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INHIBITION OF PLATELET THROMBOXANE SYNTHETASE BY SULFASALAZINE

WILLIAM F. STENSON* and ELIZABETH LOBOS
Division of Gastroenterology, Jewish Hospital of St. Louis and Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

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Abstract—Sulfasalazine is a potent antiinflammatory drug used in the treatment of ulcerative colitis. The mechanism of action of sulfasalazine is unknown but a recent study [W. F. Stenson and E. Lobos, *J. clin. Invest.* **69**, 494 (1982)] demonstrated that sulfasalazine, at therapeutic concentrations, blocks human neutrophil lipoxygenase, suggesting that its antiinflammatory effects may be mediated in part by the inhibition of the synthesis of the chemotactic lipids 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and leukotriene B_4 (LTB_4). In the present study the effect of sulfasalazine on metabolism of exogenous arachidonic acid by human platelets was investigated. Sulfasalazine inhibited platelet thromboxane synthetase (IC_{50} = 0.9 mM) and partially inhibited cyclooxygenase. A methylated analog of sulfasalazine also inhibited thromboxane synthetase (IC_{50} = 0.3 mM) and partially inhibited cyclooxygenase. Neither of the cleavage products of sulfasalazine (5-aminosalicylate and sulfapyridine) inhibited thromboxane synthetase although 5-aminosalicylate blocked cyclooxygenase (IC_{50} = 5 mM). Neither sulfasalazine nor the methylated analog nor the cleavage products inhibited platelet lipoxygenase. This is in contrast to the inhibitory effects of sulfasalazine on neutrophil 5-lipoxygenase. The concentration of sulfasalazine in the colons of treated patients is several-fold greater than the IC_{50} for thromboxane synthetase.

Fatty acid cyclooxygenase and n − 8 lipoxygenase are the initial enzymatic steps in the two pathways of arachidonic acid metabolism in the human platelet. The sole known product of the lipoxygenase pathway in platelets is 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) which is reduced to 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). The major products of the cyclooxygenase pathway in platelets are thromboxane B_2 (TxB_2) and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT). Prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ are also formed but in much smaller amounts. The production of HHT and TxB_2 can be selectively blocked by inhibition of thromboxane synthetase [1, 2]. Aspirin and other non-steroidal antiinflammatory drugs have been found to inhibit cyclooxygenase [3] and under certain circumstances the lipoxygenase pathway [4].

Sulfasalazine is an antiinflammatory agent used in the treatment of ulcerative colitis. Bacteria in the colon split sulfasalazine into 5-aminosalicylate and sulfapyridine. Most of the sulfapyridine is absorbed but both sulfasalazine and 5-aminosalicylate are poorly absorbed. In patients treated with sulfasalazine, the concentrations of sulfasalazine and 5-aminosalicylate in the stool are 2 and 10 mM, respectively [5], while the blood concentrations are much lower; the peak serum concentration of sulfasalazine after a 2 g oral dose has been reported to be 50 μM [6] while the serum concentration of 5-aminosalicylate is below the limits of detection.

The mechanism of action of sulfasalazine is known. Several studies have suggested that its therapeutic activity is related to its effects on arachidonic acid metabolism. Increased levels of prostaglandins and prostaglandin metabolites have been found in the stool, serum and urine of patients with inflammatory bowel disease [7]. Treatment with sulfasalazine results in a decrease in the levels of these products. Similarly increased amounts of PGE_2, prostacyclin and TxB_2 are secreted by colonic mucosal explants from patients with inflammatory bowel disease [8]. Incubation of these explants *in vitro* with sulfasalazine and other antiinflammatory drugs diminishes the synthesis of arachidonate metabolites. Other studies have revealed that sulfasalazine inhibits the enzymes that metabolize prostaglandins [9]. This effect would increase rather than decrease prostaglandin levels.

We recently reported that sulfasalazine, at concentrations found in the colons of treated individuals, inhibits neutrophil 5-lipoxygenase *in vitro* and thus diminishes the synthesis of 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and leukotriene B_4 (LTB_4) [10]. 5-HETE and LTB_4 are potent chemotactic compounds thought to be important in the amplification and modulation of the inflammatory response.

In the present study, we have examined the effects of sulfasalazine in the metabolism of arachidonic acid in platelets. We chose to study arachidonate metabolism in platelets for several reasons: (1) to determine whether the inhibitory effects of sulfasalazine on neutrophil 5-lipoxygenase extend to platelet n − 8 lipoxygenase, (2) to determine the effects of sulfasalazine on thromboxane synthetase in view of the report of increased levels of TxB_2 production by mucosal explants from inflammatory

* Reprint requests should be sent to: William F. Stenson, M.D., Division of Gastroenterology, Box 8124, Washington University School of Medicine, 660 South Euclid, St. Louis, MO 63110, U.S.A.

bowel disease, and (3) to define the effects of sulfasalazine and 5-aminosalicylate on cyclooxygenase. Although both of these compounds have been reported to inhibit prostaglandin synthesis, they have never before been studied in a well-defined population of homogeneous cells where their effects on cyclooxygenase could be separated from effects on other enzymes involved in arachidonic acid metabolism.

In this study we have demonstrated that sulfasalazine and methylsulfasalazine (2-hydroxy-5-[[4-(3-methyl-2-pyridinyl amino) sulfonyl phenyl]azo]-benzoic acid) inhibited thromboxane synthetase and partially inhibited cyclooxygenase. 5-Aminosalicylate inhibited cyclooxygenase but not thromboxane synthetase. Neither sulfasalazine nor 5-aminosalicylate inhibited platelet n − 8 lipoxygenase.

MATERIALS AND METHODS

Materials. Sulfasalazine, methylsulfasalazine, 5-aminosalicylate, and sulfapyridine were gifts of Pharmacia AB, Uppsala, Sweden. Sulfasalazine, methylsulfasalazine, and sulfapyridine were dissolved in 0.1 N NaOH and then diluted in buffer and the pH adjusted to 7.0. 5-Aminosalicylate was dissolved directly in buffer. [1-^{14}C]Arachidonic acid (58 mCi/mmole) was from Amersham, Arlington Heights, IL. [1-^{14}C]PGH_2 was a gift of Dr. Phillip Needleman of the Department of Pharmacology, Washington University.

Metabolism of [^{14}C]*arachidonic acid.* Blood was obtained from human volunteers, and washed platelet suspensions were prepared as described by Baenziger and Majerus [11]. Platelets (2×10^8/ml) were suspended in buffer (Tris–HCl) 50 mM (pH 7.0); NaCl, 100 mM; glucose, 1 mg/ml) and incubated with or without inhibitors in a volume of 1 ml for 1 min; [1-^{14}C]arachidonic acid (5 μM) was added, and the incubation was continued for 5 min. The reaction was stopped, and the lipids were extracted by the addition of 2.5 vol. of ice-cold chloroform–methanol–1% formic acid (1.2:1.2:0.1). The extracted lipids were applied to silica gel G TLC plates and developed in solvent system I (benzene–ether–ethanol–acetic acid, 50:40:2:0.2) and in solvent system II (benzene–dioxane–acetic acid, 60:30:3) [12]. Solvent system I separates arachidonic acid ($R_f = 0.88$), 12-HETE ($R_f = 0.64$) and HHT ($R_f = 0.59$). Solvent system II separates thromboxane B_2 ($R_f = 0.47$), PGE_2 ($R_f = 0.37$) and $PGF_{2\alpha}$ ($R_f = 0.24$). The identities of the HHT and 12-HETE bands were confirmed by gas chromatography–mass spectroscopy. The mass spectrum of the methyl ester trimethylsilyl derivative of HHT showed prominent ions (*m/e*) at 351, 335, 295, 276, and 225, while the methyl ester trimethylsilyl ether derivative of 12-HETE showed prominent ions (*m/e*) at 391, 375, 335, and 295. These data are in agreement with published spectra [13]. The radioactive areas on the TLC plates were identified by autoradiography and comigration with authentic standards. The radioactive zones were scraped, eluted, and counted in a liquid scintillation counter.

Metabolism of [1-^{14}C]PGH_2. Platelets (1×10^8/ml) were incubated in buffer in the presence and absence of inhibitors for 5 min at 37°. [1-^{14}C]PGH_2 (50,000 cpm, sp. act. 58.4 mCi/mmole) was added to the incubation mixture, and the incubation was continued for 10 min. The final incubation volume was 0.4 ml. Lipids were extracted and separated by thin-layer chromatography with solvent system II.

Metabolism of [^{14}C]*arachidonic acid in the presence of benzylimidazole.* In some experiments, platelets were treated with benzylimidazole to block thromboxane synthetase. Platelets (2×10^8/ml) were incubated with benzylimidazole (100 μM) for 5 min at 37°, then buffer or sulfasalazine or methylsulfasalazine was added to the incubation mixture, and the incubation was continued for 5 min. [^{14}C]Arachidonic acid (5 μM) was then added, and the incubation was continued for an additional 5 min. Lipids were extracted and separated, and the radiolabeled compounds were identified as described above.

RESULTS

Table 1 shows the distribution of radioactivity after incubation of [^{14}C]arachidonic acid (5 μM) with human platelets for 5 min. HHT, TxB_2, and 12-HETE were the only metabolites made in significant quantities. Figure 1 shows the effects of sulfasalazine, methylsulfasalazine, 5-aminosalicylate and sulfapyridine on the metabolism of exogenous arachidonic acid in platelets. Sulfasalazine inhibited the synthesis of thromboxane B_2 with an IC_{50} of 0.9 mM. As the production of thromboxane B_2 and HHT progressively declined, the production of PGE_2 and $PGF_{2\alpha}$ increased. This pattern is consistent with inhibition of thromboxane synthetase and the diversion of PGH_2 into the synthesis of PGE_2 and $PGF_{2\alpha}$ [2]. There was also an increase in the synthesis of 12-HETE, consistent with diversion of arachidonic acid away from cyclooxygenase and into lipoxygenase. Methylsulfasalazine had the same effects as sulfasalazine but with a lower IC_{50} (0.3 mM) for thromboxane B_2 synthesis.

5-Aminosalicylate blocked platelet cyclooxygenase with an IC_{50} of 5 mM. There was parallel inhibition of thromboxane B_2 and HHT. As the concentration of 5-aminosalicylate was increased, there was an

Table 1. Metabolism of exogenous [^{14}C]arachidonic acid by human platelets*

	% of Total recovered radioactivity
Unmetabolized arachidonic acid	8.1 ± 1.1
Esterified into phospholipids	9.9 ± 1.3
12-HETE	22.3 ± 4.2
HHT	38.0 ± 2.9
TxB_2	20.0 ± 1.9
PGE_2	1.2 ± 0.5
$PGF_{2\alpha}$	0.5 ± 0.1

* Human platelets (2×10^8/ml) were incubated with [1-^{14}C]arachidonic acid (5 μM) for 5 min at 37°. The lipids were extracted and separated by thin-layer chromatography as described in Materials and Methods. Results are expressed as mean ± S.E.; N = 11.

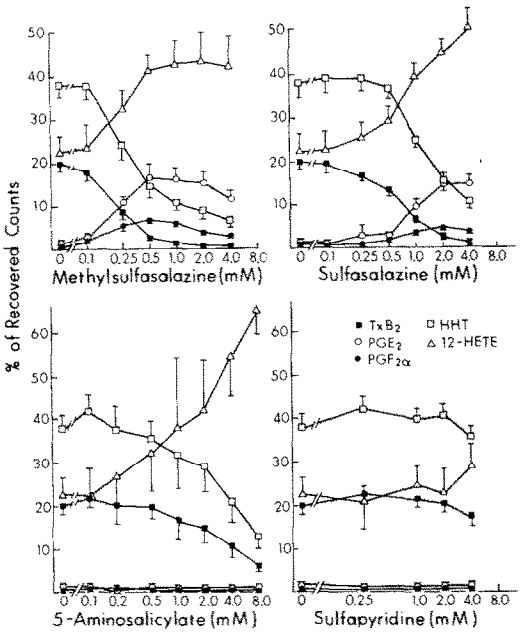

Fig. 1. Effects of sulfasalazine, metabolites and analogs on arachidonic acid metabolism. Platelets (2×10^8/ml) were incubated in the presence and absence of inhibitors for 5 min at 37°. [^{14}C]Arachidonic acid (5 μM) was added, and the incubation was continued for 5 min. The lipids were extracted and separated by thin-layer chromatography as described in Materials and Methods. The radiolabeled metabolites were quantitated by scraping the appropriate bands from the thin-layer plates and counting them in a liquid scintillation counter. Key: (□) HHT; (■) thromboxane B_2; (△) 12-HETE; (○) PGE_2; and (●) PGF_2. Results are the mean ± S.E.M. for five experiments.

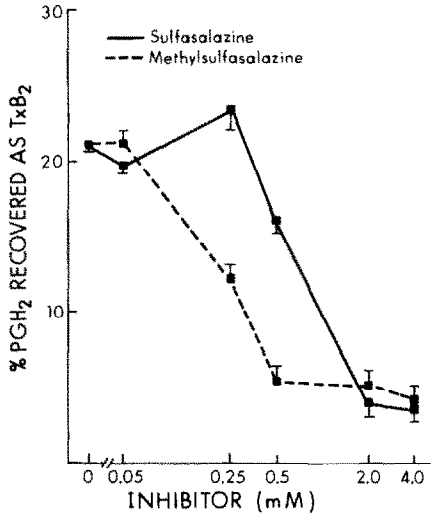

Fig. 2. Effects of sulfasalazine and methylsulfasalazine on PGH_2 metabolixm in platelets. Platelets (1×10^8/ml) were incubated in the presence and absence of inhibitors for 5 min at 37°. PGH_2 (50,000 cpm, sp. act. 58.4 mCi/mmole) was added to the incubation mixture in a final volume of 0.4 ml, and the incubation continued for 10 min. Lipids were extracted with chloroform–methanol and subjected to TLC using solvent system I. Results are means ± S.E.M. for three experiments. In the absence of inhibitors, the distribution of recovered radioactivity was: TxB_2, 21.0 ± 0.08%; PGE_2, 19.8 ± 2.3%; $PGF_{2\alpha}$, 6.4 ± 1.0%; HHT, 42.8 ± 2.2%; and PGD_2, 10.0 ± 1.0%.

increase in the synthesis of 12-HETE indicating a diversion of arachidonate from cyclooxygenase to lipoxygenase. In the presence of 5-aminosalicylate the synthesis of PGE_2 and $PGF_{2\alpha}$ remained at very low levels rather than increasingly markedly as was seen in the presence of sulfasalazine and methylsulfasalazine. The absence of an increase in prostaglandin synthesis is consistent with inhibition of cyclooxygenase rather than thromboxane synthetase. Selective inhibition of cyclooxygenase is also seen with other salicylates including aspirin [3]. The absence of an effect of sulfasalazine on platelet lipoxygenase is in marked contrast to its total inhibition of neutrophil lipoxygenase [5].

The data shown in Fig. 1 suggest that 5-aminosalicylate is a cyclooxygenase inhibitor. The data suggest that sulfasalazine and methylsulfasalazine inhibit thromboxane synthetase, but the increase in 12-HETE synthesis raises the possibility that sulfasalazine and methysulfasalazine may also affect cyclooxygenase.

Two experiments were done to separate the effects of sulfasalazine and methylsulfasalazine on thromboxane synthetase from their effects on cyclooxygenase. In one experiment, platelets were incubated with [^{14}C]PGH_2 in the presence and absence of these drugs. As shown in Fig. 2, sulfasalazine blocked the synthesis of thromboxane B_2 from PGH_2 with an IC_{50} of 0.9 mM. Methylsulfasalazine inhibited thromboxane synthetase with an IC_{50} of 0.3 mM. The use of [^{14}C]PGH_2 rather than [^{14}C]arachidonic acid as the substrate, bypassed cyclooxygenase and allowed the direct demonstration of the inhibition of thromboxane synthetase by these two drugs.

To determine if sulfasalazine and methylsulfasalazine also inhibit cyclooxygenase, platelets were first incubated with benzyl imidazole to block thromboxane synthetase and then were incubated with and without these two drugs before incubation with [^{14}C]arachidonic acid (Fig. 3). If sulfasalazine and methylsulfasalazine affect only thromboxane synthetase, then they should have no effect on arachidonate metabolism in platelets whose thromboxane synthetase is already blocked with benzylimidazole. If, however, sulfasalazine and methylsulfasalazine also inhibit cyclooxygenase, then one would except that incubation with these compounds would result in a decrease in the synthesis of PGE_2 and $PGF_{2\alpha}$ and an increase in 12-HETE. As seen in Fig. 3, incubation with sulfasalazine or methylsulfasalazine resulted in some decrease in the synthesis of PGE_2 and $PGF_{2\alpha}$ and an increase in 12-HETE; however, the highest concentration of these compounds tested, 4 mM (which is near the limits of solubility at pH 7), inhibited PGE_2 and $PGF_{2\alpha}$ synthesis by less than 50%, demonstrating that, although these compounds did affect cyclooxygenase, the effects were seen only at doses much higher than the dose required to blocked thromboxane synthetase.

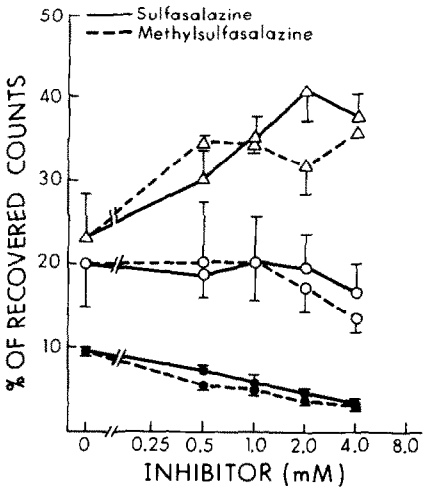

Fig. 3. Effects of sulfasalazine and methylsulfasalazine on arachidonate metabolism in benzylimidazole-treated platelets. Platelets (2×10^8/ml) were incubated with benzylimidazole (100 μM) for 5 min at 37°. Then buffer or sulfasalazine or methylsulfasalazine was added, and the incubation was continued for 5 min. [^{14}C]Arachidonic acid (5 μM) was added, and the incubation was continued for an additional 5 min. Lipids were extracted with chloroform–methanol and separated by TLC using solvent systems I and II. Platelets incubated with benzylimidazole but without other inhibitors metabolized arachidonic acid to: 12-HETE, 23.2 ± 5.3%; HHT, 12.6 ± 0.3%; PGF_{2a}, 9.4 ± 0.3%; PGE_2, 20.0 ± 5.3%; TxB_2, 2.7 ± 0.1%; and PGD_2, 3.7 ± 0.9%. The remainder of the radioactivity was in phospholipids and unmetabolized arachidonic acid. Results are the mean and S.E.M. for two experiments. Key: (△) 12-HETE; (○) PGE_2; and (●) PGF_{2a}.

DISCUSSION

The major goal of this study was to define better the effects of sulfasalazine on arachidonic acid metabolism. Platelets were chosen because their pathways of arachidonate metabolism are well defined and it is possible to use them to delineate the effects of sulfasalazine and its metabolites on specific enzymes. In this study we demonstrated that sulfasalazine and a methyl analog inhibited platelet thromboxane synthetase. Sulfasalazine and its methyl analog also inhibited cyclooxygenase, but only weakly. 5-Aminosalicylate, one cleavage product of sulfasalazine, blocked cyclooxygenase but not thromboxane synthetase. Sulfapyridine, the other cleavage product, and no effect on arachidonate metabolism. None of the drugs tested blocked platelet n − 8 lipoxygenase although sulfasalazine blocks neutrophil lipoxygenase [10].

It was not the purpose of this study to determine which of the pharmacologic effects of sulfasalazine and 5-aminosalicylate account for their therapeutic efficacies in inflammatory bowel disease, nor was the study designed to determine whether the major therapeutic agent is sulfasalazine or 5-aminosalicylate [14]. However, this study may give some insight into both of these questions. This is the first demonstration of sulfasalazine inhibiting thromboxane synthetase. An earlier study demonstrated increased levels of thromboxane B_2 secreted by explants of colonic mucosa from patients with inflammatory bowel disease [8]. However, the cell of origin of the thromboxane measured in that study is unknown. Thromboxane A_2 has proinflammatory properties, including the induction of platelet aggregation. It is possible that inhibition of thromboxane synthesis could contribute to the diminution of the inflammatory response in inflammatory bowel disease. The concentration of sulfasalazine in the colons of treated patients is about 2 mM [5], which is considerably more than the dose required to inhibit thromboxane synthesis. However, the serum concentration of sulfasalazine is less than the IC_{50} for thromboxane synthesis so one would not expect a prolongation of the bleeding time. How much of the antiinflammatory effects of sulfasalazine in ulcerative colitis can be related to inhibition of thromboxane synthesis is unknown.

This study contains the clearest evidence to date of inhibition of cyclooxygenase by 5-aminosalicylate. The concentration of 5-aminosalicylate in the colon that is greater than the IC_{50} for inhibition of cyclooxygenase [15]. This finding is consistent with the suggestion that the therapeutic effect of 5-aminosalicylate is secondary to the inhibition of cyclooxygenase. This study also shows that the parent molecule, sulfasalazine, is a relatively poor inhibitor of cyclooxygenase, compared to 5-aminosalicylate. In addition, the dose of sulfasalazine required for even partial inhibition of cyclooxygenase is a good deal higher than that required for inhibition of platelet thromboxane synthetase or neutrophil lipoxygenase.

This study does not help determine if the therapeutic effects of sulfasalazine are induced by the entire molecule or by the cleavage product, 5-aminosalicylate. However, it reaffirms that sulfasalazine has pharmacologic properties not shared by either of its cleavage products, in this case the inhibition of thromboxane synthetase.

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