Activation of a 15-Lipoxygenase/Leukotriene Pathway in Human Polymorphonuclear Leukocytes by the Anti-inflammatory Agent Ibuprofen*

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Human peripheral blood polymorphonuclear leukocytes (PMNs) metabolized [14C]arachidonic acid predominantly by lipoxygenase pathways. The major products were 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and 15-HETE. These and other lipoxygenase products, including their derived leukotrienes, have been implicated as mediators of inflammatory and allergic reactions. In human platelets, the nonsteroidal anti-inflammatory drug ibuprofen inhibited production of the cyclooxygenase product thromboxane B2 (I50 = 65 μM), whereas the lipoxygenase product 12-HETE was not appreciably affected even at 5 mM ibuprofen. The 5-lipoxygenase of human PMNs (measured by 5-HETE formation) was inhibited by ibuprofen but was about six times less sensitive (I50 = 420 μM) than the platelet cyclooxygenase. The unexpected observation was made that the human PMN 15-lipoxygenase/leukotriene pathway was selectively activated by 1–5 mM ibuprofen. Metabolites were identified by ultraviolet spectroscopy, by radioimmunoassay, or by retention times on high pressure liquid chromatography in comparison with authentic standards. The major product was 15-HETE; and in all of 19 donors tested, 15-HETE formation was stimulated up to 20-fold by 5 mM ibuprofen. Other identified products included 12-HETE and 15- and 12-hydroperoxyeicosatetraenoic acid. Activation of the 15-lipoxygenase by ibuprofen occurred within 1 min and was readily reversible.

The effects of aspirin, indomethacin, and ibuprofen on the PMN 15-lipoxygenase were compared in six donors. Ibuprofen produced an average 9-fold stimulation of the enzyme, whereas aspirin and indomethacin resulted in an average 1.5- and 2-fold enhancement, respectively.

Many cells metabolize arachidonic acid via two distinct pathways, the cyclooxygenase and the lipoxygenase. Oxidation of arachidonic acid via the cyclooxygenase pathway leads to prostaglandins, thromboxanes, and prostacyclin, whereas lipoxygenase-catalyzed metabolism produces polyunsaturated hydroxy fatty acids and leukotrienes (1, 2). These metabolites probably have important roles in inflammatory and allergic reactions (3). PMN1 leukocytes contain both 5- and 15-lipoxygenases (4–6). The 5-lipoxygenase pathway produces 5-HETE, diHETEs, LTE4, LTD4, and LTE5 (2). LTB4 is a very potent chemokinetic agent for neutrophils (5-HETE is about a 100-fold less active), whereas LTC4, LTD4, and LTE4 are the active components of slow reacting substance of anaphylaxis (2, 7). The 15-lipoxygenase pathway initially forms 15-HPETE which is either reduced to 15-HETE (by cellular peroxidases) or metabolized to other products including those of the 15-series leukotrienes (8, 9). 15-HPETE has been reported to inhibit vascular prostacyclin synthesis (10) and platelet aggregation (11). Other studies indicate that both 15-HPETE and 15-HETE mediate several cellular lipoxygenases as well as the human platelet cyclooxygenase pathway.

MATERIALS AND METHODS

Blood was obtained from human donors who had not taken any aspirin-like compounds during the preceding 2 weeks. PMN leukocytes from human heparinized blood were isolated using a modified Hypaque-Ficoll technique (22). Cell purity was ≥95% as determined by Wright's stain. The PMNs were resuspended in Dulbecco's phosphate-buffered saline, pH 7.0, containing 1 mM glucose at a concentration of 20–25 × 10^6 cells/ml. Human platelets were obtained using citrate/dextrose as the anticoagulant, centrifuging for 8 min at 120 x g, and removing the platelet-rich plasma. After the addition of 1 volume of ice-cold Tris/EDTA/NaCl buffer, pH 7.4, the platelet pellet was isolated by centrifugation at 1500 x g for 10 min at 4 °C. Platelets were resuspended in Krebs-Henseleit buffer at a concentration of 3.3 × 10^6 cells/ml.

Unlabeled arachidonic acid was obtained from NuCheck Prep, Elysian, MN, and [1-14C]arachidonic acid was from Amersham.

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1 The abbreviations used are: PMN, polymorphonuclear leukocytes; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LT, leukotriene; NSAID, nonsteroidal anti-inflammatory drug; SP-HPLC, straight phase high pressure liquid chromatography; RIA, radioimmunoassay.
Searle. Ibuprofen was a gift from The Upjohn Co. 5-HETE, 12-HETE, 12-HPETE, 15-HETE, and 15-HPETE were prepared as described previously (6, 13). 8-HETE and 9-HETE were obtained by singlet oxygen oxidation of arachidonic acid (23). 5,15-diHETE and 8,15-diHETE were prepared as described (12, 24). LTB4 was gift from Dr. J. Rokach, Merck-Frost, Canada, Inc. Indomethacin was purchased from Sigma, and aspirin from the Monsanto Chemical Co. Anti-PMN antibody to 15-HETE was a gift from Dr. D. H. Hwang, Louisiana State University, Baton Rouge, LA. [3H]15-HETE was generously provided by New England Nuclear.

Ethanolic solutions of the NSAIDs were prepared prior to each experiment. Using this procedure, no precipitation of the drugs was observed when the ethanolic solutions were added to the PMN suspensions. For most experiments, PMN leukocytes (0.5 ml) were preincubated with different concentrations of ibuprofen, aspirin, indomethacin, or ethanol vehicle for 2 min at 37 °C. [14C]Arachidonic acid (35-55 μCi/mol, 16.4 μM final concentration) was then added and the incubation continued for 5 min. The reaction was terminated by the addition of 1.5 ml of methanol. The cells were removed by centrifugation, and the supernatant was extracted with 3 ml of chloroform. The chloroform extract was evaporated under N2, and the residue was spotted on Silica Gel TLC plates. The plates were developed either in petroleum ether/ether/acetic acid (50:50:1, Solvent A) or in chloroform/methanol/acetic acid/water (90:8:1:0.8, Solvent D). Labelled products were located by autoradiography, scraped, and counted in a liquid scintillation counter. Experiments with platelets were carried out in a similar manner using TLC Solvent B for chromatographic analysis.

SP-HPLC analyses were performed on a Hewlett-Packard 1084A instrument equipped with a constant wavelength detector (254 nm) and fitted with a Lichrosorb-100 column (1.0 × 25 cm, Alltech Associates, Inc.). The column was eluted with solvent system (90% hexane/acetate acid (999:1, Solvent C) and 10% hexane/isopropyl alcohol/acetate acid (899:100:1, Solvent D)) at a flow rate of 4 ml/min. After 15 min, a linear solvent gradient was established to reach the composition of 10% Solvent C and 90% Solvent D by 30 min after injection. Fractions (0.3 min) were collected and aliquots were assayed for radioactivity by liquid scintillation counting. To some samples to be analyzed, authentic HETE, diHETE, and LTB4 standards were added to correlate HPLC retention times of the standards with those of the radioactive products formed in the incubation mixtures. In larger scale incubations, the product mixture was subjected to SP-HPLC, and the major product fraction eluting between 8 and 13 min was collected. This fraction was reduced with NaBH4 to convert any HPETE to HETE (25). An aliquot was rechromatographed on SP-HPLC. The peak with retention time of 11.1 min had disappeared, and there was a concomitant increase in the peaks with retention times of 9.2 and 10.1 min.

In those experiments where the metabolites were assayed for 15-HETE content by RIA, the chloroform extracts of the arachidonic acid metabolites or HPLC fractions were first evaporated under N2. The residues were redissolved in ethanol, and aliquots of the ethanolic solutions were then assayed by RIA as described (26).

To determine the distribution of radioactivity in cellular lipids, the PMN incubation mixtures were extracted with chloroform/methanol (2:1). After evaporation of the chloroform extract, the residue was chromatographed on silicic acid as described (27). Two fractions were obtained, a neutral fraction containing glycerides, unreacted arachidonic acid, and arachidonic acid metabolites and a polar fraction containing phospholipids. The radioactive component of the glyceride and phospholipid fractions was identified as [14C]arachidonic acid by alkaline deacylation (28). Both the glyceride and phospholipid subclasses were separated by TLC in Solvent A and chloroform/methanol/concentrated ammonium hydroxide/water (65:35:2.5:2.5, respectively). Appropriate standards were run on the same plate. Labelled products were located by autoradiography, scraped, and counted.

RESULTS

Human peripheral blood PMN leukocytes metabolized [14C]arachidonic acid to [14C]5-HETE (1.8 ± 0.19%, 19 donors), 5,12-diHETE isomers, including LTB4 (0.42 ± 0.05%), and [14C]15-HETE (0.98 ± 0.18%) as shown in Fig. 1, left. Less than 0.2% of the exogenous arachidonic acid is metabolized via the cyclooxygenase pathway (results not shown). Furthermore, 6.9% of the radioactivity on the TLC plates was in the tricacylglyceride band (the radioactive component was [14C]arachidonic acid; results not shown), and 4.3% of the radioactivity was at the origin (phospholipids). Pretreating the PMNs with increasing concentrations of ibuprofen (up to 1 mM) prior to the addition of [14C]arachidonic acid resulted in progressively decreased formation of [14C]5-HETE with little effect on [14C]15-HETE formation (Fig. 2). The concentration of ibuprofen resulting in half-maximal inhibition of the 5-lipoxygenase was found to be 0.42 ± 0.06 mM (13 donors). When the PMNs

FIG. 1. Activation of human PMN 15-lipoxygenase by ibuprofen. Autoradiographic profile of [14C]arachidonic acid metabolites formed by human peripheral blood PMN leukocytes (25 × 10^6/ml) in the absence (left) or presence (middle, 1 mM; right, 5 mM) of ibuprofen. The products were extracted and separated by TLC (Solvent A) as described under "Materials and Methods." AA, arachidonic acid; TG, triglycerides.

FIG. 2. Comparative effects of ibuprofen on human platelet cyclooxygenase and on human PMN 5- and 15-lipoxygenase activities. Platelet cyclooxygenase activity was assayed by measuring formation of thromboxane B2 (TXB2) (C). PMN 5-lipoxygenase activity was measured by 5-HETE formation (A) and 15-HETE production (B) as an indicator of 15-lipoxygenase activity. [14C]Arachidonic acid was used as substrate in all experiments. Detailed procedures are described under "Materials and Methods." The data point for 15-HETE stimulation by 5 mM ibuprofen is an average of seven determinations. Values are means ± S.E.
were pretreated with ibuprofen, 1 mM (Fig. 1, middle) and 5 mM (Fig. 1, right), metabolism of [14C]arachidonic acid to [14C]15-HETE progressively increased with the concomitant appearance of several minor new bands with Rf values of 0.33 (unidentified) and 0.10 (diHETEs). The distribution of radioactive products formed by the 5 mM ibuprofen-treated cells was 0.46% triglycerides, 19.4% 15-HETE, 0.20% 5-HETE, 2.5% diHETE, and 1.4% at the origin. In addition to inhibiting incorporation of [14C]arachidonic acid into triglycerides, ibuprofen suppressed incorporation of [14C]arachidonic acid into phospholipids. Ibuprofen (5 mM) decreased incorporation of [14C]arachidonic acid into phosphatidycholine by 94%, into phosphatidylethanolamine and phosphatidylserine by 76% (each). Trypan blue staining of PMNs treated with 5 mM ibuprofen indicated no loss in viability compared to untreated cells. SP-HPLC analysis of the product mixture obtained after PMNs were treated with 5 mM ibuprofen and [14C]arachidonic acid revealed one major peak and several minor peaks (Fig. 3). The major peak co-migrated with authentic 15-HETE (retention time 10.1 min). The retention times of two other peaks corresponded to authentic 12-HETE (9.2 min) and a mixture of 12- and 15-HPETE (11.1 min; these HPETEs are not resolved under the HPLC conditions used). Further support for the assignment of these HPETEs to the 11.1-min peak that this peak disappeared when the sample was reduced with NaBH4 (25), and a concomitant increase in the 9.2 and 10.1 peaks was observed. In a representative experiment, the relative proportions of 12-HETE, 15-HETE, and 12/15-HPETE formed (no [14C]12-HETE formation was observed by autoradiographic analysis from 106 platelets/ml under these experimental conditions). Ultraviolet spectroscopy and RIA (see below) confirmed the assignment of 15-HETE.

The effect of ibuprofen on the formation of 15-HETE from endogenous arachidonic acid was investigated. The product mixture obtained from [14C]arachidonic acid and ibuprofen-treated PMNs was analyzed by SP-HPLC. HPLC fractions eluting in the 12/15-HETE region were collected and combined, and the mass amount of 15-HETE was assayed by RIA using antiserum specific for 15-HETE (26). The specific activity of the 15-HETE formed was greater than 90% of the specific activity of the starting [14C]arachidonic acid. Furthermore, 21 ± 3 ng of 15-HETE were formed (measured by RIA) by ibuprofen-treated PMNs (1.9 × 106 cells) and exogenous arachidonic acid, whereas only 0.31 ± 0.12 ng of 15-HETE was produced by untreated PMNs and added arachidonic acid. In the absence of exogenous arachidonic acid, less than 0.1 ng of 15-HETE was formed.

The time course of ibuprofen stimulation of the PMN 15-lipoxygenase is shown in Fig. 4. Ibuprofen was either added before (left) or after (right) the addition of [14C]arachidonic acid to the PMNs. All incubations were terminated 5 min after the addition of arachidonic acid since preliminary studies (not shown) indicated that a plateau in product formation was reached at this time. The results indicate that the stimulation of the 15-lipoxygenase by ibuprofen occurs rapidly and reaches a maximum when the time interval between the addition of ibuprofen and arachidonic acid substrate is less than 1 min. Fig. 5 shows that the stimulation of the PMN 15-lipoxygenase by ibuprofen is reversible. Cells treated with ibuprofen exhibited a markedly enhanced formation of [14C]15-HETE and decreased production of [14C]5-HETE relative to the control. Cells treated with ibuprofen, then washed with 1% bovine serum albumin to remove ibuprofen (29) showed 5- and 15-lipoxygenase activities comparable to control values. Readdition of ibuprofen to the washed PMNs again resulted in increased formation of [14C]15-HETE and negli-
FIG. 5. Reversibility of ibuprofen activation of human PMN 15-lipoxygenase, 15-HETE and 5-HETE formation from [14C]arachidonic acid (16 μM, 44 μCi/μmol) in untreated human PMNs (0.5 ml, 11 × 10⁶) or PMNs (4 ml, 22 × 10⁶/μl; a 0.5-ml aliquot was removed for product analysis) pretreated with ibuprofen (5 mM) for 2 min. Products were quantitated as described under "Materials and Methods." The remaining PMNs (0.5 ml), which had been pretreated with ibuprofen, were then washed with 1% bovine serum albumin to remove the original ibuprofen. After resuspending these washed cells in buffer (22 × 10⁶/ml), one aliquot (0.5 ml) was incubated with [14C]arachidonic acid (16 μM) and a second 0.5-ml aliquot was treated with ibuprofen (5 mM) prior to the addition of arachidonic acid.

**TABLE I**

Influence of 5 mM ibuprofen on the incorporation of [14C]arachidonic acid into human PMN cellular lipids and the lipoxygenase-catalyzed metabolism of [14C]arachidonic acid

<table>
<thead>
<tr>
<th>Cellular lipids</th>
<th>[14C]Arachidonic acid incorporation into:</th>
<th>Control Ibuprofen</th>
<th>Control Ibuprofen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diglycerides</td>
<td>0.42</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>0.73</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Phospholipids</td>
<td>1.92</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3.07</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>[14C]5-HETE metabolism into:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[14C]5-HETE</td>
<td>1.29</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>[14C]15-HETE</td>
<td>0.70</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Stimulation of [14C]15-HETE formation from [14C]arachidonic acid is described under "Materials and Methods." Values obtained are relative to controls without the drug.

- Concentration range tested was 0.2-5 mM. At 5 mM, ibuprofen exhibited highest stimulation of the 15-lipoxygenase.
- Concentration range tested was 0.2-8 mM. At 5 mM, aspirin exhibited highest stimulation except for subjects G. K. and J. V., where 0.05 and 1 mM, respectively, were found to be the optimum concentrations.
- Concentration range tested was 0.05-0.7 mM and was not further extended due to insolubility of indomethacin at higher concentrations. At 0.7 mM, indomethacin exhibited highest stimulation of the 15-lipoxygenase except for subject J. V. where 0.05 mM was found to be the optimum concentration.

When the effect of ibuprofen on the 12-lipoxygenase and cyclooxygenase pathways in human platelets was examined, it was found that production of the lipoxygenase metabolite 12-HETE was not appreciably affected even at 5 mM (results not shown). However, ibuprofen inhibited the cyclooxygenase pathway, and the drug concentration required for half-maximal inhibition of thromboxane B₂ formation was about 65 μM (Fig. 2).

**DISCUSSION**

The present investigation shows that ibuprofen is about six times more effective in inhibiting the human platelet cyclooxygenase pathway (I₅₀ = 65 μM) than the human peripheral blood PMN leukocyte 5-lipoxygenase (I₅₀ = 420 μM; Fig. 2). This correlates with other reports that ibuprofen is an effective cyclooxygenase inhibitor in sheep vesicular glands (17).

When human PMNs were treated with 5 mM ibuprofen (and in certain donors, even with 1 mM), the stimulation of the PMN 15-lipoxygenase was selective since the PMN 5-lipoxygenase activity was inhibited by at least 80% in 13 donors tested. Furthermore, the platelet cyclooxygenase was also completely inhibited at these drug levels. The major metabolites formed from exogenously added [14C]arachidonic acid were 15-HETE, although other metabolites formed included 12-HETE, 15-HPETE, and 12-HPETE. Very little 15-HETE (and probably other metabolites) was formed from endogenous arachidonic acid under these experimental conditions. The presence of the HPETEs in the product mixture could be due to a partial inhibition of the cellular peroxidases by ibuprofen since other NSAIDs have been reported to inhibit these enzymes (30). Since purified reticulocyte lipoxygenase oxygenates arachidonic acid at both the n-6 and n-9 positions in a 4:1 ratio (31, 32), the formation of 12-HETE and 12-HPETE is probably due to dual lipoxygenase activities of the human PMN 15-lipoxygenase. However, the possibility that ibuprofen stimulates a separate PMN 12-lipoxygenase enzyme cannot be excluded. The amount of 5-HETE formed...
in the presence of ibuprofen (Fig. 3) represents residual 5-
lipooxygenase activity and correlates well with that obtained
by TLC autoradiographic analysis. Since untreated PMNs
produce much less LTB4 than 5-HETE, it is not surprising
that no LTB4 (or other 5,12-diHETEs) was detected when
ibuprofen-treated PMNs were analyzed by HPLC. Although
ibuprofen also inhibited incorporation of [14C]arachidonic
acid in cellular lipids, this decreased incorporation cannot
account for the observed increase in 15-lipoxygenase meta-
obolites. The activation of the 15-lipoxygenase by ibuprofen
occurred within 1 min of exposure to this drug and was
reversible. These observations suggest that the enhancement
was not due to increased protein synthesis or covalent modi-
fication of the enzyme. It should be noted, however, that the
concentration of ibuprofen needed to activate the 15-lipoxy-
genase is at least an order of magnitude greater than that
found in human serum following administration of a normal
pharmacological dose of ibuprofen (29).

In comparing the relative amounts of ibuprofen-induced
activation of the 15-lipoxygenase in PMNs from 19 different
subjects, eight donors showed an 11-29-fold increase and six
exhibited a 5–10-fold increase, whereas in five subjects 15-
HETE formation was increased 2–4-fold. Since ibuprofen was
recently reported to inhibit the soybean 15-lipoxygenase (33),
these results suggest that the mammalian 15-lipoxygenase
may have different properties from the plant enzyme. When
the effects of aspirin and indomethacin were compared to
ibuprofen, it was found that with five of six donors, ibuprofen
is 4–11-fold more effective than either aspirin or indometha-
cin. Studies are in progress to determine which functional-
ities are essential for this activation process.

Recently, several naturally occurring compounds have been
implicated as activators of lipooxygenase enzymes. For exam-
ple, 15-HETE was reported to enhance the 5-lipoxygenase in
PT-18 mast/basophils, whereas both 12-HPETE and ATP
stimulated the 5-lipoxygenase in PMN leukocytes (12,34,35).
The present report indicates that the 15-lipoxygenase in
human PMNs is normally in a relatively inactive state. No
mechanism has yet been described for the selective regulation
of the 15-lipoxygenase pathway. It is possible that ibuprofen
interacts with a physiological activation process that is nor-
mally present in these cells. Alternatively, ibuprofen may
mimic the action of endogenous activator or possibly displace
a naturally occurring inhibitor of the 15-lipoxygenase.

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