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**Research Article**

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# Sulfasalazine Inhibits the Synthesis of Chemotactic Lipids by Neutrophils

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**ABSTRACT** Neutrophils metabolize arachidonic acid through the lipoxygenase pathway to 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,12 diHETE). 5-HETE and 5,12 diHETE are potent chemotactic agents and are thought to have important roles in the inflammatory response. In this study we demonstrate that sulfasalazine, at concentrations found in the stool of patients being treated for ulcerative colitis, blocks the synthesis of both 5-HETE and 5,12 diHETE by human neutrophils. A sulfasalazine metabolite, 5-aminosalicylate, also blocks the synthesis of 5,12 diHETE.

## INTRODUCTION

Sulfasalazine has been used effectively in the treatment of ulcerative colitis but its mechanism of action is unknown. Prostaglandins are important mediators of inflammation; elevated levels of prostaglandins have been found in the stool and blood of patients with ulcerative colitis and elevated levels of prostaglandin metabolites have been found in their urine (1). Organ cultures of rectal biopsies from these patients secrete increased amounts of prostaglandins (2). Treatment of ulcerative colitis patients with sulfasalazine results in decreased levels of prostaglandins in the stool and blood, and decreased levels of prostaglandin metabolites in the urine (1). These findings have led to the hypothesis that prostaglandins are major mediators of inflammation in ulcerative colitis and that sulfasalazine reduces inflammation by blocking prostaglandin synthesis (1, 2). A major argument against this hypothesis is that other drugs that inhibit prostaglandin synthesis are not useful in the treatment of ulcerative

colitis. Indomethacin, for example, blocks cyclooxygenase and reduces prostaglandin levels in the stool of patients with ulcerative colitis and also reduces the levels of prostaglandin metabolites in their urine; but indomethacin is totally ineffective (1).

The pathology of ulcerative colitis is remarkable for the accumulation of large numbers of neutrophils and other inflammatory cells in the colonic mucosa. Neutrophils metabolize arachidonic acid to prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)<sup>1</sup> and thromboxane B<sub>2</sub> through the cyclooxygenase pathway, but their major arachidonate metabolites are the lipoxygenase products 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,12 diHETE) also known as leukotriene B<sub>4</sub> (3, 4). 5-HETE and 5,12 diHETE are chemotactic agents; they are thought to have an important role in recruiting neutrophils into areas of inflammation (5, 6). Neutrophils metabolize arachidonate through the lipoxygenase pathway when stimulated with the ionophore A23187, or the chemotactic peptide formylmethionylleucylphenylalanine (3, 7). We have used ionophore-stimulated neutrophils as a test system because ionophore is the most potent stimulus of the lipoxygenase pathway in neutrophils. In this study we demonstrate that sulfasalazine and one of its metabolites, 5-aminosalicylate, block the synthesis of 5-HETE and 5,12 diHETE by human neutrophils. We suggest that the efficacy of sulfasalazine in the treatment of ulcerative colitis may relate to the inhibition of the synthesis of these chemotactic compounds.

## METHODS

*Purification of cells.* Healthy volunteer blood donors gave written consent to participate in this study, which was

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<sup>1</sup>*Abbreviations used in this paper:* 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5,12 diHETE, 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; TLC, thin-layer chromatography.

approved by the Washington University Human Studies Committee. Purified neutrophils were prepared from heparinized blood by dextran sedimentation followed by isopycnic centrifugation using a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, N. J.). Blood donors had taken no medication for at least 2 wk. Neutrophil preparations were monitored by phase microscopy and Giemsa staining and contained less than one platelet per neutrophil. More than 98% of the nucleated cells were neutrophils.

**Incubation conditions.** Neutrophils were suspended in buffer (NaCl 100 mM, Tris HCl 50 mM pH 7.4, glucose 1 mg/ml, CaCl<sub>2</sub> 1 mM) and incubated in the presence or absence of inhibitors for 10 min at 37° in a volume of 1 ml and at a cell concentration of  $1 \times 10^7$ /ml. After 10 min of

incubation [<sup>14</sup>C]arachidonic acid (10 μM) and the ionophore A23187 (1 μM) were added and the incubation continued for an additional 5 min. The incubation was terminated and the lipids extracted by the addition of 2.5 vol of ice-cold chloroform:methanol:1% formic acid (1.2:1.2:0.1).

**Identification of products.** The metabolites of [<sup>14</sup>C]arachidonic acid were separated by thin-layer chromatography (TLC) and identified by autoradiography and comigration with known standards. The TLC solvent system used was benzene/ether/ethanol/acetic acid (50:40:2:0.2). The radioactive areas on the TLC plates detected by autoradiography were scraped, and the radioactivity measured in a liquid scintillation counter. The results are expressed as percentage of total recovered radioactivity found in each product. Percent inhibition is defined as:

$$1 - \frac{\% \text{ total radioactivity in a product in the presence of the inhibitor}}{\% \text{ total radioactivity in a product in the absence of the inhibitor}} \times 100\%.$$

**Reagents.** Sulfasalazine, sulfapyridine, and 5-aminosalicylate were gifts of Pharmacia, Inc. Sulfapyridine and sulfasalazine were dissolved in small volumes of 0.1 N NaOH, diluted with buffer and the pH adjusted to 7.4. 5-Aminosalicylate was dissolved directly in buffer. A23187, Calbiochem-Behring Corp., Div. American Hoechst Corp., La Jolla, Calif.; [<sup>14</sup>C]arachidonate (56 mCi/mmol), Amersham Corp., Arlington Heights, Ill. Standards for 5-HETE and 5,12 diHETE were biosynthesized and identified by TLC, high-pressure liquid chromatography, ultraviolet absorbance, and gas chromatography-mass spectroscopy as described previously (3, 8).

## RESULTS

Human peripheral blood neutrophils stimulated with the ionophore A23187 metabolize exogenous arachidonate through the lipoxygenase pathway to 5-HETE (4.0%) and 5,12 diHETE (6.4%) (Table I). A significant portion of the exogenous arachidonate is esterified into phospholipids (20.6%) and triglycerides (19.0%).

Sulfasalazine inhibits the synthesis of 5-HETE and 5,12 diHETE in a similar fashion. The 50% inhibitory dose (ID<sub>50</sub>) for the inhibition of 5,12 diHETE synthesis is 0.9 mM, for 5-HETE it is 1.5 mM (Fig. 1) At sulfasalazine concentrations >4 mM there is almost 100% inhibition of synthesis of both products. 5-Aminosalicylate inhibits 5,12 diHETE with an ID<sub>50</sub> of 4 mM, although inhibition was not complete even at concentrations as high as 16 mM. 5-Aminosalicylate was ineffective in blocking 5-HETE production. Sulfapyridine caused partial inhibition of the synthesis of both compounds, reaching a maximum of 30% inhibition at 4–6 mM. There was no cytotoxicity, as determined by trypan blue exclusion, in any of the conditions tested. Using the same test system indomethacin at concentrations up to 50 μM did not inhibit the synthesis of 5-HETE or 5,12 diHETE (data not shown).

## DISCUSSION

In this study we have demonstrated that sulfasalazine inhibits the synthesis of 5-HETE and 5,12 diHETE by human neutrophils. The sulfasalazine metabolite 5-aminosalicylate also inhibits the synthesis of 5,12 diHETE. The ID<sub>50</sub> for sulfasalazine is 0.9–1.5 mM (36–60 mg/100 ml), which is less than the average concentration of sulfasalazine in the stool of treated patients (78 mg/100 ml) (9). The ID<sub>50</sub> for the inhibition of 5,12 diHETE synthesis by 5-aminosalicylate is 5 mM (75 mg/100 ml), which is also less than the average concentration in the stool (150 mg/100 ml) (9). Sulfapyridine was least effective in inhibiting the synthesis of these compounds. The lesser inhibitory activ-

TABLE I  
Metabolism of Exogenous [<sup>14</sup>C]Arachidonic Acid  
by Human Neutrophils

	Percentage of total recovered radioactivity in each product
Unmetabolized arachidonate	50.0±2.1
Triglycerides	19.0±1.3
5-HETE	4.0±0.8
5,12-diHETE	6.4±1.0
Phospholipids and PGE <sub>2</sub> *	20.6±1.4

$1 \times 10^7$  neutrophils were incubated at 37°C for 5 min with [<sup>14</sup>C]arachidonic acid (10 μM) and A23187 (2 μM). Lipids were extracted and separated by TLC (see Methods). Mean±SEM for three experiments.

\* PGE<sub>2</sub> is a relatively minor metabolite of arachidonate in neutrophils. It is not separated from phospholipid by the solvent system used. Phospholipids account for almost all of the radioactivity in this band (4).

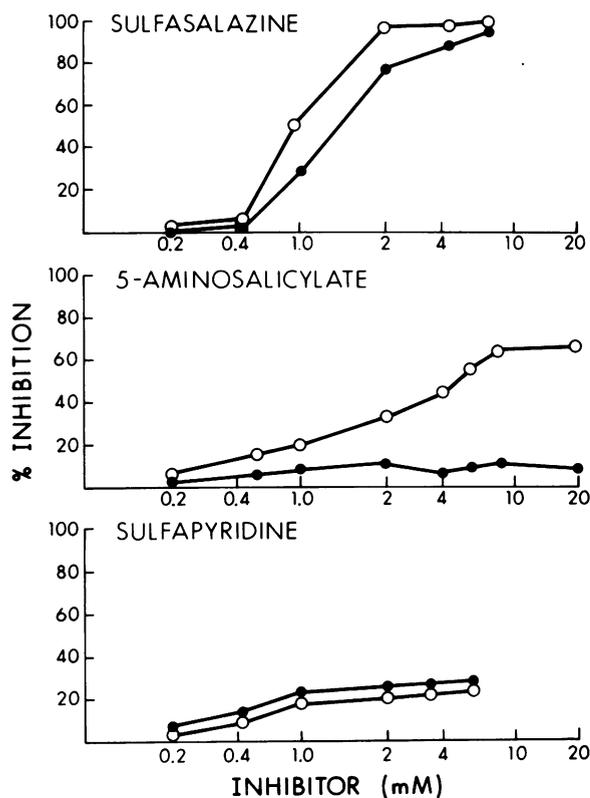


FIGURE 1 Effects of inhibitors on the synthesis of 5-HETE and 5,12 diHETE in the ionophore stimulated neutrophils. Neutrophils ( $1 \times 10^7$ /ml) were incubated at  $37^\circ$  for 10 min with or without inhibitors. [ $^{14}\text{C}$ ]arachidonate ( $10 \mu\text{M}$ ) and A23187 ( $2 \mu\text{M}$ ) were added and the incubation continued for an additional 5 min. Extraction of lipids, quantitation of 5-HETE and 5,12 diHETE, and calculation of inhibition are as described in Methods. Results are the mean of three similar experiments. ●, 5-HETE, ○, 5,12 diHETE.

ity of sulfapyridine combined with substantially lower stool concentrations (48 mg/100 ml) (9), make it unlikely that sulfapyridine is a pharmacologically important inhibitor of this pathway. Previous studies designed to determine the therapeutic component of sulfasalazine revealed that patients treated with either sulfasalazine or 5-aminosalicylate alone improved, while those treated with sulfapyridine did not (10).

The precise functions of 5-HETE and 5,12 diHETE in inflammation are not entirely defined, although their major role appears to be chemotaxis. 5,12 diHETE is a much more potent chemotactic agent than 5-HETE; 5,12 diHETE reaches a half-maximal chemotactic response for neutrophils at a concentration of 6 nM, while the half maximum concentration of 5-HETE is 400 nM (11).

Until recently salicylates and other nonsteroidal anti-inflammatory drugs were thought to exert their anti-

inflammatory effects solely by blocking cyclooxygenase and inhibiting prostaglandin synthesis. Siegel et al. (12, 13) have demonstrated that these agents may also affect the lipoxygenase pathway, although their studies did not deal specifically with 5-lipoxygenase or human neutrophils. In the present study indomethacin did not block the synthesis of lipoxygenase products by human neutrophils. The study presented here is the first to demonstrate inhibition of the lipoxygenase pathway by sulfasalazine or its metabolites. The data do not define the point at which the lipoxygenase pathway is inhibited. The similar inhibition of 5-HETE and 5,12 diHETE synthesis by sulfasalazine is consistent with inhibition at the level of the lipoxygenase, while the selective inhibition of 5,12 diHETE synthesis by 5-aminosalicylate suggests inhibition of leukotriene A synthetase. This pathway is yet to be characterized in homogenized cells; precise characterization of the site of action of these compounds will require studies in a cell-free system.

Sulfasalazine is not well absorbed from the gut. Bacteria in the colon split sulfasalazine into 5-aminosalicylate and sulfapyridine. Most of the sulfapyridine is absorbed and excreted in the urine, while most of the 5-aminosalicylate remains in the stool. The concentrations of sulfasalazine and 5-aminosalicylate in the stool are very high while the concentrations in the blood are much lower. The concentrations of these compounds in the colonic mucosa of patients with ulcerative colitis are unknown and thus the concentrations that mucosal neutrophils are exposed to is also unknown. The mucosal concentrations must be considerable, however, because these agents markedly diminish the levels of prostaglandin metabolites in the urine of these patients (1).

In this study we have shown that sulfasalazine and its metabolite, 5-aminosalicylate, block the synthesis of products of the lipoxygenase pathway, products that have important roles in recruiting inflammatory cells into sites of inflammation. We suggest that the inhibition of the synthesis of 5-HETE and 5,12 diHETE accounts for some of the antiinflammatory effects of sulfasalazine seen in ulcerative colitis.

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