Inhibition of phytohemagglutinin-induced lymphocyte mitogenesis by lipoxygenase metabolites of arachidonic acid: structure-activity relationships

Chow-Eng Low, Maureen B. Pupillo, Robert W. Bryant,[1] and J. Martyn Bailey

Department of Biochemistry, The George Washington University Medical Center, Washington, DC 20037

Abstract Phytohemagglutinin (PHA)-induced mitogenesis of mixed mouse spleen lymphocyte populations, measured by [^{3}H]thymidine incorporation, was completely inhibited by micromolar concentrations of certain hydroxyeicosatetraenoic acids (HETE's). These are lipoxygenase metabolites of arachidonic acid which are synthesized in considerable concentrations by macrophages, lymphocytes, and other components of the immune system when appropriately stimulated. In the studies described here, the structural requirements for the maximum antimitogenic activities were examined. A series of monohydroxylated HETE's were prepared using a singlet oxygen photochemical procedure or by enzymatic synthesis from arachidonic acid substrate, and isolated by HPLC. Isomers containing different numbers of double bonds were synthesized using the appropriate unsaturated fatty acid as substrate, and the functional importance of the OH and carboxylic functions was tested using various acetoxy- and carbomethoxy derivatives. A serum-free mitogenesis assay system was used for testing, which minimized binding of the fatty acids by serum proteins and increased the inhibitory potency of the various HETE's several-fold. It was found that inhibition of cell proliferation was related to: *1*) hydroxyl proximity to the center of the eicosatetraenoic acid, decreasing in the order: 9 > 11 > 12 > 15 > 8 ⋙ 5; *2*) the number of double bonds in the fatty acid chain, decreasing in the order: 15-OH, 20:4 > 15-OH, 20:3 ⋙ 15-OH, 20:2 ⋙ 15-OH, 20:0; and *3*) the 15-position functional group as well as the 1-carboxylic group, decreasing in the order: 15-hydroxy, 1-carboxylic > 15-acetoxy, 1-carboxylic ⋙ 15-hydroxy, 1-carbomethoxy > 15-acetoxy, 1-carbomethoxy. The data indicate considerable structural specificity, requiring that the inhibitor has more than two double bonds, an unshielded carboxylic acid group at the beginning of the fatty acid chain, and a hydroxyl group in close proximity to the center of the molecule. The results obtained here may be relevant to the physiological function of the mono-hydroxyeicosanoic acids under in vivo conditions.—**Low, C-E., M. B. Pupillo, R. W. Bryant, and J. M. Bailey.** Inhibition of phytohemagglutinin-induced lymphocyte mitogenesis by lipoxygenase metabolites of arachidonic acid: structure-activity relationships. *J. Lipid Res.* 1984. **25:** 1090–1095.

Supplementary key words eicosatetraenoic acids • hydroperoxyeicosatetraenoic acids • high pressure liquid chromatography

Most mammalian cells have the capacity to convert arachidonic acid (AA) to prostaglandins (PG) and thromboxanes via the cyclooxygenase pathway. In addition, many cells particularly of the hemopoietic and immune systems convert AA by lipoxygenase enzymes to a hydroperoxyeicosatetraenoic acid (HPETE) which is then reduced intracellularly to the stable hydroxyeicosatetraenoic acid (HETE). There are three main types of lipoxygenases which act on arachidonic acid depending upon which of the three active methylene groups in the molecule is the site of the original H abstraction. Enzymatic abstraction from C10 leads primarily to the synthesis of 12-hydroxyeicosatetraenoic acid (12-HETE) with only very minor amounts of the symmetrical 8-HETE being formed. This enzyme, which is present in particularly high concentration in platelets, is termed a 12-lipoxygenase. Similarly, abstraction at C7 leads to formation of 5-HETE with only minor amounts of 9-HETE being formed. Products of this 5-lipoxygenase pathway in PMNs, neutrophils, and mast cells include the leukotriene components of slow-reacting substance of anaphylaxis. Finally, abstraction at C13 leads primarily to formation of 15-HETE together with minor amounts of 11-HETE. The 15-lipoxygenases catalyzing this reaction are present in particularly high concentrations in reticulocytes, lymphocytes and neutrophils (1–3).

We have observed that 15-HETE is a potent and specific inhibitor of other lipoxygenases (vis-à-vis the cyclooxygenases), including the 12-lipoxygenase of platelets (4) and the 5-lipoxygenase leukotriene pathway in rabbit polymorphonuclear lymphocytes (5). Goetzl (2)

Abbreviations: AA, arachidonic acid; HEPES, 4(2-hydroxyethyl)-piperazine ethane sulfonic acid; HETE, eicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high pressure liquid chromatography; 15-OH, 20:0, 15-hydroxyeicosanoic acid; 15-OH, 20:2, 15-hydroxyeicosa-11,13-dienoic acid; 15-OH, 20:3, 15-hydroxyeicosa-8,11,13-trienoic acid; 15-OH, 20:4, 15-hydroxyeicosa-5,8,11,13-tetraenoic acid; PG, prostaglandin; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; GLC–MS, gas–liquid chromatography–mass spectrometry; TdR, thymidine.

[1] Present address: Department of Allergy and Inflammation, B-51, Pharmaceutical Research Division, Schering Plough Corporation, 60 Orange Street, Bloomfield, NJ 07003.

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has also shown that 15-HETE inhibits the 5-lipoxygenase pathway in human T-lymphocytes. We found that 15-HETE was a potent inhibitor of mitogenesis induced by the tumor promoter tetradecanoyl phorbol acetate (TPA) or by the T-cell mitogen phytohemagglutinin (6, 7). It has also been observed by Coffey and Hadden (8, 9) that 15-HETE blocks the early activation of the membrane-bound guanylate cyclase, which normally accompanies induction by TPA or PHA.

In order to obtain a better understanding of the necessary structural features of a hydroxy eicosanoid which contribute to biologic activity in this system, a number of analogs of 15-HETE were synthesized and tested for their effects on mitogenesis. The compounds used were of three types: *1*) HETE's having hydroxyl groups at carbon positions 5, 8, 9, 11, 12, or 15; *2*) 15-hydroxyeicosanoids with 0, 2, 3, or 4 double bonds; and *3*) various acetylated and methylated analogs of 15-HETE. In addition, because of the interfering effects due to binding of fatty acids by serum albumin, a serum-free mitogenesis assay was developed for testing the compounds. This paper reports a structure-activity relationship for inhibition of PHA-induced mitogenesis of mixed spleen lymphocytes by these compounds in serum-free assay.

MATERIALS AND METHODS

Materials

Soybean lipoxygenase (165,000 units/mg) was purchased from Sigma Chemical Co., St. Louis, MO. Arachidonic acid, 8,11,14-eicosatrienoic acid, and 11,14-eicosadienoic acid were purchased from Nu-Chek Prep, Inc., Elysian, MN. Male C57Bl/6 mice were purchased from Jackson Laboratories, Bar Harbor, ME. RPMI 1640 tissue culture media, HEPES, and penicillin-streptomycin antibiotics were purchased from Gibco, Grand Island, NY. Fetal bovine serum was obtained from M. A. Bioproducts, Walkersville, MD. Phytohemagglutinin was acquired from Wellcome Reagents, Ltd., Beckenham, England. Methyl-[^{3}H]thymidine of specific activity 2 μCi/mmol was supplied sterile in aqueous solution by Schwartz/Mann, Orangeburg, NY.

Preparation of hydroxy fatty acids

15-Hydroperoxyeicosatetraenoic acid (15-HPETE) was prepared by soybean lipoxygenase-catalyzed oxygenation of arachidonic acid. It was partially purified by silicic acid column chromatography and further purified by HPLC, using a Hewlett-Packard Model 1084A Chromatograph equipped with a μPorasil column (3.9 × 300 mm) and run with the solvent system hexane–2-propanol–acetic acid 987:12:1 as the mobile phase (10). The flow rate was set at 2 ml/min and a 254 nm UV detector was used to monitor the effluent. 15-Hydroxyeicosatetraenoic acid (15-HETE) was obtained by triphenylphosphine reduction of the isolated 15-HPETE (11). 12-Hydroxyeicosatetraenoic acid (12-HETE) was prepared as previously described (12). Under the above HPLC conditions, 12-HETE eluted at 3.8 min, 15-HETE at 4.2 min, and 15-HPETE at 5.6 min. The identity of these compounds was confirmed by GLC–MS after conversion to the methyl ester-trimethyl-silyl ether derivatives (4).

15-Hydroxy-8,11,13-eicosatrienoic acid (15-OH, 20:3) and 15-hydroxy-11,13-eicosadienoic acid (15-OH, 20:2) were prepared by soybean lipoxygenase-catalyzed oxygenation of 8,11,14-eicosatrienoic acid and 11,14-eicosadienoic acid, respectively, followed by triphenylphosphine reduction of the corresponding hydroperoxyl compounds. 15-Hydroxyeicosanoic acid (15-OH, 20:0) was prepared by catalytic hydrogenation of 15-HETE (13). The identity of these compounds was confirmed by GLC–MS after appropriate derivatization (5, 14). 5-Hydroxyeicosatetraenoic acid (5-HETE), 8-hydroxyeicosatetraenoic acid (8-HETE), 9-hydroxyeicosatetraenoic acid (9-HETE), and 11-hydroxyeicosatetraenoic acid (11-HETE) were obtained by singlet oxygen oxidation of arachidonic acid (which yielded the hydroperoxy compounds) followed by $NaBH_4$ reduction of the products. After partial purification by silicic acid column chromatography, the individual HETEs were isolated by HPLC. The identity of the HETEs was confirmed by GLC–MS after converting them into 15-O-trimethylsilyl methyl ester. A more detailed report of these chemical procedures will be published elsewhere.

Preparation of derivatives of 15-hydroxyeicosatetraenoic acids

15-Hydroxyeicosatetraenoic acid methyl ester (HM) was prepared by the conventional method of treating 15-HETE with ethereal diazomethane. 15-O-acetyleicosatetraenoic acid (HA) was prepared by treating 15-HETE with acetic anhydride and pyridine (15). 15-O-acetyleicosatetraenoic acid methyl ester was obtained by treating HA obtained above with ethereal diazomethane.

Mitogenesis assay

Mouse spleen mixed lymphocyte suspensions containing 12 × 10^6 cells/ml were prepared from spleens of C57Bl/6 male mice ranging from 6 to 10 weeks of age. The cells in RPMI-1640 growth medium containing penicillin, 100 μ/ml, and streptomycin, 100 μg/ml, and 20 mM HEPES were aliquotted in 50-μl portions into microtiter wells (Costar), together with the test compounds and mitogen, so that the final incubation volume in each well was 200 μl, containing 6 × 10^5 cells and 1

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μg/ml of PHA. The test compounds were added dissolved in ethanol (up to 1 μL per culture) and appropriate controls for the effect of this solvent on mitogenesis were included. The cells were incubated at 37°C, 5% CO_2 humidified air for 72 hr. At this time, methyl-[^{3}H]thymidine (1 μCi) was added to each well. Incubation was continued for a further 18 hr. Preliminary experiments had shown that these conditions were optimum for maximizing the [^{3}H]thymidine incorporation in this system. Cells were harvested and washed using a semi-automated MASH (multiple automated sample harvester, Microbiological Associates, Walkersville, MD) (16). The incorporation of [^{3}H]thymidine was measured by liquid scintillation counting. All cultures were performed in triplicate. The results are expressed in disintegrations per minute (dpm) ± standard deviation (SD). Cell viability was measured on harvested cultures by trypan blue staining.

RESULTS

Hydroxyl group position

Hydroxyeicosatetraenoic acids (HETEs) in which the hydroxyl group was situated at different positions on the fatty acid chain were obtained by singlet oxygen oxidation of arachidonic acid and tested over a concentration range of 1–30 μM. Of the eight possible mono-hydroxyl positional isomers, 11-HETE, 9-HETE, 8-HETE, and 5-HETE were purified and used. Since the isolation procedures do not separate the optical isomers from these synthetic compounds, each compound is expected to consist of a racemic mixture. 15-HETE and 12-HETE and their derivatives were obtained via enzymatic conversion of AA and were stereochemically pure.

When these compounds were added to mouse spleen lymphocyte cultures, mitogenic response as measured by the [^{3}H]TdR uptake was found to be inhibited, and the extent of mitogenic inhibition exerted by each compound differed. These results are shown in **Fig. 1** in which inhibition by different HETEs tested at 5 μM is plotted as a function of the position of the OH group in the molecule. A striking feature is that the inhibition is maximized when the OH group is closest to the center of the molecule so that 9-HETE and 11-HETE exerted the greater inhibitory effect on mitogenesis, followed by 12-HETE, 15-HETE, and 8-HETE in that order. 5-HETE was not inhibitory under these conditions. Cell viabilities averaged 50% in control cultures and were not significantly decreased by concentrations of the hydroxyeicosanoids up to 30 μM.

Number of double bonds

15-Hydroxyeicosanoids containing fewer double bonds were obtained in good yield when the appropriate substrates were treated with soybean lipoxygenase. 15-OH, 20:3 and 15-OH, 20:2 were obtained by this method. The fully saturated analog 15-OH, 20:0 was isolated following PtO_2-catalyzed hydrogenations of 15-HETE.

These compounds were added to mouse spleen lymphocyte cultures tested over a concentration range of 2 to 30 μM. The mitogenic response of the cells measured by [^{3}H]TdR uptake depended upon the concentration of the hydroxy fatty acid and was least for the fully saturated hydroxy fatty acid analog. 15-OH, 20:0 showed only a weak inhibitory effect (less than 20%) even at the highest concentration tested (30 μM). The results are shown in **Table 1.** 15-HETE was inhibitory at concentrations above 2 μM. The analog with one less double bond, 15-OH, 20:3, was also a potent inhibitor at low concentrations. The analog with two double bonds did not inhibit at low concentrations (5 μM and below), but at concentrations of 10 μM and above it produced 100% inhibition of mitogenesis. Cell viability at 72 hr in serum-free medium plus 10 μM 15-HETE (51.9 ± 1.8%) was not significantly different from controls (50.0 ± 12.1%).

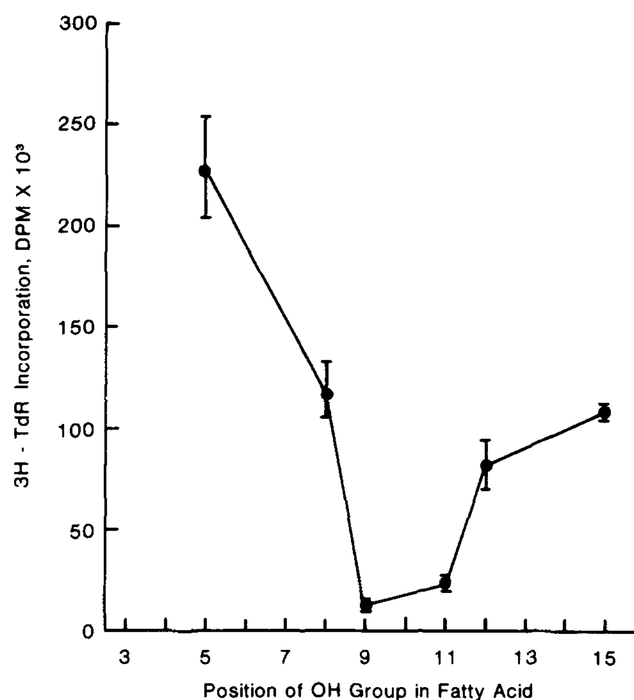

Fig. 1. Influence of functional-OH group position in eicosatetraenoic acid upon PHA-induced lymphocyte mitogenesis. Lymphocyte mitogenesis assays were carried out in 200 μL of serum-free HRPMI medium as described in Methods. The individual hydroxy-eicosatetraenoic fatty acids were added at 0 hr in 1 μL of ethanol to give a final concentration of 5 μM. Cultures were pulsed with [^{3}H]thymidine between 72 and 90 hr. Incorporation of [^{3}H]thymidine in control cultures containing 1 μL of ethanol was 217,760 ± 26,990 dpm. Results are the average of triplicate cultures.

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TABLE 1. Influence of number of double bonds in 15-hydroxyeicosanoic acids upon inhibition of lymphocyte mitogenesis

Concentration of Hydroxy Acid	$[^3H]$Thymidine Incorporation into PHA-Stimulated Lymphocytes			
	15-OH, 20:4 (15-HETE)	15-OH, 20:3	15-OH, 20:2	15-OH, 20:0
μM	*dpm*			
0	222,220 ± 59,270	222,220 ± 59,270	222,220 ± 59,270	222,220 ± 59,270
5	105,000 ± 4,910 (47)[a]	145,000 ± 12,200 (65)	206,860 ± 32,100 (93)	189,630 ± 23,900 (85)
10	56,870 ± 10,390 (26)	8,990 ± 2,000 (4)	2,380 ± 1,550 (1.0)	186,270 ± 5,190 (84)
30	759 ± 28 (0.3)	962 ± 613 (0.4)	233 ± 64 (0.1)	161,940 ± 21,190 (73)

Lymphocyte mitogenesis assays were carried out in triplicate cultures using PHA (1 μg/ml) in 200 μL of serum-free HRPMI medium as described in Methods. Fatty acids were added at 0 hr and cultures were pulsed with 1 μCi of $[^3H]$thymidine between 72 and 90 hr. Results are averaged from several experiments for each hydroxy acid concentration used.

[a] Figures in parentheses represent percent of total.

Different functional groups

The methyl ester and acetoxy derivatives of 15-HETE were isolated in good yield using the procedures described in Methods. The results of the inhibitory studies with these derivatives are shown in **Table 2.** 15-HETE was the most potent inhibitor while the acetoxy methyl ester analog (HAM) was the least. Since the acetoxy derivative (HA) is only slightly less inhibitory than 15-HETE, while the methyl ester (HM) is a much less potent inhibitor, the presence of a carboxylic group at the 1-position seems to be more important than the presence of a hydroxyl group at the 15-carbon of the fatty acid chain.

Influence of serum versus serum-free medium

The inhibitory effects of the hydroxy eicosanoids on mitogenesis were first observed in the standard medium for such assays which contain 5% serum. Because binding of fatty acids by serum albumin will reduce their effective concentrations, the experiments described in this paper were carried out in a serum-free system. Omission of serum from the test medium did not result in any major decrease in mitogenesis in control cultures. For example, in a typical experiment, the dpm of $[^3H]$thymidine incorporated into cells in the 5% serum medium was 256,488 ± 37,409, while the same cells in the serum-free medium incorporated 222,219 ± 34,261 dpm during the same period. The presence of serum however, as expected, significantly increased the I_{50} concentrations of fatty acids required to produce 50% inhibition of mitogenesis from 5 and 6 μM to 18 and 30 μM, respectively, for 15-HETE and 15-OH, 20:3 and from less than 10 μM to greater than 100 μM for the more saturated 15-OH, 20:2 analog. The fully saturated 15-OH, 20:0 fatty acid, prepared by catalytic hydrogenation of 15-HETE, had essentially no inhibitory activity in either the serum-free or serum-containing medium under the test conditions used.

DISCUSSION

Previous experiments on the inhibitory effect of the lymphocyte product 15-HETE on PHA- and PMA-induced mitogenesis of murine spleen lymphocytes were

TABLE 2. Influence of different functional group derivatives of 15-HETE on inhibition of spleen lymphocyte mitogenesis

Concentration of Hydroxy Fatty Acid	$[^3H]$Thymidine Incorporation into PHA-Stimulated Lymphocytes			
	15-HETE	HA	HM	HAM
μM	*dpm*			
0 (Control)	222,220 ± 59,270	222,220 ± 59,270	222,220 ± 59,270	222,220 ± 59,270
5	105,000 ± 4,910 (47)[a]	161,090 ± 11,370 (72)	184,710 ± 6,080 (83)	230,110 ± 43,830 (104)
10	56,870 ± 10,390 (26)	99,600 ± 10,730 (45)	189,450 ± 43,750 (85)	174,910 ± 29,810 (79)
30	759 ± 28 (0.3)	296 ± 102 (0.1)	113,220 ± 3,340 (51)	132,970 ± 3,030 (60)

Lymphocyte mitogenesis assays were carried out as described in Methods. The hydroxyeicosanoid derivatives were added at 0 hr in 3 μL of ethanol. HA, HM, and HAM refer to the acetoxy, methyl, and acetoxy-methyl derivatives, respectively, of 15-HETE. Cultures were pulsed with $[^3H]$thymidine between 72 and 90 hr. Results are the average of triplicate incubations.

[a] Percent of control.

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carried out in medium containing 5% fetal bovine serum (6, 7). Since serum contains albumin which binds fatty acids, the present studies were conducted in serum-free medium. In both types of growth media the general pattern of the inhibitory effects ran closely parallel, except that in the absence of serum the inhibitory effects were observed at much lower concentrations. The concentrations for half-maximum inhibition by the 15-hydroxyeicosanoids were reduced at least 3- or 4-fold in serum-free medium.

The order of inhibitory potency for the 15-hydroxy eicosanoids was 15-OH, 20:4 (15-HETE) > 15-OH, 20:3 ≫ 15-OH, 20:2 ≫ 15-OH, 20:0. This order was observed in the presence or absence of serum. Thus, the hydroxyeicosanoids show a clear pattern of inhibitory behavior with increasing number of double bonds at all concentrations and under all conditions tested.

Upon conversion of the 15-hydroxyl function to a 15-acetoxyl group, the resulting eicosanoid exhibits a very similar inhibitory capacity to the parent compound (15-HETE). Although this may indicate that a hydroxyl function per se is not essential, some substituent in the fatty acid chain is essential since the parent compound arachidonic acid (20:4) was inactive (7). When the carboxylic function is shielded by methylation, however, a dramatic decrease in inhibitory effect is observed. An observation of interest in this situation is that the presence of serum did not reduce the potency of the functional group derivatives to the same extent as the underivatized compounds, suggesting that they may exhibit decreased binding to serum proteins.

The position of the hydroxyl group in the polyunsaturated fatty acid chain is also very important for maximum inhibitory activity. In previous studies in serum medium, 15-HETE was considerably more potent than 12-HETE. In the present studies in serum-free medium, the potency of each was considerably enhanced and as shown in Fig. 1, 12-HETE was a slightly better inhibitor. Maximum inhibitory activity however is apparently obtained when the OH group is closest to the center of the molecule and maximum inhibitory potency was found for 9-HETE and 11-HETE. 8-HETE was much less active and 5-HETE was inactive when tested under the same conditions. It would thus be of particular interest to test 10-HETE in which the OH group is closest to the center of the 20 carbon fatty acid chain. Unfortunately this compound is formed as only a minor product in the singlet oxygen procedure used to prepare these compounds and was therefore not available for testing.

The physiological implication of these results is of interest. Several of the positional isomers are produced by specific cell types. These include 12-HETE which is synthesized in particularly large amounts by blood platelets (4) and 15-HETE which is produced in lymphocytes (12) and is also a major product of the 15-lipoxygenase in reticulocytes (3).

We have shown recently that 11-HETE is produced along with 15-HETE in a ratio of approximately 2:1 by vascular smooth muscle cells. These two compounds accumulated in amounts approximating 20 and 10 picomoles per mg of cells, respectively, in a 60-sec period following stimulation of vascular smooth muscle cells by thrombin (17). Assuming an 85% water content, the concentrations of 11-HETE and 15-HETE in the cell will thus approach 25 μM and 12.5 μM, respectively. Since the extracellular water space in vascular tissues is of the same order of magnitude as intracellular space, concentrations of these hydroxyeicosanoids which are well above the observed I_{50} values could thus be expected to occur. In other cell systems still higher concentrations of hydroxyeicosanoids are formed. Thus glycogen-elicited peritoneal macrophages synthesized 5-HETE and 15-HETE in concentrations exceeding 55 μM of intracellular water (5). Rabbit reticulocytes have a particularly active 15-lipoxygenase which composes almost 2% of the total protein in these cells (18). We have observed the accumulation of 15-HETE to a concentration of approximately 4 μM in suspensions of rabbit reticulocytes (4% hematocrit) incubated with arachidonic acid (3). This indicates that concentrations approximating 100 μM could potentially arise intracellularly or extracellularly in tissues such as spleen which are particularly rich in reticulocytes during active reticulocytosis. In a similar manner the observed concentration of 12-HETE formed in platelets aggregated in dilute (1:100) suspensions by arachidonic acid can exceed 5 μM (4). This suggests that concentrations of 12-HETE in platelet thrombi or platelet plugs may considerably exceed this figure. Other mechanisms for secreting hydroxyeicosanoids in physiologically significant concentrations from cellular lipids may be implied by the recent observations that these compounds are readily esterified and incorporated into the complex lipid components of the macrophage cell membrane (19, 20), and could presumably be released by stimulation of appropriate lipases. Concentrations of 12-HETE in the range of 30–70 μM have recently been reported in psoriatic skin (21).

The mechanism of action of the hydroxyeicosanoids in inhibiting mitogenesis has not been established. It is known, however, that they act primarily by blocking activation processes that occur in the early period following exposure of lymphocytes to the mitogen. They are much less effective when added later in the mitogenesis assay (6, 7). In addition, Coffey and Hadden (8, 9) have found that micromolar concentrations of 15-HETE, but not 5-HETE, inhibit induction of the membrane-bound guanyl cyclase which occurs within minutes

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following exposure of spleen lymphocytes to mitogenic agents such as PHA and PMA.

We have shown that 15-HETE is a potent inhibitor of cellular lipoxygenases in many cells at concentrations similar to those which block mitogenesis (4, 5). It is possible, therefore, that products of these lipoxygenase pathways may be involved in further transmission of the mitogenic signal in the spleen lymphocyte system.

Manuscript received 15 February 1984.

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