Packard Tricarb liquid scintillation counter). The dpm for each band was calculated and expressed as a percentage of the total dpm on the thin layer chromatography plate. After subtraction of boilded tissue blanks, the data were calculated as percentage $[^{14}C]$.AA conversion/100 mg mucosa and expressed as percentage conversion.

HPLC RESOLUTION OF THE 11-, 12-, 15-HETE BAND
Bulk incubation of homogenates of mucosa from control and inflamed colon with $[^{14}C]$.AA were performed as above, maintaining the same tissue/substrate ratio. The resulting 11-, 12-, 15-HETE band (resolved using solvent system ‘A’) was scraped from the thin layer chromatography plate and eluted with methanol (20 ml). The eluant was dried under nitrogen and the residue redissolved in 50 $\mu$l of solvent (see below) for injection into the high performance liquid chromatography (HPLC) column.

The dissolved residue was injected into a straight-phase Zorbx Sil Column (Dupont 4.6x250 mm) previously equilibrated (3 ml/min) with solvent (n-hexane:isopropyl alcohol:acetic acid, 992:7:1, v/v/v) and standardised using authentic mono HETEs (11-, 12-, and 15-). Elution of metabolites was monitored by ultraviolet absorbance (280 and 254 nm). Fractions were collected every 30 seconds and radioactivity measured. Authentic 15-HETE was added to extracted samples as an internal standard.

DRUGS AND CHROMATOGRAPHIC STANDARDS
The arachidonic acid metabolites were characterised by co-chromatography with the following standards: (1) Authentic standards of PGD$_2$, PGE$_2$, PGF$_{2 \alpha}$, TXB$_2$, and 6-keto PGF$_{1 \alpha}$ stored in ethanol at $-20^\circ$C. They were visualised on the thin layer chromatography plate by phosphomolybdic acid spray and heating to 100$^\circ$C. (2) $[^{14}C]$.AA cyclo-oxygenase and lipoxygenase metabolites formed by rabbit polymorphonuclear leucocytes$^{20}$ (Fig. 1) and radiolabelled TXB$_2$ formed by incubating human washed platelets with $[^{14}C]$.AA. (3) The lipoxygenase products 5-, 11-, 12-, and 15-HETE prepared in the Chemical Research Laboratory, Wellcome Research Laboratories, Beckenham, by oxygenation of arachidonic acid at room temperature.$^{15,21}$ The HETEs were prepared by reduction of the corresponding hydroperoxy derivatives with triphenyl phosphate followed by HPLC purification. The identity of the HETEs was confirmed by GC-MS of their methyl ester, trimethylsilyl ether derivatives. The products were quantified by ultraviolet spectrophotometry at 237 nm and stored in methanol (1 mg/ml) at $-20^\circ$C.

BW755C (3-amino-1[1-[trifluoromethyl]-phenyl]-2-pyrazoline), as the hydrochloride (mol wt 266), was synthesised by Drs F C Copp and C V Denyer of the Chemical Research Laboratories, Beckenham. Indomethacin (mol wt 358) was from Sigma Chemical Company and [1$^{14}$C].arachidonic acid (58 mCi/mmol stored at $-20^\circ$C in toluene) was from Amersham International Ltd. All solvents and chemicals were of analytical grade.

STATISTICAL ANALYSIS
Inhibition of the conversion of $[^{14}C]$.AA to radioactive products by indomethacin or BW775C was calculated as a percentage of its paired control and expressed as mean ± SEM of (n) values. Statistical significance was calculated using Student’s t test for paired data, where p<0.05 was considered significant.

Results
The homogenates of control human colonic mucosa converted 6.4±0.9% (mean ± SEM, n=5) of the added $[^{14}C]$.AA to radioactive products (Fig. 1). The predominant cyclo-oxygenase products formed, as characterised by their chromatographic mobility using solvent system ‘B’ (Fig. 2) were PGE$_2$>PGF$_{2 \alpha}$>PGD$_2$>TXB$_2$>6-keto PGF$_{1 \alpha}$.

The formation of these cyclo-oxygenase products was inhibited concentration-dependently by the cyclo-oxygenase inhibitor indomethacin (0.3–8 $\mu$M) and by the dual cyclo-oxygenase-lipoxygenase inhibitor$^{20,22}$ BW775C (1–38 $\mu$M). Concentrations of indomethacin (2.8 $\mu$M) and BW775C (19 $\mu$M) which were close to the IC$_{50}$ values (concentration causing 50% reduction) for inhibition of these cyclo-oxygenase products were chosen for further studies, and the results are shown in Table 1.

The predominant lipoxygenase products, characterised using solvent system ‘A’ (Fig. 3), had a chromatographic mobility corresponding with 11-, 12-, or 15-HETE (which ran together). Whereas indomethacin (2.8 $\mu$M) failed to reduce significantly the formation of these products, BW775C (19 $\mu$M) inhibited their production by 75±9%, n=5 (p<0.001; Table 1).

Smaller amounts of other products were also separated using solvent system ‘A’. These were (a) product II, a band with a chromatographic mobility (R$_{aa}$, 0.83) slightly greater than the 11-, 12-, 15-HETE band, whose production was inhibited by BW775C (19 $\mu$M) but not by indomethacin (2.8-
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Fig. 2 Formation of radiolabelled cyclo-oxygenase metabolites of [14C]-arachidonic acid by homogenates of human colonic mucosa. Levels of prostanoids, separated on TLC using solvent system ‘B’, are expressed as percentage conversion of [14C]-AA. The results are shown as mean ± SEM of five experiments using control tissue.

Table 1 Inhibition of [14C]-arachidonic acid metabolism in homogenate of human colonic mucosa by BW755C and indomethacin

<table>
<thead>
<tr>
<th>TLC band</th>
<th>BW755C (19 µM)</th>
<th>Indomethacin (3 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product 11</td>
<td>56±13*</td>
<td>11±28</td>
</tr>
<tr>
<td>11-, 12-, 15-HETE</td>
<td>75±9†</td>
<td>30±17</td>
</tr>
<tr>
<td>HHT</td>
<td>54±13*</td>
<td>66±6†</td>
</tr>
<tr>
<td>‘5-HETE’</td>
<td>89±3‡</td>
<td>64±2‡</td>
</tr>
<tr>
<td>Product VI + ‘LTB4’</td>
<td>76±16*</td>
<td>70±9†</td>
</tr>
<tr>
<td>PGs + TXB2</td>
<td>55±18*</td>
<td>64±10‡</td>
</tr>
</tbody>
</table>

Results, shown as % inhibition of [14C]-AA conversion to various products separated on TLC, are mean ± SEM of five experiments using control tissue.

* p<0.05  † p<0.01  ‡ p<0.001.

μM as shown in Table 1; (b) a product corresponding to HHT (a cyclo-oxygenase product) whose synthesis was inhibited by both indomethacin and BW775C; (c) a product corresponding to 5-HETE which was greatly reduced by both inhibitors, although the inhibition by BW775C was significantly greater (p<0.01; Table 1); (d) two other products, one with the chromatographic mobility of leukotriene B4 (LTB4) and another of novel mobility (product VI, Raa=0.31). When scraped and pooled together, these products comprised 12±3% of total products formed (n=5) and were inhibited by either indomethacin or BW775C (Table 1). In two experiments in which the two products were scraped separately they made up 8% and 4% of the total products formed, respectively.

CONVERSION OF [14C]-AA BY INFLAMED MUCOSA

A similar profile of products, separated on thin layer chromatography, were synthetised by the inflamed
tissue from the two patients with ulcerative colitis and that from control tissue from the five patients with colonic carcinomas. There appeared to be greater conversion of \(^{14}\text{C}\)-AA, however, by the inflamed mucosa both to the products separated using solvent system 'A' and to the cyclo-oxygenase products separated using solvent system 'B' (Table 2).

**HPLC of the 11-, 12-, 15-HETE Band**

The radioactivity co-chromatographing with the 11-, 12-, 15-HETE thin layer chromatography band was further resolved using HPLC techniques (Fig. 4). The predominant product in two homogenates of control colonic tissue was 12-HETE, with only small amount of 15-HETE, whereas in two other control tissues, both 12-HETE and 15-HETE could be detected. Overall, 12-HETE comprised 40±10% and 15-HETE comprised 24±10% \((n=4)\) of the total radioactivity in this thin layer chromatography band. Both 12-HETE and 15-HETE were formed in approximately equal quantities in homogenates of inflamed mucosa from a patient with ulcerative colitis (Fig. 4). Only very small quantities of 11-HETE were formed by these tissues, and although a number of other products were also resolved by HPLC, their identity requires further clarification.

**Discussion**

The present data show that arachidonate products of the lipoxygenase metabolic pathway can be formed by homogenates of human colonic mucosa. The products have been characterised both by their chromatographic mobility, using thin layer chromatography and HPLC, and by their selective inhibition with BW755C. In contrast with non-steroid anti-inflammatory drugs such as indomethacin which inhibit only the cyclo-oxygenase pathway, BW755C also inhibits the lipoxygenase pathway\(^{20,22}\) and thus can be used as a pharmacological tool to study these pathways. The predominant arachidonic acid metabolites separated by thin layer chromatography had the chromatographic mobilities of PGE\(_2\), PGF\(_{2\alpha}\), and 11-, 12-, 15-HETE. The synthesis of PGE\(_2\) and PGF\(_{2\alpha}\) as major cyclo-oxygenase products of arachidonic acid metabolism in human colon has previously been shown in both cultured biopsies and homogenates of human colon.\(^2-5\) In our studies, separation of the 11-, 12-, 15-HETE band by HPLC indicated that 12-HETE was the major identifiable mono-HETE formed by uninfamed colonic mucosa. This supports the recent finding of Bennett *et al*\(^{22}\) who, using GC-MS techniques, identified a product formed by normal human colonic mucosa.

**Table 2** Comparison of the profile of radiolabelled metabolites from \(^{14}\text{C}\)-arachidonic acid, separated on TLC using solvent system 'A' and 'B' formed by homogenates of human colonic mucosa from control tissue \((n=5)\) and from inflamed tissue from patients with ulcerative colitis \((n=2)\). Results shown as percentage conversion, are the mean of \((n)\) experiments.

<table>
<thead>
<tr>
<th>TLC band</th>
<th>Uninflamed</th>
<th>Inflamed</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system 'A'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.48</td>
<td>0.91</td>
<td>1.9</td>
</tr>
<tr>
<td>11-, 12-, 15-HETE</td>
<td>2.25</td>
<td>6.38</td>
<td>2.8</td>
</tr>
<tr>
<td>HHT</td>
<td>0.48</td>
<td>1.41</td>
<td>2.7</td>
</tr>
<tr>
<td>5-HETE</td>
<td>0.21</td>
<td>0.59</td>
<td>2.8</td>
</tr>
<tr>
<td>VI, VII</td>
<td>0.76</td>
<td>2.35</td>
<td>3.1</td>
</tr>
<tr>
<td>PGs + TXB(_2)</td>
<td>2.24</td>
<td>6.24</td>
<td>2.8</td>
</tr>
<tr>
<td>Total</td>
<td>6.4</td>
<td>17.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Solvent system 'B'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGD(_2)</td>
<td>0.42</td>
<td>0.87</td>
<td>2.1</td>
</tr>
<tr>
<td>PGE(_2)</td>
<td>0.85</td>
<td>2.31</td>
<td>2.7</td>
</tr>
<tr>
<td>TXB(_2)</td>
<td>0.23</td>
<td>0.92</td>
<td>4.0</td>
</tr>
<tr>
<td>PGF(_{2\alpha})</td>
<td>0.71</td>
<td>2.18</td>
<td>3.1</td>
</tr>
<tr>
<td>6-keto PGF(_{1\alpha})</td>
<td>0.14</td>
<td>0.33</td>
<td>2.4</td>
</tr>
<tr>
<td>Total</td>
<td>2.3</td>
<td>6.6</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Fig. 4** Resolution of major lipoxygenase products from \(^{14}\text{C}\)-arachidonic acid metabolism by homogenates of human colonic mucosa using HPLC technique. Results show products from control tissue and from inflamed tissue of patient with colitis. The position of authentic 12-HETE, 15-HETE, and 11-HETE are shown by arrows, whereas hatched bar represents 15-HETE internal standard.
from endogenous precursor corresponding to 12-HETE, although they did not quantify its production. The detection of 12-HETE by mucosal homogenates in both studies, as with TXB₂, may reflect formation by entrapped platelets, which are known to convert efficiently arachidonic acid to this lipoxygenase product as well as to TXB₂, but their formation by other cell types from the colonic mucosa cannot be excluded.

The production of 15-HETE by human colonic mucosa is a novel finding. As patients with ulcerative colitis rarely come to operation while untreated, only a single HPLC experiment could be performed on the products formed by inflamed tissue. Thus any conclusions concerning the differences between inflamed and control mucosa are necessarily limited. The 15-HETE found in homogenates of the inflamed and uninfamed mucosa may reflect lipoxygenase activity derived from leucocytes which have infiltrated the mucosa. Although the major lipoxygenase product generated by intact rabbit neutrophils is 5-HETE, in broken-cell preparations the predominant product formed is 15-HETE. Furthermore, 15-HETE is the major mono-HETE formed in intact human polymorphonuclear leucocytes incubated with arachidonic acid and in an enriched fraction of human eosinophils, as well as in rabbit peritoneal tissue and human lung.

The increased capacity of inflamed colonic mucosa to form prostaglandins and thromboxane has been shown previously. We have now confirmed these observations, using the conversion of [¹⁴C]-AA as an index of cyclo-oxygenase activity, but in addition an increase in the formation of radiolabelled lipoxygenase products was also observed. Whether this increase is entirely due to the greater number of inflammatory cells in the inflamed mucosa, or to an increase in the capacity of other cells in the mucosa to metabolise arachidonic acid requires further investigation.

We have not yet been able to establish whether synthesis of 5-HETE or LTB₄ (5, 12-di-HETE) can occur in human colonic mucosa as the bands corresponding to these products were inhibited by indomethacin as well as by BW755C. As the 5-HETE band was inhibited less by indomethacin, however, than by BW755C, this band may consist of a mixture of both lipoxygenase and cyclo-oxygenase products. There were somewhat smaller amounts of another unidentified metabolite (product II) which also appeared to be a lipoxygenase product as its formation was inhibited by BW755C but not by indomethacin. Although our findings on arachidonate metabolism by broken-cell preparations of human colonic mucosa may give limited information about its metabolism in intact mucosa in situ, such studies form a necessary basis for further investigation.

The synthesis of lipoxygenase products by human colonic mucosa has implications for the pathogenesis and treatment of human inflammatory bowel diseases. Sulphasalazine and 5-amino salicylic acid inhibit synthesis of 5,12-di-HETE (LTB₄) by human isolated neutrophils stimulated with calcium ionophore. In our experiments, these products did not appear to be major products of arachidonic acid metabolism in human colonic mucosa although it is possible that this is the result of using a broken cell preparation, a procedure required to enable more efficient conversion of [¹⁴C]-AA. The effect of sulphasalazine and its degradation products on the synthesis of 12- and 15-lipoxygenase products is present under investigation.

Both 12-HETE and 15-HETE have chemotactic or chemokinetic properties and induce secretion of specific granule contents from human polymorphonuclear leucocytes although they are less potent than 5-HETE and 5,12-di-HETE. Furthermore, recent studies indicate that 15-HETE can activate leukotriene biosynthesis in a mast cell line. These products therefore have the potential to contribute to inflammatory processes in the human bowel. We have shown that indomethacin, which appears to be of no value as an anti-inflammatory agent in ulcerative colitis, does not inhibit synthesis of the lipoxygenase product by human colonic mucosa, whereas BW775C does. It will therefore be important to compare the potency of sulphasalazine and its active constituents as inhibitors of the lipoxygenase pathways found in human colonic mucosa and to consider whether dual cyclo-oxygenase/lipoxygenase pathway inhibitors might be of value as anti-inflammatory agents in ulcerative colitis and other inflammatory conditions of the gastrointestinal tract.

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