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REGULATION OF LEUKOCYTE AND PLATELET LIPOXYGENASES BY HYDROXYEICOSANOIDS

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Abstract—During allergic and inflammatory reactions, arachidonic acid is oxidized by lipoxygenases to a variety of biologically active products, including leukotrienes. The mechanisms for regulation of the different lipoxygenase activities are not well defined. We report here that [^{14}C]arachidonic acid metabolism by the 5- and 15-lipoxygenase activities in rabbit leukocytes and the 12-lipoxygenase in rabbit platelets is inhibited by various hydroxyeicosatetraenoic acids (HETEs). 15-HETE was the most effective inhibitor of the 5- and 12-lipoxygenases, whereas similar inhibitory potencies were observed for 5-HETE and 12-HETE acting on the 15-lipoxygenase. These three enzyme pathways were all least sensitive to their own product HETEs. To determine which structural characteristics of 15-HETE are essential for inhibition of the 5-lipoxygenase, various derivatives were prepared and purified by high pressure liquid chromatography, and their structures were confirmed by gas chromatography–mass spectrometry. The inhibitory potencies of 15-HETE analogs with different degrees of unsaturation were in the order of three double bonds > 4 > 2 > 0. 15-Hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) was four times more potent than 15-HETE. The 15-acetoxy, 15-keto and methyl ester derivatives were of comparable activity to 15-HETE, and the 15-acetoxy methyl ester derivative was less potent. Based upon the observed patterns of inhibition, we postulate that complex interregulatory relationships exist between the various lipoxygenases, and that cells containing these lipoxygenases may interact with each other via their lipoxygenase metabolites.

Arachidonic acid metabolism via the lipoxygenase pathway occurs in many cells and tissues, and lipoxygenase products have been implicated as important mediators of inflammatory reactions [1]. Oxygenation of arachidonic acid by the 5-lipoxygenase enzyme, present in leukocytes and macrophages, lead to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE)† which is the initial precursor of the biologically important leukotrienes [2, 3]. Leukocytes also convert arachidonic acid via the 15-lipoxygenase pathway to 15-HETE and a new group of leukotrienes derived from 15-HPETE, whereas platelets contain the 12-lipoxygenase which produces 12-HPETE [4, 5]. Pure mammalian lipoxygenases are not yet available [6, 7], and these enzymes are usually studied in their cellular environment. The lipoxygenase catalyzed oxygenation of arachidonic acid to the particular HPETE is the rate-limiting step in the production of HETEs, leukotrienes or other metabolites from arachidonic acid. The HPETE is readily converted by cellular peroxidases and other enzymes to more stable metabolites which are then assayed. Among the various lipoxygenase products, HETEs have been reported to stimulate chemotaxis and chemokinesis of neutrophils and eosinophils and to induce neutrophil aggregation and degranulation [8–10]. We previously reported that 15-HETE is the only naturally occurring inhibitor yet described for both the platelet 12-lipoxygenase and leukocyte 5-lipoxygenase activities [11, 12]. It is thus possible that various HETEs may affect one or more lipoxygenases, and a scheme of such possible interactions, based on the results reported in this paper, is depicted in Fig. 1. The 12-, and the 5- and 15-lipoxygenase pathways predominate in platelets and leukocytes respectively [2, 4]. In this study, we compared the effects of 5-, 12- and 15-HETE on these lipoxygenases and determined which functional groups in 15-HETE are important for its inhibitory activity.

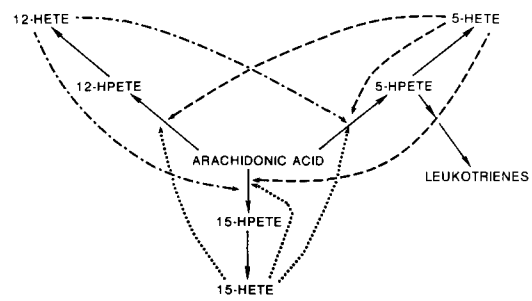

Fig. 1. Proposed scheme indicating the regulatory interrelationships between 5-, 12- and 15-lipoxygenases. It should be noted that 15-HPETE is a potent inhibitor of both the 5- and 12-lipoxygenases [11].

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† Abbreviations: GC–MS, gas chromatography–mass spectrometry; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high pressure liquid chromatography; LTB_4, leukotriene B_4; TMS, trimethylsilyl; and TXB_2, thromboxane B_2.

MATERIALS AND METHODS

Arachidonic acid, 8,11,14-eicosatrienoic acid and 11,14-eicosadienoic acid were purchased from NuChek Prep., Inc., Elysian, MN. [1-^{14}C]Arachidonic acid was obtained from the Amersham Corp., Arlington Heights, IL, and ricinoleic acid and soybean lipoxygenase were bought from the Sigma Chemical Co., St. Louis, MO. 5-HETE, 12-HETE, 15-HPETE and 15-HETE were prepared as described [11, 12]. 15-HETE was converted to the methyl ester with ethereal diazomethane, to the 15-acetate derivative by treatment with acetic anhydride and pyridine [13], to the 15-acetate methyl ester by acetylation of the 15-HETE methyl ester and to the 15-keto derivative by oxidation of 15-HETE methyl ester with MnO_2 [14] followed by saponification to the free acid with lithium hydroxide in dimethoxyethane–water [15]. 15-Hydroxy-8,11,13-eicosatrienoic acid (15-OH-20:3) and 15-hydroxy-11,13-eicosadienoic acid (15-OH-20:2) were obtained from soybean lipoxygenase catalyzed oxygenation of 8,11,14-eicosatrienoic acid and 11,14-eicosadienoic acid, respectively, followed by triphenylphosphine reduction of the corresponding hydroperoxy compounds. 15-Hydroxyeicosanoic acid (15-OH-20:0) was prepared by catalytic hydrogenation of 15-HETE [16]. All products were purified by preparative HPLC, characterized by ultraviolet spectrometry and derivatized for GC–MS analysis [12]. Hydroxy fatty acids were identified as the methyl ester trimethylsilyl ether derivatives, the acetoxy compounds were characterized as the methyl esters, and the 15-keto product was derivatized to the *O*-methyl oxime methyl esters [13]. The mass spectrum of 15-OH-20:3 (Me ester TMS ether) is shown in Fig. 2. The 15-OH-20:2 derivative exhibited prominent ions (*m/e*) with the relative abundance and probable mode of origin in parentheses at 410 (11, M), 395 (1, M-15, loss of Me), 379 (1, M-31, loss of OMe), 339 (29, M-71, loss of C_5H_{11}), 320 (1, M-90, loss of Me_3SiOH), 225 (26, M-185, loss of $(CH_2)_9COOMe$), 130 (67) and 73 (100). The derivative of 15-OH-20:0 was identified as previously described [12]. Methyl 15-acetoxy-5,8,11,13-eicosatetraenoate gave 316 (3, M-60, loss of CH_3COOH) as the only diagnostic high mass ion. The methyl ester *O*-methyl oxime derivative of 15-keto-5,8,11,13-eicosatetraenoic acid gave prominent ions at 361 (41, M), 330 (80, M-31, loss of OMe), 274 (13), 220 (28) and 180 (100, M-181, loss of $(CH_2CH = CH)_2(CH_2)_3COOMe$). Rabbit and human platelets and glycogen-elicited peritoneal polymorphonuclear leukocytes were isolated as reported [11, 12].

Platelet 12-lipoxygenase activity was measured by oxygen consumption measurements [17] and formation of 12-HETE from [1-^{14}C]arachidonic acid. Platelets (10^8) in 0.5 ml Dulbecco's phosphate-buffered saline (pH 7.0) containing 11 mM D-glucose were preincubated for 10 min at 37° with different concentrations of various HETEs. [1-^{14}C]Arachidonic acid (15 μM final concentration, 0.4 μCi) was then added. The incubations were terminated after 5 min by acidification with 10% formic acid and addition of 1.6 ml methanol, and the mixture was extracted with 3.2 ml chloroform. After evaporation of the chloroform phase under N_2, the residue was chromatographed on preactiviated Silica Gel G TLC plates using chloroform–methanol–acetic acid–water (90:8:1:0.8) as developing solvent. The [1-^{14}C]12-HETE band was localized by autoradiography, scraped from the TLC plates, and counted in a liquid scintillation counter.

Polymorphonuclear leukocyte 5-lipoxygenase activity was measured by the formation of 5-HETE or LTB_4 from [1-^{14}C]arachidonic acid, whereas the 15-lipoxygenase activity was determined by the conversion of arachidonic acid to [^{14}C]15-HETE. The standard incubation mixture consisted of polymorphonuclear leukocytes ($10–20 \times 10^6$) in 0.5 ml of Dulbecco's phosphate-buffered saline (pH 7.0), 11 mM D-glucose and different concentrations of HETE at 37°. The concentrations of various HETEs, analogs and derivatives used in testing their inhibitory potencies on a particular lipoxygenase were

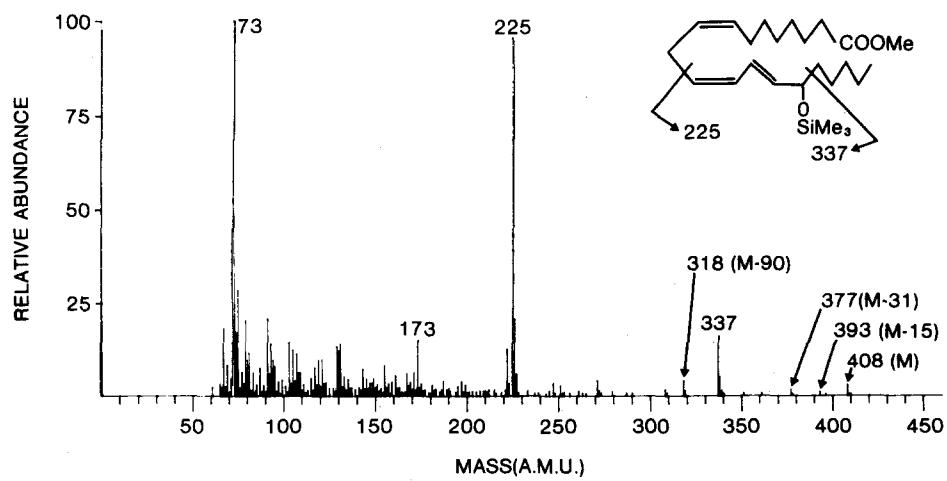


Fig. 2. Mass spectrum of the methyl ester trimethylsilyl ether derivative of 15-hydroxy-8,11,13-eicosatrienoic acid (15-OH-20:3).

usually in the range of 0.5 to 3 times the I_{50}. The maximum concentration of the fatty acid derivative tested resulted in 75–95% inhibition of lipoxygenase metabolite formation (relative to the control). After 5–7 min, [1-^{14}C]arachidonic acid (15 μM final concentration, 0.4 μCi) was added. The reaction was stopped by adding 1.6 ml methanol. The cells were removed by low speed centrifugation, and the methanolic solution was extracted with 3.2 ml chloroform. The chloroform layer was evaporated under N_2, the residue was applied to preactivated Silica Gel G TLC plates, and the plates were developed in petroleum ether–diethyl ether–acetic acid (50:50:1) together with authentic standards of 5-HETE and 15-HETE. Radioactive zones were localized by autoradiography, scraped and counted. Chemical characterization of [1-^{14}C]5-HETE, [1-^{14}C]LTB$_4$ and [1-^{14}C]15-HETE was carried out as previously described [11, 12].

Measurements of the effects of various HETEs on soybean 15-lipoxygenase activity were carried out in a 1 cm pathlength cuvette containing 1.0 ml of 0.1 M Tris buffer (pH 8.5) at 25°. Appropriate amounts of HETE and arachidonic acid (39–43 μM final concentration) were added to the cuvette, the contents were stirred, and the cuvette was placed in a Beckman Acta CIII spectrophotometer. After 1 min, the reaction was started by addition of 0.75 μg soybean 15-lipoxygenase, and the change in absorbance at 234 nm was recorded. The results were confirmed using [1-^{14}C]arachidonic acid and measuring 15-HPETE formation after 2 min.

RESULTS AND DISCUSSION

Oxygenase activity can be assayed directly by measuring oxygen consumption. In preliminary experiments with human platelets, oxygenase activity was measured by both oxygen consumption as well as product formation from [^{14}C]arachidonic acid. The results shown in Fig. 3 indicate that 15-HETE inhibited both the rate and extent of oxygen consumption. Furthermore, this inhibition correlated with decreased [^{14}C]12-HETE formation after 5 min, whereas [^{14}C]TXB$_2$ formation was not appreciably affected. On the basis of these results, subsequent work with these and other cell types was carried out with [^{14}C]arachidonic acid, and the reactions were stopped after 5 min.

Table 1. Comparative inhibition of rabbit platelet 12-lipoxygenase activity by various HETEs and ricinoleic acid*

Inhibitor added	Concentration of inhibitor required for half-maximal inhibition (μM)
15-HETE	0.93 ± 0.26
5-HETE	21 ± 0.83
12-HETE	> 100
Ricinoleic acid	> 150

* The 12-lipoxygenase activity was measured by the formation of 12-HETE from [1-^{14}C]arachidonic acid as described under Materials and Methods. All values are given as the means ± S.E. and are the results of at least three experiments.

Rabbit platelets produced 7.46 ± 0.49 μM [^{14}C]12-HETE from arachidonic acid (15 μM). Pretreatment of platelets with various HETEs followed by incubation with [^{14}C]arachidonic acid resulted in a concentration-dependent inhibition of the 12-lipoxygenase activity as measured by [^{14}C]12-HETE formation. Table 1 compares the concentrations of exogenously added HETEs required for half-maximal inhibition (I_{50}). 15-HETE was about 22 times more potent than 5-HETE, whereas 12-HETE showed very little inhibitory activity even at concentrations as high as 100 μM.

Rabbit polymorphonuclear leukocytes metabolized [^{14}C]arachidonic acid to [^{14}C]5-HETE and [^{14}C]LTB$_4$ via the 5-lipoxygenase pathway and to [^{14}C]15-HETE by the 15-lipoxygenase. These metab-

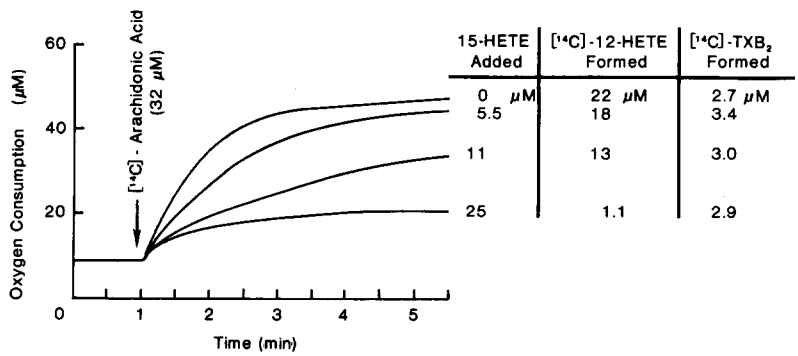


Fig. 3. Inhibition of arachidonic acid oxygenation and [^{14}C]12-HETE formation in human platelets by 15-HETE. A washed platelet suspension (3.3 mg protein) in 2 ml of medium containing 1.8 μM antimycin A, 137 mM NaCl, 2.7 mM KCl, 11 mM dextrose and 25 mM Tris–HCl (pH 7.6) at 30° was preincubated for 2 min with different concentrations of 15-HETE. Arachidonic acid (32 μM final concentration, 4.5 μCi/μmole) was added, and oxygen consumption was measured. After 5 min, the reactions were stopped, the products were extracted, and the formation of radioactive metabolites was measured as described under Materials and Methods. The four curves correspond to different concentrations of 15-HETE as indicated in the first column of the table. Note that at the highest concentration of 15-HETE, the remaining oxygen consumption was primarily due to the unaffected cyclooxygenase activity.

Table 2. Inhibition of the 5- and 15-lipoxygenase pathways in rabbit polymorphonuclear leukocytes by different HETEs*

Inhibitor added	Concentration (μM) of inhibitor required for half-maximal inhibition of: 5-Lipoxygenase [^{14}C]5-HETE	5-Lipoxygenase [^{14}C]LTB$_4$	15-Lipoxygenase [^{14}C]15-HETE
15-HETE	3.7 ± 0.50	5.1 ± 1.1	20 ± 3.6
12-HETE	6.8 ± 1.4	6.8 ± 1.1	9.4 ± 1.0
5-HETE	14 ± 3.4	13 ± 3.4	10 ± 3.3

* The conversion of [1-^{14}C]arachidonic acid into 5-HETE and LTB$_4$ via the 5-lipoxygenase pathway and into 15-HETE by the 15-lipoxygenase was measured as described under Materials and Methods. All values are given as the means ± S.E. and are the results of at least five experiments.

olites have been previously isolated, purified by HPLC, and characterized by gas chromatography–mass spectrometry [11, 12]. The concentrations of these metabolites formed from arachidonic acid (15 μM) were [^{14}C]5-HETE, 1.59 ± 0.18 μM, [^{14}C]LTB$_4$, 1.14 ± 0.15 μM and [^{14}C]15-HETE, 0.36 ± 0.046 μM. The inhibitory effects of various HETEs on the 5-lipoxygenase are presented in Table 2. 15-HETE was a more effective inhibitor than 12-HETE which was more potent than 5-HETE. The I_{50} values for [^{14}C]LTB$_4$ inhibition were nearly identical to those found for [^{14}C]5-HETE production. The inhibitory effectiveness of these exogenously added HETEs on the leukocyte 15-lipoxygenase activity was different than that found for the 5-lipoxygenase. The 15-lipoxygenase was most sensitive to 5-HETE and 12-HETE inhibition, whereas 15-HETE was the least potent inhibitor. These HETEs were also tested for their inhibitory activities towards soybean 15-lipoxygenase as measured spectrophotometrically by the initial rates of conjugated diene formation from arachidonic acid. Very little difference in inhibitory potencies was observed: 5-HETE (I_{50}, 28 ± 3.4 μM), 15-HETE (I_{50}, 36 ± 3.6 μM) and 12-HETE (I_{50}, 37 ± 4.9 μM).

To determine which structural characteristics of 15-HETE are essential for its inhibitory activity on 5-lipoxygenase activity, various derivatives were prepared and purified by HPLC, and their structures were confirmed by GC–MS. Table 3 shows the different inhibitory potencies of 15-HETE analogs with different degrees of unsaturation. Changing the oxygen functionality from the 15-hydroxy to the 15-hydroperoxy group increased the inhibitory effectiveness about 4-fold, whereas the 15-keto, 15-acetoxy and 15-HETE methyl ester derivatives were comparable to 15-HETE (Table 4). The 15-acetoxy methyl ester was less potent.

Table 3. Effect of unsaturation on the inhibitory activity of various 15-hydroxyeicosanoids on the 5-lipoxygenase in rabbit polymorphonuclear leukocytes*

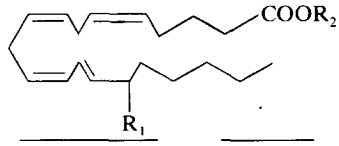
Fatty acid	Double bond positions	Concentration of fatty acid required for half-maximal inhibition (I_{50}) (μM)
15-OH-20:4	5,8,11,13	3.7 ± 0.50
15-OH-20:3	8,11,13	1.5 ± 0.47
15-OH-20:2	11,13	12 ± 3.7
15-OH-20:0		>100

* 5-Lipoxygenase activity was measured by [^{14}C]5-HETE formation from [^{14}C]arachidonic acid as described under Materials and Methods. All values are given as the means ± S.E. and are the results of at least three experiments.

The present results provide direct evidence that lipoxygenases with differing specificities exhibited differential sensitivities towards the HETE lipoxygenase products. Thus, 15-HETE and 12-HETE (and to a lesser extent 5-HETE) modulated the leukocyte 5-lipoxygenase. The 15-lipoxygenase exhibited a different profile, with 5-HETE and 12-HETE being more inhibitory than 15-HETE. The situation with the platelet 12-lipoxygenase was again different, where the 15-HETE was a far more potent inhibitor than 5-HETE with little demonstrable inhibitory activity for 12-HETE at concentrations below 100 μM. Despite obvious difficulties in determining the actual concentrations of arachidonic acid and the various HETEs likely to be encountered in their cellular environment, the results obtained in these *in vitro* experiments suggest that a complex interdependency exists between various lipoxygenase pathways. A scheme indicating the possible interregulatory relationships between 5-, 12- and 15-lipoxygenases is depicted in Fig. 1. Since one or more of each of these lipoxygenases have been reported to be present in peripheral blood cells such as leukocytes, lymphocytes, platelets and reticulocytes [2, 5, 18, 19], our data suggest that these cells may interact with each other via their lipoxygenase metabolites. The HETEs appear to inhibit only the lipoxygenase catalyzed conversions rather than any subsequent enzymatic steps since similar I_{50} values for [^{14}C]5-HETE and [^{14}C]LTB$_4$ formation were obtained. 15-OH-20:3 was a more potent inhibitor than 15-HETE. Although 15-HETE could be a substrate for the leukocyte 5-lipoxygenase, 15-OH-20:3 cannot be, because it lacks the 5,6-double bond. It thus seems likely that these hydroxyeicosanoids act as inhibitors rather than competitive substrates of the lipoxygenases.

15-HPETE is a more potent inhibitor of both the 5-lipoxygenase in leukocytes and the 12-lipoxygenase in platelets than the corresponding alcohol 15-HETE

Table 4. Inhibitory activity of various 15-HETE derivatives on 5-HETE formation by rabbit peritoneal polymorphonuclear leukocytes*

Fatty acid			Concentration of fatty acid required for half-maximal inhibition (I_{50})
	R_1	R_2	(μM)
15-HETE	HO	H	3.7 ± 0.50
15-HPETE	HOO	H	0.95 ± 0.26
15-KETE	=O	H	1.7 ± 0.35
15-HETE-Me	HO	CH_3	1.9 ± 0.59
15-HETE-OAc	$CH_3C(=O)-O$	H	2.6 ± 0.45
15-HETE-OAc,Me	$CH_3C(=O)-O$	CH_3	9.2 ± 0.29

* 5-HETE formation from [1-^{14}C]arachidonic acid was measured as described under Materials and Methods. All values are given as the means ± S.E. and are the results of at least three experiments.

[11]. Similar qualitative differences between HPETEs and the corresponding HETEs have been reported in other systems. Thus, 12-HPETE was found to be more effective than 12-HETE in stimulating guanylate cyclase [20], inhibiting thromboxane synthetase [21] and modulating several human neutrophil functions such as chemotaxis and chemokinesis [9], whereas 5-HPETE was reported to be more potent than 5-HETE in the enhancement of histamine release in human basophils [22]. The hydroperoxy derivatives are unstable and are rapidly reduced by cellular peroxidases to the more stable HETEs. It is thus possible that HPETEs are the initial, more potent and shorter lived modulators, whereas the corresponding HETEs may be the secondary, less potent but longer acting mediators.

The present study indicates that a free hydroxyl or carboxyl group is not an absolute structural requirement for inhibition of the 5-lipoxygenase activity in leukocytes since the 15-acetoxy, 15-keto and methyl ester derivatives are all as effective as 15-HETE. However, the presence of at least two and preferably three double bonds appears essential.

In summary, one possible subsidiary role of the lipoxygenases, the first enzyme involved in synthesis of various leukotrienes, may be to produce H(P)ETEs which can regular the functioning of other lipoxygenases and thus provide a measure of cellular control over leukotriene production.

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