

# 5-HYDROXYEICOSANOIDS SELECTIVELY STIMULATE THE HUMAN NEUTROPHIL 15-LIPOXYGENASE TO USE ENDOGENOUS SUBSTRATE

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Mammalian lipoxygenases oxygenate free arachidonic acid to a variety of biologically active metabolites. Products of these lipoxygenase pathways include mono- and dihydroxyeicosanoids, leukotrienes, and lipoxins (1-3). Monohydroxyeicosatetraenoic acids (HETEs)<sup>1</sup> have been shown to influence cellular migration, phospholipases, fatty acid oxygenases, and secretion of cellular constituents, and to modulate several immune responses (4). 5,15-DiHETE and 8,15-diHETE have been reported to enhance FMLP-induced superoxide generation in neutrophils and to stimulate protein kinase C activity (5, 6). Leukotriene B<sub>4</sub> is a very potent chemotactic agent, leukotrienes C<sub>4</sub>, D<sub>4</sub>, and E<sub>4</sub> induce airway constriction, hypotension, and plasma extravasation, and the lipoxins have been shown to stimulate superoxide generation, inhibit the cytotoxic activity of human NK cells, and activate protein kinase C (2, 3, 6).

A number of investigators have presented evidence that certain cellular lipoxygenases exist in an inactive or cryptic state until properly stimulated. Thus, Ca<sup>2+</sup> and ATP were shown to activate the 5-lipoxygenase in leukocytes and rat basophilic leukemia cells (7, 8). We have previously demonstrated that the 5-lipoxygenase in the murine PT-18 mast/basophil cell line was stimulated by 15-HETE, a product of the 15-lipoxygenase (9). In addition, we recently reported that several HETEs were able to activate the 15-lipoxygenase in human neutrophils (10). The common feature of all these studies was that the arachidonic acid substrate was added exogenously to either cells or lipoxygenase preparations. In this report, we describe experiments designed to examine whether the human neutrophil 15-lipoxygenase can be selectively stimulated to oxygenate endogenous arachidonic acid.

## Materials and Methods

5(S)-HETE and 5(S),15(S)-diHETE were purchased from BioMol Research Labs., Inc. (Plymouth Meeting, PA). Leukotriene B<sub>4</sub> (LTB<sub>4</sub>), lipoxin A<sub>4</sub> (LXA<sub>4</sub>), and LXB<sub>4</sub> were generously provided by Dr. J. Rokach, Merck-Frosst, Quebec, Canada. 12-HETE was prepared from human platelets (American Red Cross, Washington DC), and 15-HETE was prepared using soybean lipoxygenase as previously described (11). The methyl ester of 5-HETE was prepared by treating 5-HETE with ethereal diazomethane at room temperature for 15 min.

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<sup>1</sup> *Abbreviations used in this paper:* HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; LX, lipoxin; PG, prostaglandin; RP- and SP-HPLC, reverse-phase and straight-phase HPLC.

The ester was purified by straight-phase HPLC (SP-HPLC). To verify retention times on reverse-phase (RP)- and SP-HPLC, racemic monoHETEs were prepared by singlet oxygen oxidation of arachidonic acid as previously described (12). All hydroxyeicosanoids were evaluated for purity by RP-HPLC before use.

Blood was obtained by venipuncture from human donors who had not taken aspirin or aspirin-like drugs for the previous 2 wk. Neutrophils were isolated from heparinized blood by a modified Ficoll-Hypaque technique according to the procedure of Ferrante and Thong (13). The neutrophil layer was collected and washed in assay buffer (Dulbecco's PBS, 10 mM dextrose, pH 7.4). Red cells were removed by hypotonic lysis. Cell viability was evaluated by trypan blue exclusion and was consistently >99%. Red cell contamination was <1%, and the platelet concentration was never >1:1, with respect to neutrophils, and in most cases was much less. Cells were diluted to  $2 \times 10^7$ /ml.

Neutrophils were prelabeled as follows. First, [ $^3\text{H}$ ]arachidonic acid (100 Ci/mmol, DuPont Co., Wilmington, DE) was dried under nitrogen and dissolved in ethanol before use. Ethanol concentration in the prelabeling buffer was never >0.05%. After addition of arachidonic acid (8  $\mu\text{Ci}/\text{ml}$  of cells), cell suspensions were incubated in a 37°C shaking water bath for 90 min with intermittent mixing. Cells were washed three times with equal volumes of assay buffer containing 0.1% BSA (Sigma Chemical Co., St. Louis, MO). After two washes, the amount of radiolabeled free arachidonate released into the washing buffer did not significantly change. Prelabeled neutrophils were diluted to  $2 \times 10^7$  cells per ml of assay buffer and stored at room temperature before the assay.

To test the effects of hydroxyeicosanoids on [ $^3\text{H}$ ]arachidonate-prelabeled neutrophils, these compounds were first dried under nitrogen, dissolved in buffer, and kept on ice. At time zero, hydroxyeicosanoids were added to the prelabeled neutrophils, which were placed in a 37°C shaking water bath. No additions were made to control tubes. The incubations were stopped by adding the reaction mixture to a tube containing 3 vol of methanol.

To evaluate the viability of cells after treatment with hydroxyeicosanoids, the activity of the cytosolic enzyme lactate dehydrogenase was measured in supernatants (14). Lactate dehydrogenase release of hydroxyeicosanoid-treated samples did not differ significantly (mean  $\pm$  SEM =  $101 \pm 5\%$ ,  $n = 16$ ) from control samples.

After addition of an internal standard (1  $\mu\text{g}$  prostaglandin B<sub>2</sub> [PGB<sub>2</sub>]) to the methanolic cell suspension (sample recovery typically ranged from 70 to 90%), lipids were extracted by a modified Folch procedure (10). The chloroform layer was removed and stored at -20°C until analysis. A mixture of monoHETEs was added to correlate the retention time of the neutrophil lipoxygenase products. All solvents were HPLC grade (Fisher Scientific Co., Fairlawn, NJ). After removal of chloroform from the samples by evaporation under nitrogen, the samples were dissolved in 50  $\mu\text{l}$  acetonitrile/water (65:35) immediately before injection on RP-HPLC. Separation was performed on a Zorbax (DuPont Co.) octadecasilane  $0.46 \times 25\text{-cm}$  column attached to a solvent programmer, LC 85B variable wavelength UV detector, and Series 4 liquid chromatograph (Perkin Elmer Corp., Norwalk, CT). Eluate fractions were collected with a fraction collector (2211; LKB Produkter, Bromma, Sweden). Retention times were monitored with a plotter/integrator (3369A; Hewlett-Packard Co., Palo Alto, CA). For separation of radiolabeled monoHETEs, a solvent mixture consisting of 57% acetonitrile, 33% water (pH 3.7), and 10% methanol was run isocratically for 33 min. Retention times for the major lipoxygenase products were: 15-HETE, 19.2 min; 12-HETE, 22.4 min; and 5-HETE, 25.3 min. The PGB standard eluted at 7.1 min. It should be noted that the variation in retention times in both SP- and RP-HPLC was >5%, and was proportional for each component. To quantify free [ $^3\text{H}$ ]arachidonate, the eluting solvent composition was changed to 100% methanol during a 5-min gradient and then run isocratically for 30 min. Arachidonate eluted at 47 min (the retention time of arachidonic acid was 75 min in the HPLC analyses used to separate the HETE methyl esters). To verify the arachidonic acid retention times, separate analyses were performed with authentic [ $^3\text{H}$ ]arachidonate. Lipoxygenase product formation and arachidonate levels were determined by liquid scintillation counting of 1-min fractions (0.3-min fractions in the monoHETE region) in 3a70B scintillant (Research Products International Corp., Mt. Prospect, IL) on a liquid scintillation counter (LS-3801; Beckman Instruments, Inc., Fullerton, CA). Results were corrected for recovery of the internal standard.

To further confirm the identities of the radioactive products, HETE analyses were also performed by SP-HPLC using a silicic acid column (0.46 × 25 cm) (Alltech Associates, Inc., Deerfield, IL) and a combination of solvent A (99% hexane, 0.1% acetic acid) and solvent B (89.9% hexane, 10.0% isopropanol, 0.1% acetic acid). 10% solvent B was run isocratically for 10 min, followed by a 15-min gradient to 90% B, and run isocratically at 90% B for 30 min. The major monoHETEs eluted at the following times: 12-HETE, 12.9 min; 15-HETE, 14.6 min; 5-HETE, 25.6 min.

In some experiments, cellular lipid classes were separated by silicic acid chromatography. Samples were dried, dissolved in ethyl acetate/hexane (5:95), and added to a Sep-Pak cartridge (C18; Millipore Continental Water Systems, Bedford, MA). Eluates were characterized by thin-layer chromatography. The cartridge was first eluted with 5% ethyl acetate, which removed neutral lipids, including triglycerides. Next, the eluting solvent was changed to 20% ethyl acetate in hexane, which eluted arachidonic acid and HETEs. Finally, phospholipids were eluted with methanol.

To assess the esterified monoHETE content of cellular lipids, the phospholipid sample was dried under nitrogen and treated with methanolic NaOH (0.2 M) in chloroform at room temperature for 15 min (15). The mixture was acidified, and the fatty acid methyl esters were extracted into chloroform. The samples were stored at -20°C until analysis. To separate monoHETE methyl-esters by RP-HPLC, the isocratic mode described above was extended to 60 min. The retention times of 5-, 12-, and 15-HETE remained the same (see above), and the retention times of the methyl esters were: 15-HETE-ME, 41 min; 12-HETE-ME, 46 min; and 5-HETE-ME, 54 min.

In experiments designed to quantify the mass of nonesterified arachidonic acid in unlabeled neutrophils (40–6.5 × 10<sup>7</sup> cells), chloroform-extracted lipids were analyzed by SP-HPLC using the silicic acid column (Alltech Associates, Inc.) and a modification of a recently described method (16). In this system, the composition of solvent C was 95.2% hexane, 4.8% methyl tert-butyl ether, 0.02% acetic acid; and solvent D was 70% hexane, 30% methyl tert-butyl ether, 0.02% acetic acid. 100% solvent C was run isocratically for 20 min at 1 ml/min followed by a 2-min gradient to 100% solvent D. A 30-min isocratic phase completed the solvent program. In addition, 0.1 μCi [<sup>3</sup>H]arachidonate (1 pmol) was added as an internal standard to all samples. Authentic arachidonate and [<sup>3</sup>H]arachidonate coeluted at 13.5 min. Free arachidonic acid in experimental samples was determined from the absorbance at 210–213 nm using a standard curve generated on the same day. [<sup>3</sup>H]Arachidonic acid always coeluted with unlabeled arachidonate and was used to determine recovery. The arachidonic acid peak was isolated and analyzed by capillary gas chromatography in order to confirm its identity.

In experiments designed to determine whether treatment of unlabeled neutrophils with 12-HETE could produce 15-HETE, the cells (10<sup>7</sup>) were incubated with 43-μM 12-HETE for 8 min at 37°C. Products were extracted into chloroform and analyzed by RP-HPLC. The material eluting with a retention time identical to that of authentic 15-HETE was collected and assayed by RIA as previously described (17). The crossreactivity of the antiserum to 12-HETE was 0.1%.

## Results

Human neutrophils were prelabeled by incubating cells with [<sup>3</sup>H]arachidonic acid (4 μCi/10<sup>7</sup> cells) for 90 min at 37°C. Analysis of the cells indicated that 65 ± 3% of the label was incorporated into the phospholipid fraction and 33 ± 3% into the neutral lipid fraction, and that 0.5–2% was free [<sup>3</sup>H]arachidonic acid (four separate experiments). Treatment of prelabeled neutrophils with exogenously supplied 5-HETE for 30 min at 37°C stimulated the 15-lipoxygenase, as shown by the formation of [<sup>3</sup>H]-15-HETE (Fig. 1). At concentrations of 8 or 25 μM, exogenously added 5-HETE also stimulated the neutrophil 5-lipoxygenase activity, as indicated by the formation of [<sup>3</sup>H]-5-HETE. The metabolites were identified on the basis of their retention times on RP- and SP-HPLC in comparison with authentic standards. Under

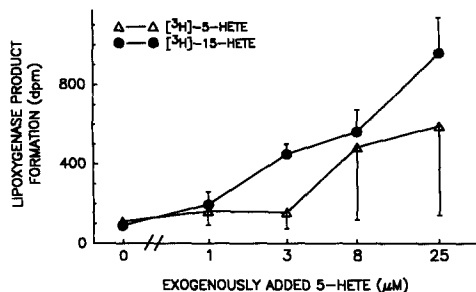


FIGURE 1. Effect of 5-HETE on the 5- and 15-lipoxygenase activities in human neutrophils. Human neutrophils ( $10^7$ ), prelabeled with [ $^3\text{H}$ ]arachidonic acid, as described in Materials and Methods, were incubated in the presence or absence of 5-HETE for 30 min at  $37^\circ\text{C}$ . Products were extracted and analyzed by RP-HPLC. Production of [ $^3\text{H}$ ]5-HETE and [ $^3\text{H}$ ]15-HETE was used to indicate the 5- and 15-lipoxygenase activities, respectively. The mean value  $\pm$  SEM from three to five experiments are presented.

these conditions,  $<0.1\%$  conversion of 5-HETE to 5,15-diHETE was observed (in three experiments, an average of  $<1$  ng of 5,15-diHETE was formed from  $4 \mu\text{g}$  of 5-HETE).

To determine which structural features in the 5-HETE molecule are important to specifically stimulate the neutrophil lipoxygenases, the effects of several analogs and derivatives of 5-HETE were examined. The results in Fig. 2 show that 5,15-diHETE was the most selective stimulator of the 15-lipoxygenase tested, whereas  $\text{LTB}_4$ , the other dihydroxyeicosanoid, and 5-HETE methyl ester were equipotent but somewhat less selective. Exogenously added 15-HETE and 12-HETE were the least potent activators. Several experiments were carried out comparing the stimulatory activities of 5-HETE,  $\text{LXA}_4$ , and  $\text{LXB}_4$ . At  $25 \mu\text{M}$ , 5-HETE was about twice as effective as  $\text{LXB}_4$  ( $8 \mu\text{M}$ ) in stimulating the neutrophil 15-lipoxygenase, and was four times more potent than  $\text{LXA}_4$  ( $8 \mu\text{M}$ ) (results not shown). A dose-response relationship of 5,15-diHETE on the neutrophil lipoxygenases indicated that concentrations as low as  $1 \mu\text{M}$  exhibit a selective stimulation of the 15-lipoxygenase (Fig. 3). In addition, the time course showed that maximal stimulation occurred after 30 min, which did not change after 1 h (Fig. 4). Time course studies with 5-HETE yielded similar results (data not shown).

During the HPLC analyses of the reaction mixtures, it was observed that free

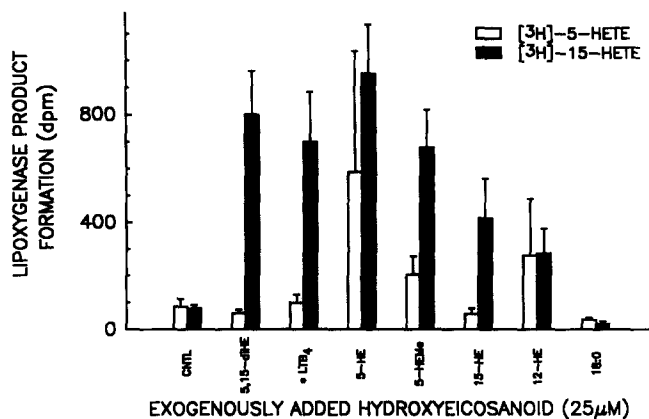


FIGURE 2. Effect of different hydroxyeicosanoids and stearic acid on the human neutrophil lipoxygenase activities. [ $^3\text{H}$ ]Arachidonic acid-prelabeled human neutrophils ( $10^7$ ) were treated in the absence or presence of different hydroxyeicosanoids or stearic acid for 30 min at  $37^\circ\text{C}$ . The fatty acid concentration used was  $25 \mu\text{M}$ , except for leukotriene  $\text{B}_4$ , which was tested at  $8 \mu\text{M}$ . The formation of [ $^3\text{H}$ ]5-HETE and [ $^3\text{H}$ ]15-HETE was quantitated by RP-HPLC and liquid scintillation counting. The results are expressed as the mean  $\pm$  SEM of three to five experiments.

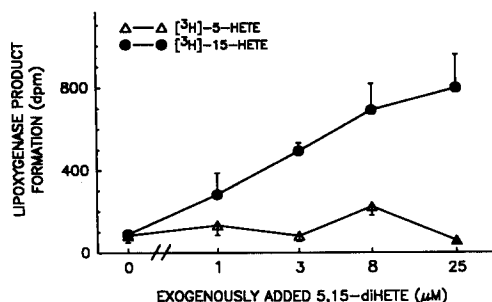


FIGURE 3. Concentration dependence of the stimulatory effect of 5,15-diHETE on the 15-lipoxygenase in human neutrophils. Human neutrophils ( $10^7$ ), prelabeled with [ $^3\text{H}$ ]arachidonic acid, were incubated with the indicated concentrations of 5,15-diHETE. [ $^3\text{H}$ ]HETE formation was measured as described in Materials and Methods. Data represent the mean  $\pm$  SEM from three to six separate experiments.

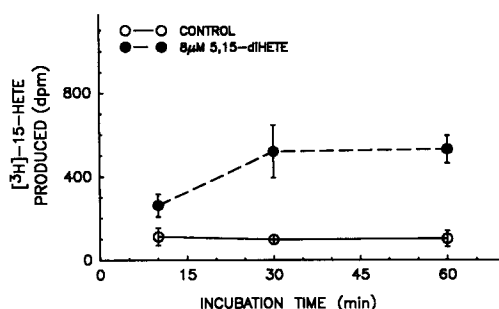


FIGURE 4. Time course of the stimulation of the cryptic 15-lipoxygenase in human neutrophils by 5,15-diHETE. Neutrophils ( $10^7$ ), prelabeled with [ $^3\text{H}$ ]arachidonic acid, were incubated at  $37^\circ\text{C}$  for various periods of time in the absence (control) or presence of  $8 \mu\text{M}$  5,15-diHETE. [ $^3\text{H}$ ]15-HETE formation, a measure of 15-lipoxygenase activity, was determined by RP-HPLC. Values are the mean  $\pm$  SEM from three different experiments.

[ $^3\text{H}$ ]arachidonic acid was the largest fatty acid component in both control samples and hydroxyicosanoid-treated cells, averaging  $43,000 \pm 15,000$  dpm/ $10^7$  cells ( $n = 9$ ; range of values, 10,000–156,000 dpm). Although almost all of the [ $^3\text{H}$ ]arachidonic acid is incorporated into cellular lipids,  $\sim 0.5$ – $2\%$  of the [ $^3\text{H}$ ]arachidonic acid remains nonesterified. It was not possible to remove this cell-associated but free fatty acid pool of arachidonic acid even after repeated washings. The data in Table I show

TABLE I  
Release of [ $^3\text{H}$ ]Arachidonic Acid from Prelabeled  
Human Neutrophils into Buffer

| Treatment            | [ $^3\text{H}$ ]Arachidonic acid released into buffer<br>$\text{dpm} \times 10^{-3}/10^7$ cells |
|----------------------|-------------------------------------------------------------------------------------------------|
| Prelabeling protocol | $403 \pm 54$                                                                                    |
| First wash           | $37 \pm 5.4$                                                                                    |
| Second wash          | $24 \pm 3.2$                                                                                    |
| Third wash           | $20 \pm 2.7$                                                                                    |

Neutrophils ( $2 \times 10^7/\text{ml}$  Dulbecco's PBS, pH 7.4, containing 10 mM dextrose) were incubated with [ $^3\text{H}$ ]arachidonic acid ( $8 \mu\text{Ci}/2 \times 10^7$  cells) for 90 min at  $37^\circ\text{C}$ . The medium was removed by centrifugation and its radioactivity content measured. The cells were resuspended in buffer (containing 0.1% BSA) at the same concentration. After 5 min, the supernatant (first wash) was removed by centrifugation and its radioactivity content was measured. The radioactivity was identified as [ $^3\text{H}$ ]arachidonic acid by thin layer chromatography. The washing procedure was repeated twice more. Values are the mean  $\pm$  SEM for five different experiments.

that after the second wash, the same amount of [ $^3\text{H}$ ]arachidonic acid was released into the washing buffer as was released after the third wash. Similar results were observed after a fourth wash (results not shown). Moreover, free [ $^3\text{H}$ ]arachidonic acid levels did not appreciably change when [ $^3\text{H}$ ]arachidonic acid-prelabeled cells were incubated at 37°C over a 60-min period (10-min incubation, 39,000  $\pm$  6,000 dpm/ $10^7$  cells [ $n = 3$ ]; 30-min incubation, 43,000  $\pm$  12,000 dpm/ $10^7$  cells; 60-min incubation, 47,000  $\pm$  6,200 dpm/ $10^7$  cells).

These results prompted us to perform several experiments using unlabeled neutrophils. The amount of nonesterified, but cell-associated, arachidonic acid in these cells was determined by Folch's extraction and SP-HPLC. The results shown in Table II indicated that the endogenous pool of free arachidonic acid in cells from four different donors ranged from 30 to 670 ng/ $10^7$  cells. No significant differences in free arachidonate were evident between control and stimulated (8  $\mu\text{M}$  5,15-diHETE) samples. In two of the donors, little increase in free arachidonate was observed between nonincubated cells and cells incubated at 37°C for 30 min. In separate experiments, unlabeled neutrophils were treated with 12-HETE (43  $\mu\text{M}$ ), followed by extraction of products and analysis by RP-HPLC. The material eluting with a retention time identical to that of authentic 15-HETE was collected and quantitated by RIA. 12-HETE-treated neutrophils produced 4.0  $\pm$  1.0 ng 15-HETE/ $10^7$  cells (three separate experiments), whereas no 15-HETE (<0.1 ng/ $10^7$  cells) could be detected in untreated cells.

### Discussion

Human neutrophils have been reported to contain the 5- and 15-lipoxygenases, and various studies have demonstrated that these cellular lipoxygenases need to be stimulated in order to metabolize exogenously supplied substrate (2, 7, 8, 10). The present studies show that endogenous free arachidonic acid can be used when the inactive 15-lipoxygenase is stimulated in both unlabeled and in [ $^3\text{H}$ ]arachidonic acid-prelabeled neutrophils, as evidenced by formation of immunoreactive 15-HETE or [ $^3\text{H}$ ]-15-HETE, respectively.

The structure-function studies indicate that stimulation of the 15-lipoxygenase

TABLE II  
*Free Arachidonic Acid Levels in Human Neutrophils*

| Donor | Control cells                     |        | 5,15 diHETE-treated cells |
|-------|-----------------------------------|--------|---------------------------|
|       | 0 min                             | 30 min | 30 min                    |
|       | <i>ng/<math>10^7</math> cells</i> |        |                           |
| BD    | 30                                | 36     | 27                        |
| ME    | 63                                | 77     | 95                        |
| RN    | 47                                | 82     | 90                        |
| TS    | 670                               | -      | 470                       |

Human peripheral blood neutrophils (unlabeled, 4.0–6.5  $\times 10^7$ ) were isolated and incubated at 37°C for 30 min in the absence (control, 30 min) or presence of 8  $\mu\text{M}$  5,15-diHETE. Other control samples were not incubated at 37°C, but were immediately extracted into chloroform (0-min control). After the incubation was terminated, the lipids were extracted and free arachidonic acid was quantitated by SP-HPLC as described in Materials and Methods.

is not a general property of fatty acids since stearic acid was ineffective. The position of the hydroxyl group seems to be important since 5-hydroxyeicosanoids were appreciably more potent stimulators than either 12- or 15-HETE. The low amount of [ $^3\text{H}$ ]-5-HETE formation in the presence of exogenous 15-HETE may be due to the previously reported inhibitory activity of 15-HETE on the neutrophil 5-lipoxygenase (18). The effectiveness of 5-HETE methyl ester indicates that a free carboxyl functionality is not essential. The presence of a second hydroxyl group appears to increase the selectivity of stimulating the 15-lipoxygenase relative to the 5-lipoxygenase. Since  $\text{LXA}_4$  and  $\text{LXB}_4$  were less potent than either 5-HETE or 5,15-diHETE, this suggests that the presence of three hydroxyl groups and/or different positions and stereochemistries of the double bonds may adversely affect the stimulatory potencies of hydroxyeicosanoids on the 15-lipoxygenase. The relative inability of most of these hydroxyeicosanoids to stimulate the 5-lipoxygenase and the observation that FMLP was ineffective in stimulating the 15-lipoxygenase (results not shown) suggest that the mechanisms of stimulation of the 5- and 15-lipoxygenase are different.

Transesterification data indicate that 98–99% of the [ $^3\text{H}$ ]arachidonic acid was esterified into cellular lipids (results not shown), and about two-thirds of this [ $^3\text{H}$ ]arachidonic acid was found in the phospholipid fraction. No significant increase in [ $^3\text{H}$ ]arachidonic acid release was observed when prelabeled cells were treated with 5,15-diHETE relative to control cells. This suggests that 5,15-diHETE did not stimulate the neutrophil phospholipase, which makes it unlikely that any HETEs esterified into cellular lipids would be liberated. Although it is possible that other hydroxyeicosanoids could stimulate cellular phospholipases to release free [ $^3\text{H}$ ]arachidonic acid, this is considered unlikely in view of the report by Chang et al. (19), who found that 5-, 12-, and 15-HETE inhibited the rat neutrophil phospholipase  $\text{A}_2$ . A more probable source of substrate is a pool of cell-associated but nonesterified arachidonic acid. Washing the cells did not remove this pool, since the amount of [ $^3\text{H}$ ]arachidonic acid released after several washes reached a plateau level. The size of this pool was  $\sim 40,000$  dpm (or  $\sim 180$  fmol/ $10^7$  cells), which suggests that the hydroxyeicosanoids shown in Fig. 2 metabolized  $\sim 0.5$ –3% of substrate. It should be noted that a similar pool of free arachidonic acid associated with the cell pellet of prelabeled neutrophils has recently been described (20). Analyses of unlabeled neutrophils confirmed the presence of an endogenous pool of free arachidonic acid. This pool size in four donors ranged from 30 to 670 ng/ $10^7$  cells, with a mean of 200 ng. This value suggests that treatment of unlabeled neutrophils with exogenous 12-HETE resulted in a 2% conversion to 15-HETE, well within the range found with prelabeled neutrophils. Whether this pool of cell-associated but nonesterified arachidonic acid is normally present *in vivo* or is released during the cell isolation and/or washing procedures cannot be ascertained.

Various studies have demonstrated that HETEs are incorporated into membranous lipids (21, 22). To determine whether exogenously added 5,15-diHETE influenced the incorporation of [ $^3\text{H}$ ]arachidonic acid metabolites into neutrophilic phospholipids, cellular phospholipids were isolated and separated from other lipid classes. The phospholipids were then transesterified by treatment with methanolic sodium hydroxide and the fatty acid methyl esters analyzed by RP-HPLC. The analyses showed that >99% of the tritium-labeled methyl esters were methyl [ $^3\text{H}$ ]arachidonate, with the remainder consisting primarily of [ $^3\text{H}$ ]-HETE methyl esters. In 5,15-diHETE

(8  $\mu\text{M}$ )-treated neutrophils, no significant change (relative to controls) in the amount of either [ $^3\text{H}$ ]-5-HETE or [ $^3\text{H}$ ]-15-HETE incorporated into phospholipids was observed (results not shown).

In conclusion, the results of the present study provide additional support for the hypothesis that certain cellular lipoxygenases, such as the neutrophil 15-lipoxygenase, exist in cryptic states. Furthermore, 5-hydroxyeicosanoids, the metabolites that are produced by the other neutrophil lipoxygenase, are capable of stimulating the 15-lipoxygenase. This further confirms previous observations that complex interrelationships exist between cellular lipoxygenases and lipoxygenase metabolites (18). It is possible that 5-hydroxyeicosanoids may mimic an unidentified physiological activator of the cryptic 15-lipoxygenase.

### Summary

When human neutrophils, prelabeled with [ $^3\text{H}$ ]arachidonic acid, were incubated with 5S,15S-dihydroxyeicosatetraenoic acid (5,15-diHETE), a dose-dependent increase in the 15-lipoxygenase product [ $^3\text{H}$ ]-15-HETE was observed relative to untreated cells. Typically, a fivefold increase in [ $^3\text{H}$ ]-15-HETE formation was obtained upon exposure of these cells to 3  $\mu\text{M}$  5,15-diHETE. There was no appreciable enhancement of the 5-lipoxygenase metabolite [ $^3\text{H}$ ]-5-HETE. Product identities were confirmed by comparing retention times on straight- and reversed-phase HPLC with authentic standards, and RIA. Other 5-hydroxyeicosanoids, such as 5-HETE, 5-HETE methyl ester, and leukotriene  $\text{B}_4$ (5S,12R-diHETE), were equally effective in stimulating the formation of [ $^3\text{H}$ ]-15-HETE, but exogenously added lipoxin  $\text{A}_4$ , lipoxin  $\text{B}_4$ , 15-HETE, and 12-HETE were much less potent, whereas stearic acid was ineffective. The diHETEs also showed a greater selectivity in activating the 15-lipoxygenase relative to the 5-lipoxygenase.

A likely source of substrate for the 15- and 5-lipoxygenases is a pool of cell-associated but noncovalently bound arachidonic acid. In [ $^3\text{H}$ ]arachidonic acid-prelabeled neutrophils, the amount of free [ $^3\text{H}$ ]arachidonic acid ranged between 50 and 700 fmol/ $10^7$  cells, whereas unlabeled neutrophils contained 100–2,200 pmol/ $10^7$  cells of nonesterified arachidonic acid. The exogenously added hydroxyeicosanoids induce a 0.5–3% conversion of this substrate pool to product.

These findings indicate that the 15-lipoxygenase in human neutrophils is a cryptic enzyme that needs to be stimulated in order to metabolize endogenous substrate. It is possible that 5-hydroxyeicosanoids may mimic an as yet unidentified physiological activator of the 15-lipoxygenase.

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