Aspirin-like drugs interfere with arachidonate metabolism by inhibition of the 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid peroxidase activity of the lipoxygenase pathway

(sodium salicylate/indomethacin/12-hydroxy-5,8,10,14-eicosatetraenoic acid)

MARVIN I. SIEGEL, RANDY T. MCCONNELL, AND PEDRO CUATRECASAS

Molecular Biology Department, The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

Communicated by George H. Hitchings, May 16, 1979

ABSTRACT Aspirin, indomethacin, and sodium salicylate are anti-inflammatory, analgesic, and antipyretic. Whereas aspirin and indomethacin inhibit prostaglandin synthetase (cyclo-oxygenase; 8,11,14-eicosatetraenoate, hydrogen-donor: oxygen oxidoreductase, EC 1.14.99.1), salicylate does not. However, all three drugs affect the metabolism of arachidonate via the lipoxygenase pathway by inhibiting the conversion of 12-hydroperoxy- to 12-hydroxy-5,8,10,14-eicosatetraenoic acid.

There have been numerous attempts to correlate the anti-inflammatory, antipyretic, and analgesic effects of substances like aspirin, indomethacin, and sodium salicylate with their ability to interfere with the activity of endogenous substances. Ever since Vane's (1, 2) classic studies 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 the production of arachidonate metabolites via the enzyme prostaglandin synthetase (fatty acid cyclo-oxygenase; 8,11,14-eicosatetraenoate, hydrogen-donor:oxygen oxidoreductase, EC 1.14.99.1). While Vane's group was examining the action of indomethacin and aspirin in lung (1) and spleen (2), Smith and Willis (3) demonstrated the selective inhibition of prostaglandin production in platelets from humans who had taken either aspirin or indomethacin. It has not been entirely clear, however, whether the entire spectrum of pharmacological activities of aspirin-like drugs can be explained solely on the basis of inhibition of prostaglandin biosynthesis. In addition, the mechanism of action of sodium salicylate has been an enigma because it does not appear to inhibit prostaglandin synthesis in vitro despite the fact that it exhibits potent effects not too dissimilar from those evoked by aspirin (4). Furthermore, aspirin is rapidly (i.e., within minutes) hydrolyzed to salicylic acid in the body (5), and it is difficult to detect inhibition of prostaglandin synthetase in other tissues with doses sufficient to inactivate platelet cyclo-oxygenase (6–8). It has been shown that the prostaglandin synthetase of intact tissues, other than platelets, is relatively refractory to aspirin and inhibition requires very much higher concentrations over protracted time periods (7–9). It therefore seems probable that, in addition to inhibition of cyclo-oxygenase activity, aspirin and indomethacin may have in common some other biochemical effect that is also shared by sodium salicylate.

Hamberg and Samuelsson originally demonstrated (10) that human platelets can convert exogenously added arachidonate into 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) as well as to fatty acid cyclo-oxygenase products. The existence of a lipoxygenase pathway distinct from cyclo-oxygenase ac-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Activity was supported by the fact that platelet suspensions produced 3 times more HETE from arachidonate in the presence of aspirin or indomethacin despite the virtually total inhibition of cyclo-oxygenase activity by these substances (11). In fact, when horse platelets are stimulated by aggregating agents, approximately 75% of the arachidonate released from endogenous phospholipids by the action of phospholipases is metabolized via lipoxygenase (12, 13).

Although the possible biological activities of the products of the metabolism of arachidonate via lipoxygenase have not been elucidated, HETE, the end product of this metabolic route in human platelets (10, 14), appears to be chemotactic for human polymorphonuclear leukocytes (15, 16). In addition, a lipid fraction from Escherichia coli having chemical and chromatographic properties similar to HETE has chemotactic activity with human polymorphs and rabbit alveolar macrophages (17). The unstable lipoxygenase product (14), 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE), may also play as yet unrecognized, important biological functions. For example, various fatty acid hydroperoxides that are analogs of HPETE have been shown to inhibit the formation of prostacyclin via prostacyclin synthetase in porcine aorta (18) and other tissues (19). In addition, the release of anaphylactic mediators from perfused guinea pig lung is enhanced by hydroperoxy-fatty acids (20, 21).

Because of the possible physiological and pathological importance of HPETE and HETE, we speculated that aspirin, indomethacin, and sodium salicylate might also affect the metabolism of arachidonate via lipoxygenase, thereby perhaps contributing to these drugs' anti-inflammatory, analgesic, or antipyretic activities or to certain adverse side effects seen with these drugs. In order to test this hypothesis, a study of various tissues was begun to determine whether aspirin, indomethacin, and sodium salicylate have any effects on arachidonate metabolism via the lipoxygenase pathway.

MATERIALS AND METHODS

Arachidonic acid was obtained from P-L Biochemicals. [1-14C]Arachidonate (57 Ci/mole, 1 Ci = 3.7 × 1010 becquerels) was from Amerham. Precast thin-layer chromatography plates, Sil G-25 without gum, were purchased from Brinkmann (Westbury, NY). Burroughs Wellcome (Research Triangle Park, NC) provided aspirin, and sodium salicylate was from Allied Chemical (Morristown, NJ). Indomethacin was from Merck, Sharp & Dohme. All other reagents were of the highest quality available.

Human platelets were prepared from subjects who had received no aspirin-like compounds during the preceding 4 weeks.

Abbreviations: HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid.
Blood was collected in 0.15 vol of the anticoagulant, citrate dextrose, and centrifuged for 20 min at 500 X g at room temperature; the platelet-containing plasma was withdrawn from above the pelleted erythrocytes. After addition of EDTA to a final concentration of 1 mM, the platelet-containing plasma was cooled to 0°C and centrifuged at 2000 X g for 20 min. The pelleted platelets were resuspended in isotonic buffer for experiments requiring intact cells (see figure legends). Alternatively, the pelleted platelets were resuspended and lysed by freeze-thawing three times in 25 mM Tris-HCl (pH 7.7). The frozen-thawed homogenate was centrifuged at 0°C for 1 hr at 100,000 X g in order to obtain cytosol.

Assays were conducted in 25 mM Tris-HCl (pH 7.4) at 37°C unless otherwise noted. All assays were performed under conditions of protein concentration and time of assay that elicit linear kinetics (unpublished observations). Reactions in 0.1 ml of mixtures containing various concentrations of [1-14C]arachidonate were initiated by the addition of the enzyme solution. In order to terminate the assay, we added 2.4 ml of a chloroform/methanol, 1:1 (vol/vol) mixture and 0.9 ml of 0.1% formic acid, mixed the suspension on a Vortex, and immediately cooled it in ice. After centrifugation at 500 X g for 10 min at 0°C, the organic layer was withdrawn and evaporated under dry N2. The residue was dissolved in a minimal volume of chloroform/methanol, 1:2 (vol/vol) and spotted on silica thin-layer plates. Chromatograms were developed with an ascending solvent consisting of liginor/diethyl ether/glacial acetic acid, 50:50:1 (vol/vol). Products were located by autoradiography; the appropriate regions of the thin-layer plates were scraped and radioactivity was measured in the suspensions in Bray's counting solution in a liquid scintillation counter.

The two products formed from arachidonate by the action of the enzymes of the lipoygenase pathway were located by autoradiography after thin-layer chromatography as described above. The one migrating with an RF of 0.33 cochromatographed with biologically produced HETE identified previously (12, 13). The second product had an RF of 0.37. After reduction with sodium borohydride in methanol, this product cochromatographed with HETE. When reaction mixtures were subjected to high-pressure liquid chromatography on a Spectra Physics 3500 high-pressure liquid chromatograph equipped with a Spectra Physics 4.6 mm X 25 cm 5u Spherisorb silica column and eluted with hexane/2-propanol/glacial acetic acid, 99:1:81 (vol/vol) at 1 ml/min, two products were observed. The first, eluting with a retention time of 18 min, cochromatographed with HETE on thin-layer chromatography and yielded a gas chromatograph/mass spectrum characteristic of HETE (10, 14). The second, eluting at 28 min, cochromatographed on thin-layer chromatography with the putative HPETE identified above. After reduction with borohydride, this compound cochromatographed with HETE on thin-layer and high-pressure chromatography systems and yielded the same gas chromatograph/mass spectrum described above. Finally, addition of the putative HPETE to reaction mixtures containing lipoygenase and peroxidase activities gave rise to a product cochromatographing with HETE. Therefore, these data are consistent with this reducible compound being HPETE.

RESULTS AND DISCUSSION

When platelets, prepared by differential centrifugation from freshly drawn human blood (12), were lysed in hypotonic medium by repetitive freeze-thawing, greater than 60% of the lipoygenase activity appeared in the 100,000 X g supernatant (unpublished observations). The ability of this lipoygenase preparation to metabolize arachidonate was markedly influenced by the presence of aspirin or indomethacin. As illustrated in Fig. 1, these drugs had pronounced effects on the fate of the lipoygenase product, HPETE. The drugs clearly inhibited the conversion of HPETE to HETE via a putative HPETE peroxidase activity in the lysed platelet supernatant. The result of this action of aspirin and indomethacin was an accumulation of HPETE at the expense of enzymatic HETE production.

Fig. 1 also illustrates the fact that low doses of aspirin (<1 mM) or indomethacin (<50 ÂµM) increased the total rate of conversion of arachidonate via lipoygenase (i.e., sum of HPETE and HETE). Because less than 10% of the substrate was metabolized (unpublished observation), this effect was probably not the result of increased availability of substrate due to diversion of arachidonate from another metabolic route to lipoygenase. The theoretical possibility that this activation could have resulted from a release of inhibition of lipoygenase caused by cyclo-oxygenase products is extremely unlikely in view of the fact that this preparation was essentially devoid of cyclo-oxygenase activity. However, the increased rate of arachidonate metabolism via lipoygenase in the presence of drugs that cause an elevation of HPETE levels could be explained by the observation that exogenously added 6 ÂµM HPETE stimulated lipoygenase activity severalfold (data not shown). Therefore, HPETE may be a feedback regulator of the lipoygenase pathway.

This effect of aspirin and indomethacin occurred not only in lysed, isolated preparations of lipoygenase free of cyclo-oxygenase, but also in intact human platelets (Fig. 2). When intact platelets were exposed to various concentrations of these drugs, the metabolism of exogenously added [1-14C]arachidonate via lipoygenase was altered markedly. At concentrations equivalent to those necessary to inhibit cyclo-oxygenase activity as measured by 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) production in vitro (Fig. 2) and in vivo (5, 22), aspirin and indomethacin established a new higher steady-state concentration of HPETE. It is interesting that at low drug concentrations a transient elevation of HETE was observed. This increased HETE production could be due to increased substrate availability, release from inhibition by cyclo-oxygenase products, or stimulation of lipoygenase by HPETE as discussed above. Because HPETE peroxidase activity was probably not

![Graph showing the effect of aspirin and indomethacin on arachidonate metabolism via the lipoygenase pathway in 100,000 X g supernatants of lysed human platelets. Human platelets were prepared by centrifugation and lysed and the 100,000 X g supernatant was prepared. Assays were conducted for 5 min at 37°C in 50 mM Tris-HCl (pH 7.4) containing 80 ÂµM [1-14C]arachidonate and various concentrations of aspirin and indomethacin. Reactions were terminated and analyzed. Incubation of 80 ÂµM [1-14C]arachidonate for 5 min in 50 mM Tris-HCl (pH 7.4) at 37°C in the presence of boiled platelet preparations gave rise to no auto-oxidation products of the unsaturated fatty acid in the presence or absence of indomethacin or aspirin. O, HETE; ■, HPETE plus HETE.](image-url)
rate limiting, the increased lipoxygenase activity was reflected in the elevated amounts of HETE.

Further evidence for the significance of this metabolic effect was derived from experiments in which platelets labeled with [1-14C]arachidonate in their phospholipids were challenged with the aggregating agent, thrombin, which released the labeled, endogenous arachidonate (12, 13) (Fig. 3). Indomethacin affected the metabolism of this released arachidonate by initially diverting substrate from cyclo-oxygenase to the lipoxygenase, as reflected by higher HETE production. However, it was also apparent that indomethacin inhibited the conversion of HPETE to HETE. As observed with lysed human platelet supernatants (Fig. 1) and intact cells using exogenously added arachidonate (Fig. 2), in the presence of indomethacin there was an increase in HPETE and relatively less HETE production.

The effects of aspirin and indomethacin were not limited to human platelets, but were also observed in other species and tissues, such as 3T3 fibroblasts, rat neutrophils, and rat macrophage homogenates (not shown). For example, in rat neutrophil preparations the ratio of HPETE to the total lipoxygenase activity, as measured by the sum of HPETE and HETE, was 0.22 in the absence of drugs, 0.41 at 10 μM indomethacin, and 0.97 at 400 μM indomethacin.

One of the major dilemmas in the rationalization of the mechanism of action of anti-inflammatory, analgesic, and antipyretic drugs according to the prostaglandin synthetase hypothesis has been the explanation of the mode of action of sodium salicylate. Whereas aspirin and indomethacin inhibit cyclo-oxygenase activity, salicylate does not appear to have this effect *in vitro* (1, 3, 4, 25). However, as illustrated in Fig. 4, sodium salicylate shares with indomethacin and aspirin the pronounced effect on the metabolism of arachidonate via the lipoxygenase pathway. The half-maximal inhibition of HETE production and the concomitant rise in the amount of HPETE produced occurred at approximately 100 μM sodium salicylate.

Because aspirin, when taken orally, irreversibly inhibits human platelet cyclo-oxygenase activity (3), it was of interest to determine whether aspirin would irreversibly inhibit HPETE peroxidase activity. As shown in Table 1, a 1-g oral dose of aspirin irreversibly inhibited cyclo-oxygenase but not HPETE peroxidase activity when human platelets were assayed with exogenously added arachidonate.

The reversible nature of the inhibition of HPETE peroxidase activity by aspirin, indomethacin, and sodium salicylate was
Blood was drawn from a healthy human volunteer before and 2 hr after a 975-mg oral dose of aspirin, and platelets were prepared by differential centrifugation. Assays were conducted with intact platelets in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl with exogenously added 80 µM [1-14C]arachidonate and the products were identified and quantitated. Values are HPETE/(HPETE + HETE). All ratios are ±0.05.

* pmol/min per mg of 12-hydroxy-5,8,10-heptadecatrienoic acid produced.

also demonstrated by direct experiments (Table 2). When human platelets were preincubated, lysed, and subjected to gel filtration to remove free drug, the reversibility of aspirin, indomethacin, and salicylate inhibition became apparent. The results also show that after these treatments addition of fresh drug was still effective in the platelet preparations. Therefore, although indomethacin and aspirin, but not salicylate, are irreversible inhibitors of cyclo-oxygenase, all of these drugs share the common property of reversible inhibition of HPETE peroxidase activity. In the presence of these inhibitors, the rate of conversion of HPETE to HETE decreases to a rate that is equivalent to that of the nonenzymatic decomposition of the hydroperoxy-fatty acid. It is conceivable, therefore, that with these drugs the increase in HPETE, or an as yet unknown, biologically active metabolite of the hydroperoxy-fatty acid, may be of greater clinical importance than the inhibition of HETE formation.

Because of the similarity of the effects of indomethacin, aspirin, and sodium salicylate on HPETE peroxidase activity, it is tempting to speculate that at least some of the pharmacological actions of these drugs have in common may be related to their ability to influence arachidonate metabolism via the lipooxygenase pathway. For example, because prostacyclin is hyperalgesic (24, 25) and HPETE analogs inhibit prostacyclin synthetase (18, 19), part of the analgesic activity of these drugs may be explained by their common ability to elevate HPETE at the expense of HETE, thereby aiding in the inhibition of prostacyclin production. In addition, because HETE appears to be chemotactic (15, 17), inhibition of its production may be in part related to the drugs' anti-inflammatory activity [i.e., blocking the recruitment of phagocytic cells (26, 27)]. Finally, because the release of anaphylactic mediators from perfused guinea pig lung is enhanced by hydroperoxy-fatty acids (20), the acute allergic responses to aspirin and indomethacin observed in certain sensitive individuals (28) may be the result of these patients' extreme sensitivity to HPETE peroxidase inhibition or the resultant rise in HPETE concentration. Because currently the pharmacological properties of HPETE or potential metabolites are largely unknown, it is difficult to speculate further on the possible consequences of elevated levels.

The difficulties in detecting HPETE may explain why this is the first report of aspirin-like drugs interfering with arachidonate metabolism by inhibition of the HPETE peroxidase activity of the lipooxygenase pathway. Because of the instability of the hydroperoxy-fatty acid in aqueous solutions, rapid extraction of assay mixtures with organic solvents is necessary. In addition, HPETE and HETE cochromatograph on the frequently used (12, 13) prostaglandin thin-layer chromatography system with silica developed with the upper phase of ethylacetate/iso-octane/acidic acid/water, 90:50:20:100 (vol/vol). Therefore, the amount of HETE produced observed in previous reports was probably the sum of HPETE and HETE.

As the functions of HPETE and HETE become clarified in normal and pathologic processes, it may be possible to better understand the biochemical basis and the relative importance of these substances and the prostaglandins in the anti-inflammatory, analgesic, antipyretic, and other actions of the aspirin-like drugs. Furthermore, new insights into the chemical bases of various pathologic processes may be forthcoming upon detailed study of the lipooxygenase pathway and its products.

We thank Drs. H. White, R. Vinegar, E. Lapetina, and K. Chandraboze for valuable discussions and Ms. F. Siegel for electron microscopy of platelet preparations.

Table 1. Effect of aspirin on arachidonate metabolism in human platelets

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Additions in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aspirin, Indomethacin, Salicylate,</td>
</tr>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Control</td>
<td>0.39 (151)*</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.31 (70)*</td>
</tr>
</tbody>
</table>

Table 2. Reversibility of inhibition of HPETE peroxidase activity

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Additions in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aspirin, Indomethacin, Salicylate,</td>
</tr>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Control</td>
<td>0.08</td>
</tr>
<tr>
<td>Aspirin, 2 mM</td>
<td>0.17</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Salicylate, 2 mM</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Human platelets obtained by centrifugation were preincubated for 1 hr at room temperature in 50 mM Tris-HCl, pH 7.4/0.1 M NaCl/1 mM EDTA/1 mg of glucose per ml in the absence or presence of indomethacin, aspirin, or sodium salicylate. Platelets were pelleted by centrifugation and frozen-thawed three times in 50 mM Tris-HCl (pH 7.4). Aliquots (0.5-ml) were applied to 0.7 x 20 cm Sephadex G-25 (medium) columns that had been equilibrated with 50 mM Tris-HCl (pH 7.4) and were eluted with the same buffer. The protein eluting in the breakthrough fractions was assayed in 50 mM Tris-HCl (pH 7.4) in the presence or absence of the various drugs. Values are HPETE/(HPETE + HETE). All ratios are ±0.05.