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# ASPIRIN-LIKE DRUGS INHIBIT ARACHIDONIC ACID METABOLISM VIA LIPOXYGENASE AND CYCLO-OXYGENASE IN RAT NEUTROPHILS FROM CARRAGEENAN PLEURAL EXUDATES

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Summary: Aspirin-like drugs inhibit the metabolism of arachidonic acid via lipoxygenase and cyclo-oxygenase in rat neutrophils from carrageenan pleural exudates. These non-steroidal anti-inflammatory drugs inhibit the formation of 11-hydroxy- and 15-hydroxy-eicosatetraenoic acid (HETE) as well as prostaglandins. In addition, the concentration- and time-dependent irreversible inhibition of lipoxygenase by aspirin and indomethacin parallels closely the patterns observed for inhibition of cyclo-oxygenase. The results suggest that some common steps may exist for the synthesis of HETE and prostaglandins from arachidonic acid in rat neutrophils. The ability of aspirin-like drugs to inhibit the formation of the chemotactic hydroxy-fatty acids may contribute to their anti-inflammatory activity.

INTRODUCTION: There have been numerous attempts to correlate the effects of non-steroidal anti-inflammatory drugs with their ability to interfere with the activity of endogenously produced substances or mediators. Ever since Vane's classic studies (1,2) on the mode of action of aspirin and indomethacin, the most logical explanation for the mechanism of action of these drugs has been the inhibition of arachidonic acid metabolism via prostaglandin synthetase (fatty acid cyclo-oxygenase; 8,11,14-eicosatetraenoate, hydrogen donor; oxygen oxidoreductase, E.C. 1.14.99.1). It has not been entirely clear, however, whether the whole spectrum of pharmacological activities of aspirin-like drugs can be explained by inhibition of prostaglandin biosynthesis.

In addition to being the precursor of prostaglandins, arachidonic acid can be metabolized via lipoxygenases to yield hydroperoxy-eicosatetraenoic acids (HPETE) which are then degraded to produce hydroxy-eicosatetraenoic acids (HETE) (3-4). While the possible biological roles of the products of arachidonate metabolism via lipoxygenase have only begun to be examined and elucidated, HETE appears to be chemotactic for human polymorphonuclear leukocytes and rabbit

The abbreviations are: HETE, hydroxy-eicosatetraenoic acid; HPETE, hydroperoxy-eicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; $PGG_2$, prostaglandin $G_2$; BW755C, 3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline.

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alveolar macrophages in vitro (5-8). In addition, it has been reported recently that in human platelets non-steroidal anti-inflammatory drugs inhibit the conversion of 12-HPETE to 12-HETE via a putative HPETE peroxidase activity (9,10). Furthermore, HPETE has been shown to be an effective inhibitor of platelet cyclo-oxygenase and a potent stimulator of lipoxygenase (11). It was therefore of interest to investigate the effects of anti-inflammatory drugs on arachidonic acid metabolism via lipoxygenase in rat neutrophils from carrageenan induced pleural exudates; these cells are presumably actively involved in acute inflammatory responses.

MATERIALS AND METHODS: Neutrophils (85 to 95% pure) were isolated from the pleural cavity of male Sprague Dawley rats three hours after the intrapleural injection of 500 µg of carrageenan (Marine Colloids Inc., lot number RENJ 8254) in 0.25 ml of pyrogen-free water (12). The neutrophils were isolated from the pleural exudates by centrifugaton at 4° for 10 min at 200 x g. The cell pellet was resuspended in 17 mM Tris·HCl buffer, pH 7.2, containing 0.75% $NH_4Cl$ to lyse contaminating erythrocytes (13), followed by centrifugation at 4° for 5 min at 200 x g. The pelleted neutrophils were resuspended in 50 mM Tris·HCl, pH 7.4, containing 100 mM NaCl and 1 mM EDTA.

Arachidonic acid metabolism in these rat neutrophils was determined by incubating 7 x $10^6$ intact cells for 2 minutes at 37° with [1-$^{14}$C]arachidonic acid (Amersham, 55 µCi/µmole) in 0.1 ml of 50 mM Tris·HCl, pH 7.4, containing 100 mM NaCl and 1 mM EDTA. Assays (in duplicate) were terminated by the addition of 0.9 ml 0.1% formic acid and 2.4 ml of a chloroform:methanol (1:1 v/v) mixture. The suspension was vortexed, immediately cooled in ice, centrifuged, and the organic layer withdrawn. The evaporated extract was resuspended in a minimum volume of chloroform:methanol (1:2 v/v) and spotted on silica thin layer plates (Sil G-25, without gypsum, Brinkmann). Chromatograms were developed with an ascending solvent of ligroine: diethyl-ether:glacial acetic acid (40:60:1 v/v/v). Products were located by autoradiography and the appropriate regions of the thin layer plates were scraped and counted in a liquid scintillation counter. Metabolites were identified by co-chromatography with authentic standards on thin layer plates and by high-pressure liquid chromatography (9,10,14,15).

Aspirin and BW 755C were from Burroughs Wellcome. Sulindac and indomethacin were from Merck, Sharp and Dohme, ibuprofen from Upjohn, naproxen from Syntex and phenylbutazone from Ciba-Geigy. Allied Chemical provided sodium salicylate. All other reagents were of the highest quality available.

RESULTS: Neutrophils isolated from three-hour carrageenan exudates (12) actively metabolize arachidonic acid via cyclo-oxygenase and lipoxygenases. By comparison to chemically synthesized compounds (14,15), the lipoxygenase products formed have been identified as 11-HETE and 15-HETE. Under these conditions, these cells do not metabolize arachidonic acid to 5- or 12-HETE. The formation of HHT has been used as a measure of arachidonic acid metabolism via cyclo-oxygenase. The production of these metabolites is linear for approximately two minutes and it is half-maximally saturated at 40 µM exogenously added [1-$^{14}$C]arachidonic acid (data not shown).

In order to investigate whether non-steroidal anti-inflammatory drugs affect the metabolism of arachidonic acid via lipoxygenase as well as cyclo-oxygenase in neutrophils, cells were preincubated for various times in the presence

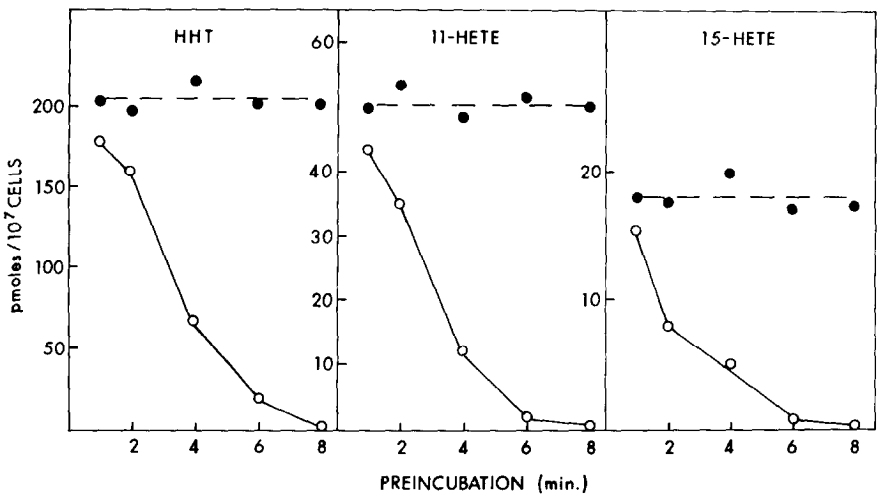


Figure 1. Time dependent inhibition by aspirin of neutrophil arachidonic acid metabolism. Rat neutrophils were preincubated at room temperature with (○) or without (●) 100 μM aspirin in 50 mM Tris·HCl, pH 7.4, containing 100 mM NaCl and 1 mM EDTA. At various times the cells were assayed for their ability to metabolize exogenously added 40 μM [1-$^{14}$C]arachidonic acid. Values are ± 10%.

of 100 μM aspirin. As illustrated in Figure 1, inhibition of cyclo-oxygenase by aspirin is time dependent. Half-maximal inhibition of HHT production from exogenously added arachidonic acid occurs after approximately three minutes preincubation with 100 μM aspirin. In addition, the metabolism of arachidonate via lipoxygenase, as measured by production of 11-HETE and 15-HETE, is also markedly inhibited by aspirin with a similar time course (Figure 1). When neutrophils are preincubated with 1.0 μM indomethacin, a time course of inhibi-

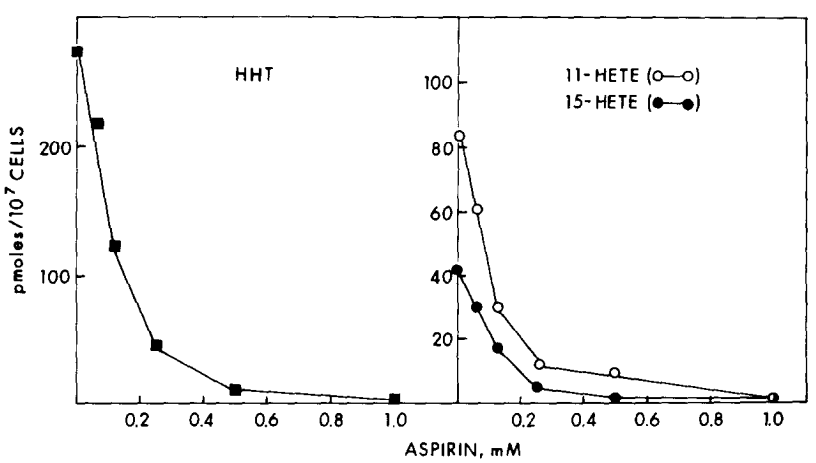


Figure 2. Concentration dependence of aspirin inhibition of neutrophil arachidonic acid metabolism. Rat neutrophils were preincubated for 4 minutes at room temperature with various concentrations of aspirin and then assayed by the addition of 40 μM [1-$^{14}$C] arachidonic acid. Values are ± 10%.

tion of both cyclo-oxygenase and lipoxygenase activities very similar to that of aspirin is observed (data not shown). Not only are the time courses of drug inhibition of lipoxygenase and cyclo-oxygenase similar, but the dependence of this inhibition on aspirin concentration is also similar for both pathways (Figure 2). Under these conditions, the half-maximal inhibition of these metabolic routes occurs at approximately 100 μM aspirin. That is, preincubation of neutrophils with 100 μM aspirin for 4 minutes inhibits by approximately 50% the production of HHT, 11-HETE and 15-HETE from exogenously added [1-$^{14}$C]arachidonic acid (Figure 2). Similarly, the inhibition of these pathways by indomethacin is dependent upon the drug concentration in a pattern very similar to that of aspirin illustrated in Figure 2. Under these conditions, half-maximal inhibi-

TABLE I

Effect of Non-Steroidal Anti-Inflammatory Drugs on Arachidonate Acid Metabolism in Rat Neutrophils

| | Arachidonic Acid Metabolite Produced | | |
|---|---|---|---|
| Drug Added | 15-HETE | 11-HETE | HHT |
| | | % inhibition | |
| 0.5 mM Aspirin | 84.4 ± 1.0[1] | 96.0 ± 0.5 | 98.9 ± 0.2 |
| 0.13 mM Aspirin | 57.0 ± 2.0 | 63.8 ± 2.0 | 55.5 ± 2.0 |
| 2.0 mM Sodium salicylate | 40.0 ± 2.0 | 34.0 ± 2.0 | 47.0 ± 2.0 |
| 0.5 mM Sodium salicylate | n.d. | 20.0 ± 3.0 | 11.5 ± 4.0 |
| 25 μM Indomethacin | 90.2 ± 1.0 | 96.4 ± 1.0 | 99.8 ± 0.1 |
| 0.5 μM Indomethacin | 20.5 ± 4.0 | 45.0 ± 3.0 | 50.5 ± 2.0 |
| 50 μM Phenylbutazone | 31.2 ± 3.0 | 67.9 ± 2.0 | 61.2 ± 2.0 |
| 200 μM Ibuprofen | 87.2 ± 2.0 | 99.8 ± 0.2 | 99.8 ± 0.2 |
| 200 μM Naproxen | 86.0 ± 2.0 | 99.8 ± 0.2 | 99.8 ± 0.2 |
| 200 μM Sulindac | 89.0 ± 2.0 | 99.8 ± 0.2 | 99.8 ± 0.2 |
| 50 μM BW 755C | 74.0 ± 2.0 | 95.7 ± 1.0 | 93.6 ± 0.5 |
| 0.5 mM Acetaminophen | n.d. | 34.9 ± 3.0 | 35.7 ± 3.0 |
| 0.5 mM Phenacetin | n.d. | 15.0 ± 3.0 | 2.4 ± 2.0 |

[1]range observed in replicate experiments

Neutrophils were preincubated with drugs for 5 minutes at room temperature in 50 mM Tris·HCl, pH 7.4, containing 100 mM NaCl and 1 mM EDTA. Metabolism of exogenously added 40 μM [1-$^{14}$C]arachidonic acid was determined as described in Materials and Methods. Neutrophils preincubated in the absence of added drugs produced 16.3 pmoles/$10^7$ cells of 15-HETE, 35.8 pmoles/$10^7$ cells of 11-HETE and 128.2 pmoles/$10^7$ cells of HHT. n.d.: not determined.

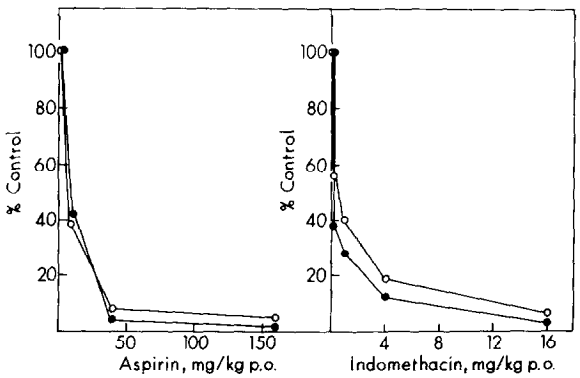


Figure 3. Inhibition of arachidonic acid metabolism in pleural exudate neutrophils following oral administration of aspirin or indomethacin to rats. Male Sprague Dawley rats were starved overnight and then given (orally) aspirin or indomethacin (5 rats per group). After 30 minutes, 500 µg of carrageenan in 0.25 ml pyrogen-free water were injected into the pleural cavity. Three hours later the rats were sacrificed, and the exudate volume and number of neutrophils recorded (12). Neutrophils were then washed and assayed for arachidonic acid metabolism. Control rats (carrageenan injection but no oral aspirin or indomethacin) had volumes of 0.81 ml and 56.9 x $10^6$ cells in the exudates. Aspirin at 10, 40, and 160 mg/kg gave 28, 62, and 91% inhibition of exudate volume and 4, 42, and 72% inhibition of cell number, respectively (mean of 5 rats at each dose). Indomethacin at 1.0, 4.0 and 16 mg/kg gave 35, 71, and 80% inhibition of exudate volume and 9, 31, and 36% inhibition of cell number, respectively. Values are ± 10%. 11-HETE (O); HHT (●).

tion of lipoxygenase and cyclo-oxygenase activities occurs with 0.5 µM indomethacin (data not shown).

Since aspirin and indomethacin, known cyclo-oxygenase inhibitors, appear to inhibit arachidonic acid metabolism via lipoxygenase as well, it was of interest to determine whether other non-steroidal anti-inflammatory drugs would also exhibit similar activity. As shown in Table I, a variety of drugs which inhibit cyclo-oxygenase also inhibit the production of 11-HETE and 15-HETE by rat neutrophils. BW 755C, which has previously been shown to inhibit both lipoxygenase and cyclo-oxygenase in horse platelets (16), also inhibits both pathways in rat neutrophils.

Because of the similarity of action of anti-inflammatory drugs on lipoxygenase and cyclo-oxygenase activities, it was important to determine whether the ability of aspirin and indomethacin to inhibit cyclo-oxygenase irreversibly (17,19) would also extend to the inhibition of lipoxygenase in neutrophils. To test this hypothesis, cells were preincubated with aspirin or indomethacin for 5 minutes at room temperature, washed by centrifugation and assayed for arachidonic acid metabolism. Clearly, preincubation with those anti-inflammatory drugs, but not with sodium salicylate, leads to irreversible inhibition of both lipoxygenase and cyclo-oxygenase activities (Table II). This irreversible inhibition of arachidonic acid metabolism occurs in intact animals as well as *in vitro* (Figure 3)

TABLE II

Irreversibility of the Inhibition by Aspirin and Indomethacin of the Lipoxygenase and Cyclo-oxygenase Activities of Rat Neutrophils

| Drug Added in Preincubation | Arachidonic Acid Metabolite Produced | | |
|---|---|---|---|
| | 15-HETE | 11-HETE | HHT |
| | pmoles/$10^7$ cells | | |
| None | 27.1 ± 2.5[1] | 54.4 ± 3.0 | 183.1 ± 4.0 |
| 500 μM Aspirin | 3.6 ± 2.0 | 4.1 ± 2.0 | 6.1 ± 2.0 |
| 25 μM Indomethacin | 7.1 ± 2.0 | 1.0 ± 1.0 | 3.1 ± 2.0 |
| 500 μM Sodium salicylate | 26.4 ± 3.0 | 54.2 ± 2.0 | 189.1 ± 4.0 |

[1]range observed in replicate experiments

Neutrophils were preincubated with or without drugs for 5 minutes at room temperature in 50 mM Tris·HCl, pH 7.4, containing 100 mM NaCl and 1 mM EDTA. After centrifugation at 200 x g for 10 minutes at 4°, cells were resuspended in the same buffer and centrifuged again. The washed neutrophils were resuspended in the same buffer and assayed with 40 μM [1-$^{14}$C]arachidonic acid.

The metabolism of exogenously added [1-$^{14}$C]arachidonic acid via both lipoxygenase and cyclo-oxygenase is markedly inhibited in neutrophils isolated from animals dosed orally with aspirin or indomethacin 30 minutes before the injection of carrageenan into the pleural cavity. This ex vivo inhibition of 11-HETE and HHT production is dependent on the dose of aspirin or indomethacin administered (Figure 3).

DISCUSSION: For several years non-steroidal anti-inflammatory drugs have been known to reduce the production of prostaglandins by inhibiting cyclo-oxygenase activity (1,2,17). Arachidonic acid, however, is metabolized not only to prostaglandins but also along a pathway (lipoxygenase) that leads to the production of straight-chain hydroxy-fatty acids (3,4,8,20,21). Some of these latter compounds appear to be chemotactic for human leukocytes and rabbit alveolar macrophages (5-8), suggesting a possible role of these substances in inflammatory reactions.

The demonstration that aspirin-like drugs alter the metabolism of arachidonic acid via lipoxygenase in human platelets (9,10) stimulated the present investigations of the lipoxygenase pathway in rat neutrophils. In contrast to the ability of aspirin-like drugs to inhibit the conversion of 12-HPETE to 12-HETE in platelets in a reversible manner (9,10), the inhibition of HETE production in neutrophils

appears to be irreversible (Figure 3 and Table II). In addition, since no 11-HPETE or 15-HPETE can be detected in these neutrophils, it appears that, while a site of action of aspirin-like drugs in human platelets is a putative HPETE peroxidase activity (9,10), in rat neutrophils there may be direct inhibition of lipoxygenase activity. Since HETE is chemotactic (5-8), inhibition of its production by aspirin-like compounds (Table I) may contribute to the anti-inflammatory action of these drugs.

The results of these studies suggest that in rat neutrophils the cyclo-oxygenase and lipoxygenase pathways (along the 11- and 15-hydroxy routes) may, at least in part, share similar enzymes, perhaps during the earliest transformations of arachidonic acid. This hypothesis is supported by the fact that aspirin and indomethacin irreversibly inhibit arachidonic acid metabolism via both cyclo-oxygenase and lipoxygenase (Table II and Figure 3), and that the time- and concentration-dependent patterns of inhibition of lipoxygenase parallel closely those of cyclo-oxygenase. Furthermore, 11-HPETE has been proposed as an intermediate in the conversion of arachidonic acid to $PGG_2$ by cyclo-oxygenase (22,23).

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