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DIFFERENTIAL EFFECTS OF ANTI-INFLAMMATORY DRUGS ON LIPOXYGENASE AND CYCLO-OXYGENASE ACTIVITIES OF NEUTROPHILS FROM A REVERSE PASSIVE ARTHUS REACTION

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SUMMARY: Rat neutrophils isolated from four-hour reverse passive Arthus reaction pleural exudates actively metabolize arachidonic acid. Production of 11-hydroxy- and 15-hydroxy-icosatetraenoic acid and 12-hydroxy-heptadecatrienoic acid is inhibited by indomethacin, benoxaprofen, BW 755C, piroxicam, ibuprofen, timegadine, and naproxen, suggesting that production of these arachidonic acid metabolites occurs at similar enzymic active sites. In addition, in the presence of the calcium ionophore A_{23187} or the non-ionic detergent, BRIJ 56, rat neutrophils also produce the lipoxygenase products 5-hydroxy-icosatetraenoic acid and leukotriene B. The production of these metabolites is calcium dependent. Moreover, the calcium ionophore A_{23187} and BRIJ 56 synergistically act to augment the metabolism of exogenously added arachidonic acid via lipoxygenase. The formation of these metabolites is inhibited by BW 755C, benoxaprofen and timegadine but not by other non-steroidal anti-inflammatory drugs tested. In fact, at doses which inhibit cyclo-oxygenase activity, indomethacin, naproxen, and ibuprofen stimulate arachidonic acid metabolism via lipoxygenase.

INTRODUCTION: A reverse passive Arthus reaction in the pleural cavity of rats affords the opportunity to measure the volume of the exudate produced and the types and numbers of cells involved in the inflammatory lesion. The RPAR reaction in the pleural cavity is complement dependent and dominated by the influx of polymorphonuclear cells (1). Peak arrival of neutrophils occurs approximately four to six hours after the reverse passive Arthus reaction is initiated, while mononuclear cells become significant only after 12 hours (1). The ability to isolate cells from the pleural cavity permits an analysis of the biochemistry of the infiltrating neutrophils.

Polymorphonuclear leukocytes metabolize arachidonic acid via both the cyclo-oxygenase and lipoxygenase pathways. Prostaglandin E_2, thromboxane

The abbreviations are: 5-HETE, 5-hydroxy-6,8,11,14-icosatetraenoic acid; LTB, 5S,12R-dihydroxy-6,8,10,14-icosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; 11-HETE, 11-hydroxy-5,8,12,14-icosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-icosatetraenoic acid; BW 755C, 3-amino-1-[m-(trifluoromethyl)-phenyl)]-2-pyrazoline; RPAR, reverse passive Arthus reaction; BSA, bovine serum alumin.

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B_2, and HHT have been reported to be formed in animal and human neutrophils (2-4) and the lipoxygenase products LTB and 5-HETE have been identified in rabbit, rat, and human leukocytes (5-7). The biological roles of the lipoxygenase products are under intense investigation in many laboratories. While the monohydroxy-icosatetraenoic acids have been reported to be chemotactic and chemokinetic for human neutrophils and alveolar macrophages (8,9), LTB possesses much more potent activity (10). In addition to chemotactic activity, lipoxygenase products stimulate the release of granules (11-15), the production of superoxide (16), and glucose transport (17,18) in target cells.

While previous studies have shown that rat neutrophils isolated from three-hour carrageenan pleural cavity exudates actively metabolize arachidonic acid via cyclo-oxygenase and, with the addition of appropriate stimuli, via lipoxygenase (4,7), a direct biochemical investigation of cells obtained with a complement induced inflammatory stimulus has not been available. The RPAR pleural cavity model affords this opportunity. Utilization of this system allows an investigation of the biochemical activities of non-steroidal anti-inflammatory drugs with respect to their effects on arachidonic acid metabolism via cyclo-oxygenase and lipoxygenase. In addition, the results of such investigations should help explain the observation that certain non-steroidal anti-inflammatory drugs exhibit differential effects on edema formation versus cell arrival at inflamed sites (19-23).

MATERIALS AND METHODS: Male Wistar-Lewis rats were injected intravenously with 5 mg BSA (Sigma A-7638) in 0.2 ml pyrogen free saline followed by an intrapleural injection of 500 µg of the IgG fraction of rabbit anti-BSA (Cappel Labs., Lot 17782) in 0.2 ml pyrogen free saline (1). Injections were made under light ether anesthesia. Four hours later, the pleural cavity exudate consisting of 85 to 95% neutrophils was removed. Neutrophils were isolated from the pleural exudates by centrifugation at 4^{o} 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 (24), followed by centrifugation at 4^{o} for 5 min at 200 x g. The pelleted neutrophils were rewashed in 50 nM Tris•HCl, pH 7.4, containing 100 nM NaCl, by centrifugation at 4^{o} for 5 min at 200 x g. The cell pellet was resuspended in 50 mM Tris•HCl, pH 7.4, containing 100 mM NaCl and 1 mM $CaCl_2$, for a final cell count of 8-12 x 10^6 intact neutrophils per 0.1 ml suspension.

Arachidonic acid metabolism was determined by incubating 0.1 ml of the above cell suspension with [1-^{14}C] arachidonic acid (Amersham, 59 µCi/µmole) as previously described (4,7). Assays in duplicate, were initiated by addition of intact cells to the complete reaction mixtures at 37^{o}. After one minute, assays were terminated by the addition of 2.4 ml of a chloroform:methanol (1:1 v/v) mixture and 0.9 ml 0.1% formic acid. The suspension was vortexed, immediately cooled on ice, centrifuged, and the organic layer withdrawn. The extract was evaported under a stream of N_2 and resuspended in 0.1 ml chloroform:methanol (1:2 v/v) for spotting on silica thin layer plates (Sil G-25, without gypsum, Brinkmann). Chromatograms were developed with an ascending solvent of

ligroine:diethylether: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. Results for 11- and 15-HETE synthesis are reported as the sum of the two products, since in all experiments these arachidonic metabolites were affected identically.

BW 755C, indomethacin, ibuprofen, naproxen, and benoxaprofen were from Schering. Timegadine was from Leo Pharmaceutical Co., BRIJ 56 was purchased from ICI, and A_{23187} from Calbiochem. All other reagents were of the highest quality available.

RESULTS: Neutrophils isolated from four-hour RPAR pleural cavity exudates actively metabolize exogenously added arachidonic acid to produce three major metabolites, HHT, 11-HETE, and 15-HETE. The production of these arachidonic acid derivatives is linear for approximately 4 minutes at 37^{0}. Half-maximal production of these compounds occurs at an exogenously added arachidonic acid concentration of 37 μM. Under these conditions, only negligible amounts of 5-HETE and LTB are produced. However, in the presence of the calcium ionophore, A_{23187}, lipoxygenase activity, as measured by 5-HETE and LTB formation, is stimulated. The non-ionic detergent BRIJ 56 is also capable of exposing this latent lipoxygenase activity. In the presence of this detergent, rat neutrophils no longer exclude trypan blue, indicating that they have become extremely permeable to exogenously added materials. Maximal production of the lipoxygenase products occurs with 10 μM A_{23187} or 0.1% BRIJ 56 (data not shown). Not only does BRIJ 56 by itself stimulate 5-HETE and LTB formation, but this non-ionic detergent also augments the production of these metabolites induced by the calcium ionophore, A_{23187} (Fig. 1A), possibly by increasing substrate availability and calcium ion concentrations inside the now permeable neutrophils. The addition of 0.1% BRIJ 56 to assay mixtures already containing 10 μM A_{23187} results in a five-fold stimulation of arachidonic acid metabolism via lipoxygenase. In contrast, the calcium ionophore and BRIJ 56, alone or in combination, have little effect on cyclo-oxygenase activity. At concentrations of both agents optimal for lipoxygenase stimulation, there is only a 40% augmentation of HHT production (Fig. 1A).

The production of 5-HETE and LTB by rat neutrophils is also dependent upon exogenously added calcium ion. In the presence of 37 μM arachidonic acid, 10 μM A_{23187}, and 0.1% BRIJ 56, maximal stimulation occurs with 0.5 mM $CaCl_2$ (Fig. 1B). As measured by HHT production, cyclo-oxygenase activity is only slightly affected by increasing calcium ion concentrations.

In order to investigate the effect of non-steroidal anti-inflammatory drugs on arachidonic acid metabolism in rat neutrophils obtained from

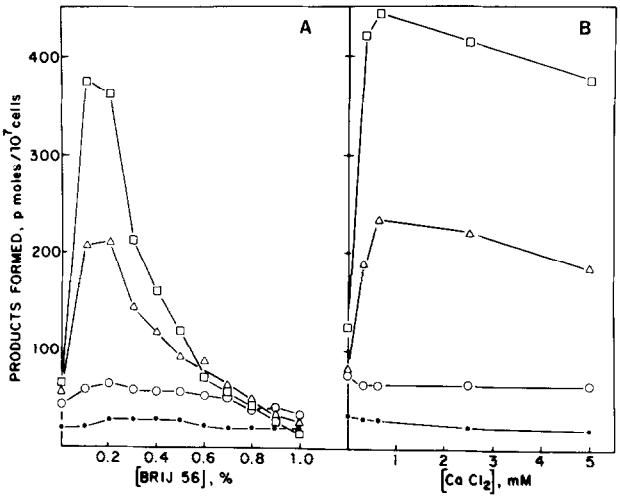


FIGURE 1. Effect of BRIJ 56 and calcium ion concentration on arachidonic acid metabolism in rat neutrophils. The amount of 5-HETE (□), LTB (△), HHT (○), and 11- and 15-HETE (●) produced by rat neutrophils incubated with 37 μM [1-^{14}C] arachidonic acid in the presence of [A] 10 μM A_{23187} and various concentrations of the non-ionic detergent BRIJ 56 or [B] 10 μM A_{23187}, 0.1% BRIJ 56, and various concentrations of $CaCl_2$ was determined as described in Materials and Methods. For [B] cells were resuspended in 50 mM Tris•HCl, pH 7.4, containing 100 mM NaCl without $CaCl_2$. Calcium chloride was then added to the assay mixtures. Values are plus or minus 10%.

four-hour RPAR pleural exudates, cells were preincubated at room temperature for various times in the presence of indomethacin. As previously reported for neutrophils obtained from carrageenan pleural cavity exudates (4), inhibition of cyclo-oxygenase activity is time dependent with half-maximal inhibition occurring after 4 minutes of preincubation with 0.4 μM indomethacin (data not shown). The production of 11-HETE and 15-HETE is similarly affected. In fact, as shown in Table I, all of the non-steroidal anti-inflammatory drugs tested inhibit the production of 11-HETE and 15-HETE at concentrations similar to those which block HHT synthesis.

The effect of indomethacin on arachidonic acid metabolism in rat neutrophils is illustrated in Fig. 2. Half-maximal inhibition of HHT, 11-HETE, and 15-HETE production occurs with 0.4 μM indomethacin. In addition, at concentrations which inhibit cyclo-oxygenase, indomethacin stimulates the metabolism of arachidonic acid via lipoxygenase (Fig. 2). This stimulation of 5-HETE and LTB production also occurs with ibuprofen and naproxen (Table I). Of the drugs tested, only piroxicam inhibits cyclo-oxygenase activity with no effect on 5-HETE and LTB synthesis (Table I).

TABLE I

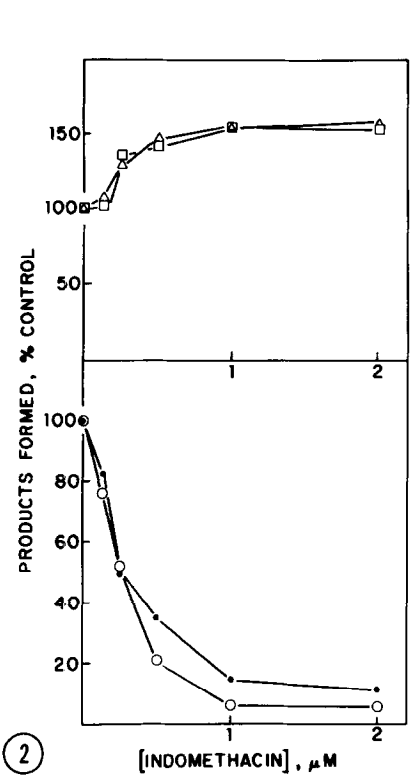
Effect of Non-Steroidal Anti-Inflammatory Drugs on Arachidonic Acid Metabolism in Rat Neutrophils

Drug	Arachidonic Acid Metabolite Produced: HHT	11- and 15-HETE	5-HETE	LTB
	Concentration for half-maximal inhibition (μM)			
Indomethacin	0.4	0.4	S	S
Piroxicam	0.1	n.d.	n.e.	n.e.
Ibuprofen	2.0	n.d.	S	S
Naproxen	0.06	0.04	S	S
Benoxaprofen	40	35	150	150
BW 755C	18	13	25	38
Timegadine	1.0	1.0	0.2	0.2

Rat neutrophils obtained from four-hour RPAR pleural cavity exudates were preincubated at room temperature with various concentrations of drug for 4 minutes and then assayed for 1 minute at 37° in the presence of 37 μM [1-^{14}C] arachidonic acid, 10 μM A_{23187} and 0.1% BRIJ 56 as described in Materials and Methods. The concentration of drug which inhibited by 50% the formation of each metabolite was determined. n.d., not determined; S, stimulated production of this product at concentrations which inhibited cyclo-oxygenase activity; and n.e., no effect. Values are ± 10%.

A second group of non-steroidal anti-inflammatory drugs inhibits both the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism in rat neutrophils. BW 755C, a potent anti-inflammatory agent (19), has previously been reported to inhibit HHT, 11-HETE, 15-HETE, 5-HETE, and LTB production in rat neutrophils obtained from three-hour carrageenan pleural exudates (4,7). BW 755C is similarly active in neutrophils obtained from RPAR exudates (Table I). In addition, as illustrated in Fig. 3, benoxaprofen, while not as potent as BW 755C, also inhibits the production of these metabolites. Both BW 755C and benoxaprofen are approximately two times more potent against cyclo-oxygenase than lipoxygenase activity (Fig. 3 and Table I).

Timegadine is a recently described anti-inflammatory drug (25) that inhibits HHT, 5-HETE, and LTB production (Fig. 4). While it is similar to BW 755C and benoxaprofen in this respect, timegadine is a more potent lipoxygenase than cyclo-oxygenase inhibitor whereas the reverse is true for the other non-steroidal drugs tested. In addition, timegadine is at least a 125-fold more potent inhibitor of lipoxygenase than either BW 755C or benoxaprofen (Table I), but does not inhibit as completely (Fig. 3 and 4).



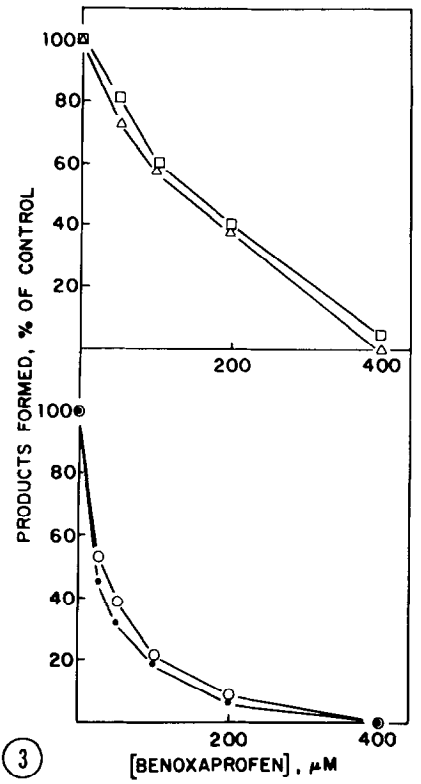


FIGURE 2. Effect of indomethacin on neutrophil arachidonic acid metabolism. Rat neutrophils were preincubated for 4 minutes at room temperature in the presence of various concentrations of indomethacin and then assayed for 1 minute at 37° by the addition of 37 μM [1-^{14}C] arachidonic acid, 10 μM A_{23187}, and 0.1% BRIJ 56 as described in Materials and Methods. HHT (○); 11-HETE and 15-HETE (●); 5-HETE (□); and LTB (△). Values are ± 10%.

FIGURE 3. Effect of benoxaprofen on neutrophil arachidonic acid metabolism. Assays were conducted as described in Figure 2 but in the presence of various concentrations of benoxaprofen. Symbols are as in Figure 2. Values are ± 10%.

DISCUSSION: The results of this study indicate that rat neutrophils isolated from four-hour RPAR pleural exudates metabolize arachidonic acid to cyclo-oxygenase and lipoxygenase products. However, significant production of 5-HETE and LTB only occurs when these cells are exposed to the calcium ionophore A_{23187} or the non-ionic detergent BRIJ 56. These two stimulators appear to act synergistically to permit substantial metabolism of arachidonic acid via the calcium dependent 5-lipoxygenase pathway, presumably by increasing substrate and calcium ion availablity (Fig. 1).

Since non-steroidal anti-inflammatory drugs inhibit the production of 11-HETE and 15-HETE at concentrations similar to those which inhibit production of traditional cyclo-oxygenase products such as HHT (Table I and

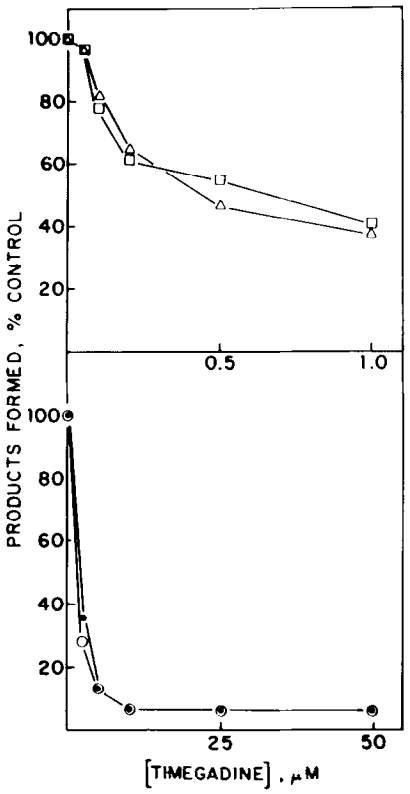


FIGURE 4. Effect of timegadine on neutrophil arachidonic acid metabolism. Assays were conducted as described in Figure 2 but in the presence of various concentrations of timegadine. Symbols are as in Figure 2. Values are ± 10%.

Fig. 2-4), it appears that these mono-hydroxy fatty acids are synthesized by similar enzymes. Mechanistically, abstraction of a hydrogen atom from position 13 of arachidonic acid is probably a common first step in the synthesis of 11-HETE, 15-HETE, and HHT. Since a similar active site is probably involved, this site would be equally inhibited by the non-steroidal anti-inflammatory drugs tested. This hypothesis is further supported by the facts that inhibition occurs at similar concentrations of drugs (Table I and Fig. 2-4) with similar time courses and, in addition, that aspirin and indomethacin have previously been shown to irreversibly inhibit the production of 11-HETE and 15-HETE, as well as HHT, in rat neutrophils from carrageenan-induced pleural exudates (4).

Lipoxygenase products appear to have biological properties which suggest that these molecules play an important role in inflammatory processes. LTB, for example, is a potent chemotactic agent which is 100- to 1000- times more effective than mono-HETE's in chemokinetic *in vitro* assays (10). In addition, leukocytes exposed to various inflammatory

stimuli metabolize arachidonic acid to 5-lipoxygenase products (26). The formation of these hydroxylated arachidonic acid derivatives is associated with the oxygen uptake, superoxide production and stimulation of hexose monophosphate shunt activity that occur when neutrophils become activated (27). The facts that rat neutrophils obtained from RPAR-induced pleural exudates produce these arachidonic acid metabolites and that newer anti-inflammatory drugs which inhibit cell arrival, as well as edema formation, at inflamed sites *in vivo* (19-23) block their production provide further support for the hypothesis that, regardless of the agent which initiates the inflammation, lipoxygenase products are intimately associated with the inflammatory process. In addition, the fact that certain anti-inflammatory drugs increase the synthesis of 5-lipoxygenase products at concentrations similar to those at which these drugs inhibit cyclo-oxygenase (Table I and Fig. 2) supports the conclusions of Higgs *et al.* (19-23) that low doses of indomethacin and aspirin excerbate inflamed sites by recruiting additional neutrophils by increasing the production of chemotactic lipids, while drugs which are effective against lipoxygenase inhibit cellular infiltration.

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